DOI:10.14753/SE.2023.2733

MELANOMA-ASSOCIATED FIBROBLASTS INCREASE INTERLEUKIN-10 PRODUCTION OF MACROPHAGES IN A CYCLOOXIGENASE-DEPENDENT MANNER

PhD thesis

Uğur Çakır, MD

Károly Rácz Doctoral School of Clinical Medicine

Semmelweis University

Supervisors:	Miklós Sárdy, MD, PhD.
	Krisztián Németh, MD, PhD.
Official reviewers:	Attila Patócs, MD, DSc.
	Andrea Ladányi, DSc.

Head of the Complex Examination Committee:	József Tímár, MD, DSc.
Members of the Complex Examination Committee:	Eszter Baltás, MD, PhD.
	Gábor Földes, MD, DSc.

Budapest
2022

DOI:10.14753/SE.2023.2733

Table of contents

DOI:10.14753/SE.2023.2733

DOI:10.14753/SE.2023.2733

List of abbreviations

AIF1: allograft inflammatory factor 1

ALM: acral lentiginous melanoma

AUC: area under curve

α-SMA: α smooth muscle actin

BMSCs: bone marrow-derived stromal cells

CAFs: cancer-associated fibroblasts

COX: cyclooxygenase

ErbB3: v-erb-b2 avian erythroblastic leukemia viral oncogene homolog3

FAP: fibroblast activation protein

FSP1: fibroblast specific protein 1

HPF: hepatocyte growth factor

IBA-1: ionized calcium binding adapter molecule 1

IDO: Indoleamine 2,3-dioxygenase

IL: interleukin

iNOS: inducible nitric oxide synthase

LMM: lentigo maligna melanoma

MAFs: melanoma-associated fibroblasts

MAPK: mitogen-activated protein kinase

MDSC: myeloid-derived suppressive cells

MSC: mesenchymal stromal cell

NK: natural killer

NM: nodular melanoma

NRG1: Neuroregulin 1

PDGFR: platelet-derived growth factor receptor

PD-Ls: programmed death ligands

PI3K: phosphoinositide 3-kinase

SSM: superficial spreading melanoma

TAMs: tumor-associated macrophages

TGF-β: transforming growth factor β

TME: tumor microenvironment

TNF-α: tumor necrosis factor-α

Tregs: regulatory T cells

DOI:10.14753/SE.2023.2733

1 Introduction

Melanoma is a malignancy of melanocytes which are located in the basal layer of epidermis. Melanocytes are derived from the neural crest, and produce several factors that promote migration and metastasis of melanoma [1,2]. Melanoma is divided into the following major subtypes based on their histopathological characteristics; superficial spreading melanoma (SSM), nodular melanoma (NM), lentigo maligna melanoma (LMM), and acral lentiginous melanoma (ALM). SSM is the most common subtype and usually refers to melanoma in a radial or horizontal growth phase. SSM is especially common among fair-skinned individuals and tends to carry a good prognosis due to a low Breslow thickness if diagnosed at an early stage. NM generally occurs in the vertical growth phase and has a poorer prognosis. LMM commonly occurs in skin showing significant signs of chronic UV radiation, and has cells individually distributed alongside the dermal–epidermal junction and skin appendages. ALM histologically presents as tumor cells in single units along the dermal–epidermal junction, as confluent foci, and commonly occurs at acral sites. ALM is a more common subtype in darker skinned ethnicities. There are also some other subtype variants defined by clinical or histological characteristics including ocular, mucosal, amelanotic, spitzoid, and desmoplastic melanoma [3].

Melanoma will have 350 171 expected new cases globally in 2025 according to GLOBOCAN with an incidence rising in developed countries with predominantly fair-skinned population [4]. An estimated 63 271 people will die globally of melanoma in 2025, according to GLOBOCAN, and melanoma accounts or over 80% of skin cancer deaths [5]. The 5-year survival rate (2011–2017) according to Surveillance, Epidemiology, and End Results Program was 93.3% for melanoma, up from 81.9% in 1975, the earliest recorded. The 5-year survival between 2011-2017 in the US was 99.4% for stage I–II melanoma, decreasing to 68.0% for stage III and 29.8% for stage IV [5]. Although overall melanoma survival rates are increasing with the introduction of new immuno- and targeted therapies, survival rates for metastatic melanomas remain low.

Low survival rates for metastatic melanoma can be not only due to tumor cells but also other cells in the tumor microenvironment (TME). Melanoma TME consists of many cells

like regulatory T lymphocytes, melanoma-associated fibroblasts (MAFs), myeloid-derived suppressor cells, tumor-associated macrophages (TAMs), $CD4^+$ and $CD8^+$ T lymphocytes. Growing evidence has demonstrated that TME plays an important role in melanoma progression [6,7]. TME influences tumorigenesis and metastasis through various biological processes. Furthermore, TME heterogeneity also plays a predictor of prognosis and sensitivity to immuno- and targeted therapies in various cancers [8].

Cancer-associated fibroblasts (CAFs) (and in case of melanoma, MAFs) are characterized by markers such as α smooth muscle actin (α-SMA), fibroblast activation protein (FAP), vimentin, fibroblast specific protein 1 (FSP1), and platelet-derived growth factor receptor (PDGFR)-α and β [9–11]. CAFs are involved in many cellular processes, such as extracellular matrix remodeling, angiogenesis, cell-to-cell interactions and some studies showed that interactions between tumor cells and CAFs can promote tumor progression, metastasis and drug resistance. Melanoma cells when co-cultured with MAFs or incubated with MAF-derived conditioned media exhibited greater invasion and migration capability [12–15]. Studies also demonstrated that MAFs' activation is essential for melanoma metastasis, inhibition of MAFs by β-catenin suppression in mice resulted in a decreased tumor mediated neo-vascularization [16]. The cross-talk between melanoma cells and the MAFs can lead to drug resistance. It was found that the role of hepatocyte growth factor (HGF) is important in the development of acquired resistance to BRAF inhibitors. HGF secreted by MAFs in co-culture systems induces activation of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways leading to resistance to BRAF inhibitor therapy [17]. Neuroregulin 1 (NRG1) secreted by MAFs may also affect resistance to BRAF inhibitors via v-erb-b2 avian erythroblastic leukemia viral oncogene homolog3 (ErbB3). ErbB3 is upregulated in melanoma treated with BRAF inhibitors and its deactivation decreases the resistance of melanoma cell lines to therapy [18]. It was also demonstrated that treatment with BRAF inhibitors boost the production of transforming growth factor β (TGF-β) by melanoma cells, which leads to MAF activation and increased fibronectin production causing resistance to BRAF therapy [19].

Furthermore, CAFs are important regulators of the anti-tumor immune response. Some studies have suggested that CAFs have immunomodulatory capabilities that potentially regulate both innate and adaptive anti-tumor immune responses [20–22]. CAFs promote recruitment and differentiation of pro-tumorogenic immune cells in TME such as myeloid-derived suppressive cells (MDSC), regulatory T cells (Tregs) or TAMs and inhibit proliferation and activity of anti-tumorogenic immune cells, like cytotoxic $CD8^+$ T cells and natural killer (NK) cells [23–28]. In melanoma, MAFs play an essential role in immune escape of tumor cells. MAFs modulate NK cell phenotype and anti-tumor cytotoxicity [20,29] and they also impair cytotoxic activity of $CD8^+$ T cells [30,31].

Macrophages are derived from bone marrow myeloid progenitor cells and are part of the mononuclear phagocytic immune system among others monocytes and tissue resident macrophages. Monocytes can be accumulated in tissues and they can differentiate into macrophages. Macrophages can exhibit different phenotypes according to the stimuli they receive in their microenvironment. Macrophage polarization can be towards either pro-inflammatory M1 or anti-inflammatory M2 subtypes [32,33]. M1 macrophages secrete high amounts of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, IL-12, and IL-23, through which they elicit anti-microbial and anti-tumor effects, while they inhibit tissue regeneration and wound healing. On the other hand, M2 macrophages produce anti-inflammatory cytokines such as IL-10 and TGF-β, through which they promote tissue repair, wound healing, angiogenesis and fibrosis [34].

Macrophages are recruited to the tumor stroma via cytokines produced by the cells of TME. Tumor-associated macrophages are present in large amounts in tumor stroma of many cancers and are associated with tumor development process [35–37]. Phenotypic characteristics of TAMs in TME are not in a steady state but rather related to current condition of tumor. TAMs that share functional similarity to pro-inflammatory M1 phenotype, are important for the early stages of the inflammatory response in tumor. On the other hand, M2-like TAMs are the predominant macrophage phenotype in TME and correlate with tumor progression and poor prognosis [38]. TAMs produce cytokines such as IL-10, TGF-β, prostaglandin E2, out of which IL-10 promotes immune escape and

progression of tumors via inhibiting the pro-inflammatory/anti-tumor milieu [39]. Furthermore, IL-10 inhibits the function and differentiation of antigen-presenting cells [40], cytotoxic $CD8^+$ T cells and NK cells, and it mediates the immunosuppressive activity of Treg cells [41,42]. Elevated IL-10 levels in serum and also elevated IL-10 mRNA levels in freshly excised tumors were found in various cancer types, including melanoma [39,43–48].

The cross-talk between the CAFs and TAMs have been intensively studied. CAFs recruit monocytes from peripheral blood and increase their adhesion to the tumor stroma, promote differentiation to tumor-associated macrophages and phenotypical change of TAMs from M1 to M2-like phenotype [49,50]. Macrophages, on the other hand, assist the epithelial-mesenchymal transformation and give rise to CAFs and TAMs also help mesenchymal stem cells differentiate into CAFs [26,51]. All these observations suggest that the cross-talk between CAFs and TAMs may result in tumor progression.

Previous works of our research group found out that bone marrow-derived stromal cells (BMSCs) cause macrophages to increase their IL-10 production in a prostaglandin E2 dependent manner [52]. Based on previous studies which showed that CAFs share characteristic similarity to mesenchymal stromal cells [53], my research focused on the immunosuppressive effects of MAFs and in particular on the cross-talk between MAFs and macrophages as well as IL-10 production assisted by this cross-talk.

DOI:10.14753/SE.2023.2733

2 Objectives

The objective of my research was to investigate the interactions between MAFs, macrophages and melanoma cells in particular to immunosuppressive IL-10 production. In order to achieve this objective, we examined if

1.1. MAFs were in close contact with macrophages in melanoma microenvironment
1.2. MAFs caused an increase of IL-10 production of macrophages,
1.3. The increase of IL-10 production were affected by MAFs cell count, macrophage phenotype and co-culture incubation time,
1.4. There were a correlation between the IL-10 increasing ability of MAFs and clinicopathological factors of melanoma,
1.5. The IL-10 increasing ability of MAFs could be enhanced when pretreated with conditioned media from BRAF inhibitor- and chemotherapy treated melanoma,
1.6. Melanoma tumor cells had improved chemoresistance against BRAF inhibitors when co-cultured with MAFs,
1.7. Indoleamine 2,3-dioxygenase (IDO), inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX) pathways played a role in MAF-mediated IL-10 production of macrophages.

3 Methods

Between 2015 and 2019, 32 stage-III/IV melanoma patients were enrolled in our study, which was conducted at the Department of Dermatology, Venereology and Dermatooncology, Semmelweis University, Budapest, Hungary. After obtaining informed consent, freshly excised tumors from melanoma patients were collected and retrospectively analyzed as approved by the Hungarian Scientific and Research Ethics Committee of the Medical Research Council (ETT TUKEB; Decree No. 32/2007, supplements 32-2/2007 and 32-3/2007). The study was conducted in accordance with the ethical standards as dictated by the Declaration of Helsinki.

3.1 Cell Culture

The human monocytic cell line THP-1 (TIB-202), and BRAF mutated human malignant melanoma cell lines SK-MEL-28 (HTB72) and MALME-3M (HTB64) were purchased from American Type Culture Collection (ATCC; Rockville, MD, USA). THP-1 monocytes were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Gibco™) medium supplemented with 10% fetal bovine serum (FBS) (Gibco™ Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1% penicillin–streptomycin (P/S) (Gibco™), and 1% l-glutamine (Gibco™). BRAF mutated melanoma cells isolated from the excised tumors (MM-55) as well as SK-MEL-28 and MALME-3M were maintained in standard Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS, 1% penicillin–streptomycin (P/S), and 1% L-glutamine. MAFs were propagated in MAF medium (DMEM supplemented with 20% FBS, 1% penicillin–streptomycin (P/S) and 1% L-glutamine), and half of the medium was refreshed every other day.

3.2 MAF Isolation and Generation of MAF-Derived Conditioned Media

MAFs were isolated from either primary or metastatic tumors of melanoma patients and characterized as previously described [30]. First, the inner tumor mass was minced into ≈1 mm^3 pieces and digested in 20 mL DMEM supplemented with 200 U/mL type IV collagenase and 0.6 U/mL dispase (Thermo Fisher Scientific, Waltham, MA, USA). MAFs were then separated from melanoma cells by utilizing a differential adhesion/trypsinization method. This protocol is based on the observation that fibroblasts such as MAFs adhere better to plastic than melanoma cells. In brief, the dispase/collagenase-digested tumor cell suspension was plated in a plastic cell culture dish. Then, 30 min later, floating cells were removed, and adherent cells were cultured (differential adhesion). Subconfluent cell cultures were trypsinized for 1 min, detached cells were removed, and still adherent cells enriched in MAFs were subcultured (differential trypsinization) [54]. Cultured

MAFs were shown to be void of the melanoma markers melan-A and gp100 and positive for fibroblast-associated protein (FAP).

MAF cultures with 75–80% confluence were washed twice in phosphate-buffered saline (PBS) and further cultured in 10 mL basal medium (BM) consisting of DMEM, 1% P/S, 1% L-glutamine, and 0.5% BSA (Sigma-Aldrich). After 48 h, conditioned media (CM) derived from MAFs was collected.

3.3 Immunostaining of Melanoma Samples for FAP and Iba-1

After surgical excision, the tissue was fixed in buffered 10% paraformaldehyde and embedded in paraffin. Sections were cut onto positively charged slides at 6 µm thickness, baked overnight in a 65 °C oven, deparaffinized, and then antigen retrieval was performed in citrate buffer (pH 9) in a microwave oven. The sections were then blocked with BSA to inhibit non-specific binding of the antibodies, and endogenous peroxidase activity was also blocked in order to not interfere with the staining procedure that followed. First, the tumor stroma was labelled using antibody to fibroblast activation protein (FAP) (ABCAM ab207178, rabbit monoclonal antibody) in 1:1000 dilution at 4 °C overnight, followed by 1 h incubation with a rabbit IgG VisUCyte HRP polymer (VC003 R&D Systems), and then an Alexa-594 conjugated Tyramide at 1:10,000 dilution. Following a second microwave session (to eliminate the primary antibody and inactivate the added HRP), the second primary antibody, Iba-1 (WAKO 019-19741), was applied to the sections at 1:2000 dilution, followed by the rabbit Visu-cyte polymer (R&D Systems, VC-003) and an Alexa-488 conjugated Tyramide (1:10,000 dilution). Finally, DAPI was used for nuclear staining. Negative controls included no primary antibody and/or no HRP conjugate. Visualization was performed with a Leica DMI6000 inverted fluorescence microscope using the LAX software.

3.4 M1/M2 Differentiation Assay

THP-1 monocytes were differentiated into macrophages of M0, M1, and M2-like phenotype, as described by *Genin et al*. [55]. First, THP-1 monocytes (2×10^5 cells/well) were plated in 96-well plates and differentiated into M0-like macrophages by 24 h incubation with 20 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich), followed by 24 h incubation in fresh RPMI 1650 medium. M0-like macrophages were polarized into M1-like macrophages by 24 h incubation with 20 ng/mL of interferon-γ (IFN-γ) (R&D System) and 10 pg/mL of lipopolysaccharide (LPS) (Sigma-Aldrich). Macrophage M2-like polarization was achieved by 72 h incubation with 20 ng/mL of interleukin 4 (PeproTech) and 20 ng/mL of interleukin 13 (PeproTech).

3.5 Cell Culture Assays

For MAF-macrophage co-culture assays, THP-1 monocytes (2×10^5 cells/well) in 96-well plates were differentiated into macrophages of various phenotypes as described above. Following a PBS wash, 5×10^4 MAF or pre-conditioned MAF cells (see below) per well were added and incubated in DMEM supplemented with 10% FBS, 1% penicillin–streptomycin, and 1% L-glutamine for 24 h. To enhance cytokine production, cells were stimulated with 1 μg/mL LPS for an additional 18 h. Lastly, the plates were centrifuged, and supernatants were collected and stored at −20 C.

For MAF titration (dose curve) assay, MAFs at 2×10^5 cells per well with a twofold decreasing titration were added to a constant number of differentiated THP-1 macrophages at 2×10^5 cells per well and incubated as described above.

For MAF monocultures, MAFs at 5×10^5 cells per well were incubated in 96-well plates with DMEM supplemented with 10% FBS, 1% penicillin–streptomycin, and 1% L-glutamine for 24 h, followed by LPS treatment, as described previously.

3.6 Cytotoxicity assay

MALME-3M and SK-MEL-28 melanoma cell line cells were transduced with the fluorescent protein expressing lentiviral supernatants produced with pRRL-EF1-eGFP-WPRE expression plasmid as described by Rádai et. al. and Windt et. al. [56,57]. After the transduction, cell lines were sorted by flow cytometry based on fluorescent intensity.

2.5×10^3 cells/well MAFs and 2.5×10^3 cells/well either green fluorescent protein (GFP) positive MALME-3M or GFP-positive SK-MEL-28 were plated in 96-well plates and either vemurafenib or dabrafenib were added in twofold dilution series starting with highest concentration of 5 μM. 72h after incubation, fluorescence was recorded by Perkin Elmer EnSpire microplate reader (Perkin Elmer, Waltham, MA, USA) at 485 nm excitation and 510 nm emission wavelengths for GFP.

Area under curve (AUC) was calculated with Graphpad Prism 7.0 software using the normalized data of fluorescence measurements described above.

3.7 Generation of Conditioned Media from Untreated and Chemotherapy or Small-molecule Inhibitor Treated Melanoma Cells

Melanoma cell cultures reaching 75–80% confluence were washed twice in PBS and further cultured in 10 mL basal medium (BM) consisting of DMEM, 1% P/S, 1% L-glutamine, and 0.5% BSA (Sigma-Aldrich). After 48 h, media conditioned by cultured cells (conditioned media, CM)

were collected. Twofold serial dilutions of CM in BM were made, and MAFs were incubated in diluted CM for 48 h. Subsequently, cells were washed in PBS. Preconditioned MAFs were used in co-culture assays as described above.

Melanoma tumor cells were treated with 1 of 5 drugs: 1 μM vemurafenib, 1 μM dabrafenib, 1 μM trametinib, 1 μM dabrafenib + 1 μM trametinib, or 500 μM dacarbazine (DTIC) for 48 h. These treatment concentrations were selected on the basis of previous cytotoxicity experiments and were demonstrated to be able to induce cell death in SK-MEL-28 and MALME-3M melanoma cell lines. Subsequently, cells were washed in PBS and incubated in fresh culture medium for 48 h. The CM from chemotherapy treated cells were collected and MAFs were incubated in them for 48 h. These pre-conditioned MAFs were used in co-culture assays as described above.

3.8 Inhibitor Assay

NS-398 (selective COX2 inhibitor), SC-560 (selective COX1 inhibitor), 1-methyl-D-tryptophan (IDO inhibitor), and L-NG-Nitro arginine methyl ester (L-NAME; iNOS inhibitor) were tested in twofold dilution series starting with 8 μM, 8 μM, 8 mM, and 8 mM, respectively. These compounds were added at the initiation of the co-culture with MAFs and macrophages and incubated overnight before addition of LPS. Supernatants were assayed for IL-10 by ELISA after 18 h of LPS treatment.

3.9 ELISA

Supernatants from macrophage and MAF co-cultures were collected and measured by the R&D Systems IL-10 ELISA kit (Quantikine; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Measurements were conducted in triplicate/quadruplicate. Absorbance was measured at 450 nm.

3.10 Statistical analysis

We examined the differences between the groups for statistical significance by Student's t-test or two-way ANOVA (with Tukey's test as post hoc test) using Prism 7.0; Graphpad Software. A p-value of <0.05 was accepted as statistically significant. ROUT test (Robust regression and Outlier removal) was performed for outliers detection using Prism 7.0; Graphpad Software. All experiments were performed in triplicate/quadruplicate.

DOI:10.14753/SE.2023.2733

4 Results

4.1 MAFs are in Intimate Contact with Macrophages In Vivo

We examined two melanoma samples with combined immunostainings exploring the spatial distribution of MAFs and macrophages within the melanoma tumor stroma. This was based on the previous studies exhibiting that intravenously injected MSCs are eventually surrounded by recipient-derived macrophages, which facilitates the interactions between these two cell types [58]. MAFs were identified with a commonly used cancer-associated fibroblast marker FAP, while macrophages were detected using ionized calcium binding adapter molecule 1, IBA-1 (also known as allograft inflammatory factor 1, AIF1), a highly specific marker used to detect tumor-associated macrophages [59,60]. We found that FAP-positive MAFs were readily identified within the cancer stroma, and interestingly, the majority of these stromal cells were surrounded by macrophages (Figure 1).

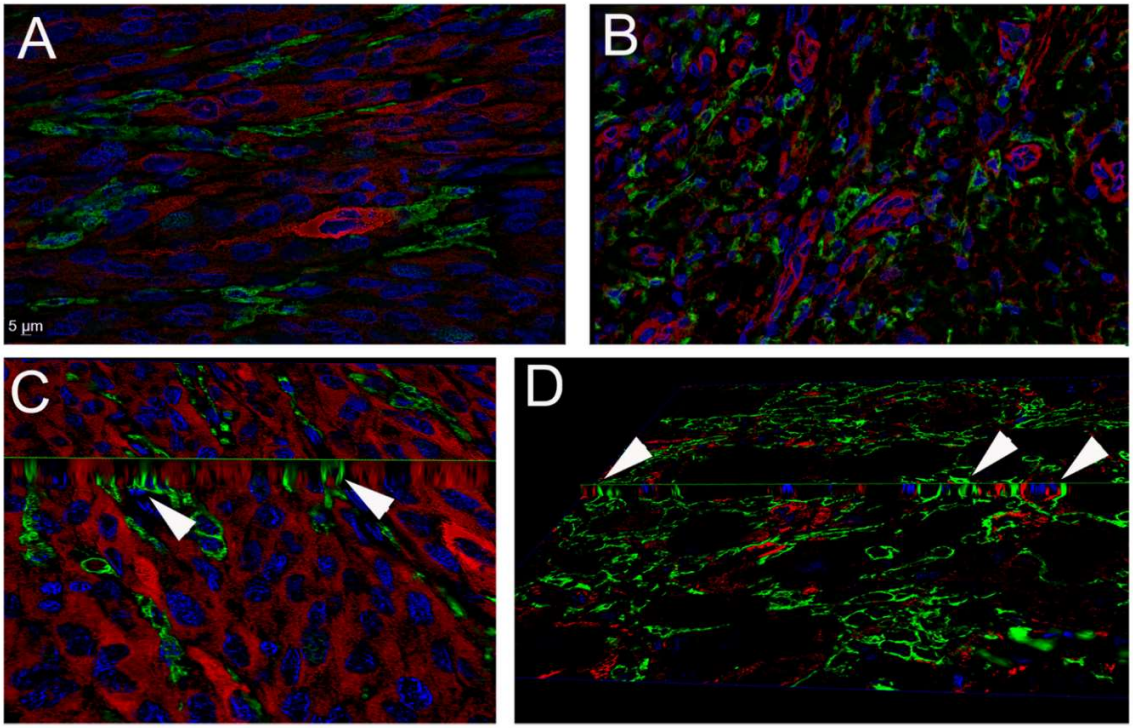

Figure 1. Immunohistochemistry of excised melanoma from two different patients. (**A**,**C**) Patient 1, (**B**,**D**) Patient 2. (**A**,**B**) The 0.5 µm thin optical sections from Z-stacks following deconvolution. (**C**,**D**) Images generated from slicing the three-dimensional Z stack; the arrows point at the intersection of the horizontal and vertical planes to demonstrate the very close connection between the membranes of Iba-1-positive macrophages (green fluorescence) and FAP-positive MAFs (red fluorescence). Blue fluorescence (DAPI) labels cell nuclei [61].

4.2 MAFs Increase IL-10 Secretion of THP-1 Cells

CAFs of other cancers were shown to increase IL-10 secretion in monocytes/macrophages [62–64]. Based on this phenomenon, we hypothesized that MAFs behave similarly. To examine this, we first co-cultured macrophage-like THP-1 cells with MAFs in various ratios. While the number of THP-1 cells was kept constant, a gradual increase in the number of added MAFs resulted in a dose-dependent elevation of THP-1-derived IL-10 output, reaching an almost fourfold increase when equal number of THP-1 cells and MAFs were co-cultured (Figure 2a). Time curve analysis between 12 h and 96 h following LPS stimulation (36 h and 120 h total of co-culture time, respectively) demonstrated a peak stimulatory effect at 24 h (Figure 2b).

DOI:10.14753/SE.2023.2733

To examine if MAFs can elicit IL-10 secretory response in various macrophage phenotypes, we pretreated monocytoid THP-1 cells with PMA or selected growth factors and co-cultured uncommitted M0, and polarized M1 or M2-like THP-1 macro-phages with MAFs. M0 and M2 macrophages both responded with a robust increase in their IL-10 production, while M1 cells showed a slight, but not significant, increase in IL-10 secretion (Figure 2c,d).

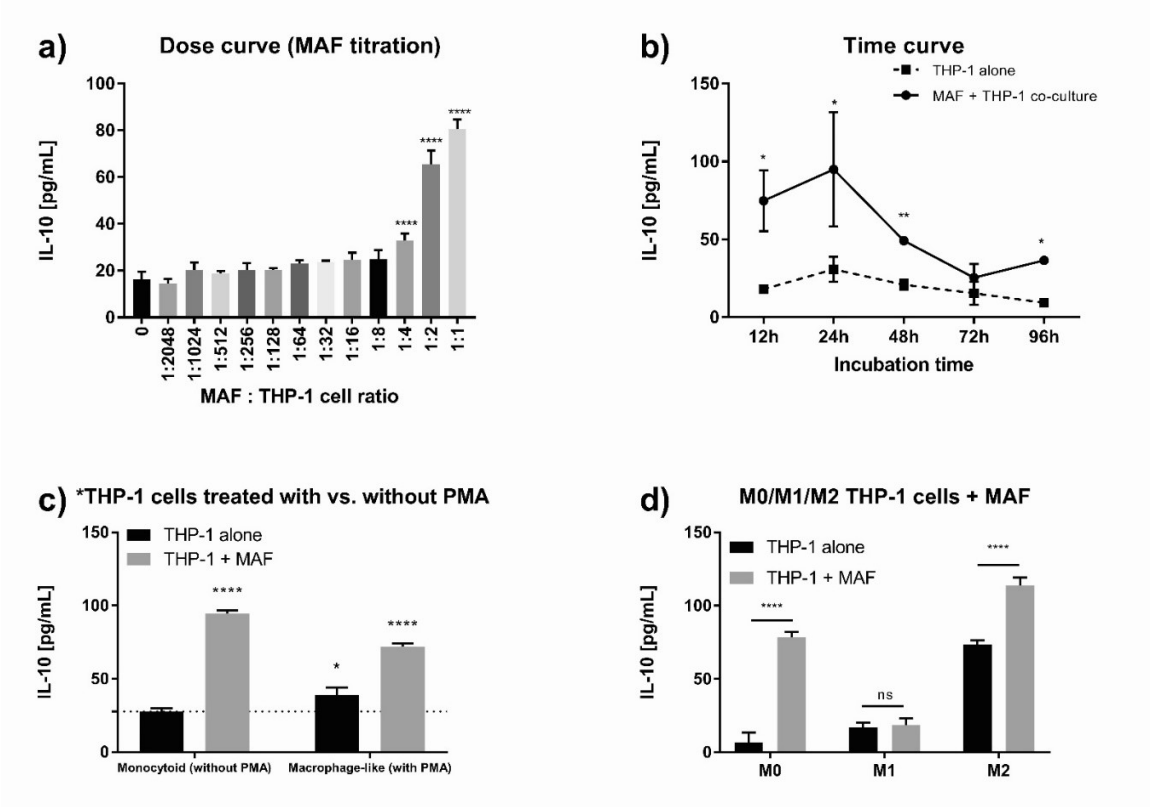

Figure 2. Effect of MAFs on IL-10 secretion in THP-1 macrophages. (a) IL-10 concentration of MAF-THP-1 co-cultures with a MAF/macrophage cell ratio between 1:2048 and 1:1, n = 4. (b) IL-10 concentration of THP-1 monoculture and MAF/THP-1 co-culture at 12 h, 24 h, 48 h, 72, and 96 h of incubation time, n = 4. (c) IL-10 concentration of monocytoid (without PMA pretreatment) and macrophage-like (with PMA pretreatment) THP-1 cells in monoculture and co-culture with MAFs, n = 6. (d) IL-10 concentration of M0-, M1-, and M2-like differentiated THP-1 macrophages in monoculture and co-culture with MAFs, n = 5. Representative data from one of two independent experiments. Error bars represent s.e.m. * $p < 0.05$, ** $p < 0.005$, and **** $p < 0.0001$ [61].

DOI:10.14753/SE.2023.2733

4.3 Thicker Melanomas Harbor More Immunosuppressive MAFs Compared to Thinner Tumors

After establishing the boosting effect of MAFs on the IL-10 production of macrophages in vitro, we wondered if the degree of immunosuppression exhibited by MAFs may correlate with well-defined clinical parameters of melanoma patients (Table 1). First, we compared the IL-10-increasing ability of MAFs collected from primary melanoma samples of various Breslow depths. Interestingly, melanoma-derived MAFs from tumors thicker than 2 mm provoked a markedly higher IL-10 output in THP-1 macrophages as compared to thinner melanomas less than 2 mm deep (Figure 3). There was no difference between primary vs. metastatic melanoma-derived MAFs, and the BRAF status of the melanomas did not seem to influence the IL-10 increasing ability of MAFs either.

DOI:10.14753/SE.2023.2733

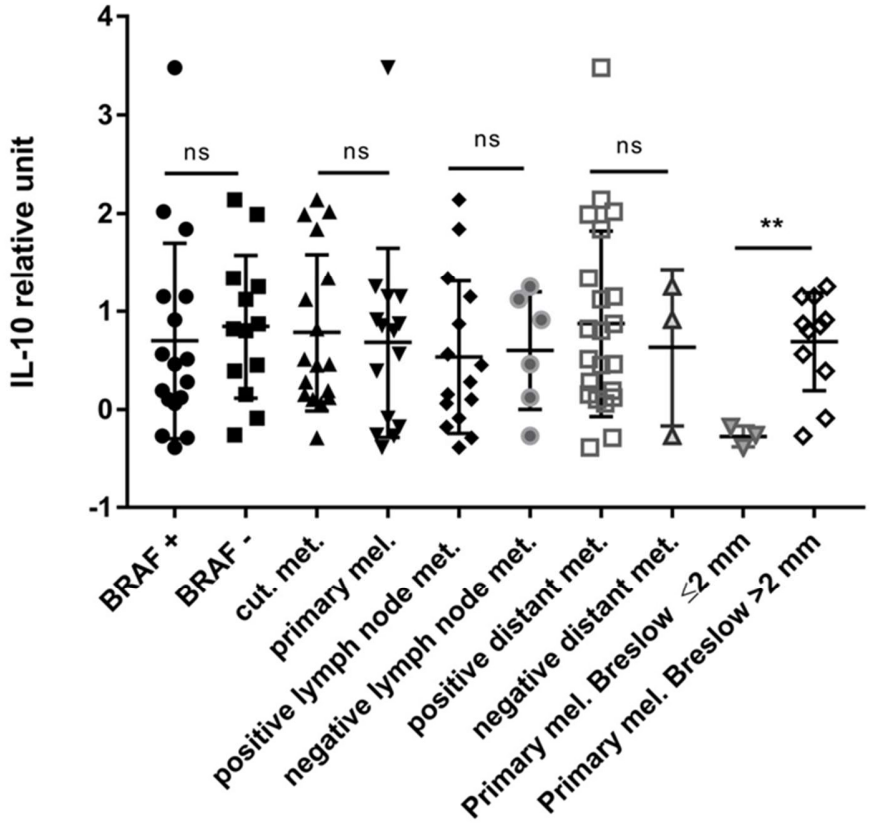

Figure 3. Clinical correlation of ex vivo IL-10 production of MAF macrophage co-cultures. Clinicopathological properties of melanomas and relative IL-10 concentration in supernatants of THP-1 macrophages co-cultured with MAFs isolated from tumors of various melanoma patients, n = 33 MAFs (isolated from 32 patients, MAFs from both primary tumor and metastasis of patient number 2 were isolated). Using ROUT test (Robust regression and Outlier removal) on Graphpad Prism 7.0 software the cases with relative IL-10 unit of 63,98 and 3,48 were identified as outliers and excluded from all and one measurement, respectively. cut.met.: cutaneous metastasis, primary mel.: primary melanoma, met.: metastasis. Data from one experiment. non-significant, ** $p < 0.005$ [61].

DOI:10.14753/SE.2023.2733

Table 1. Clinicopathological properties of MAF isolated patients and relative IL-10 concentration difference of MAF macrophage co-culture from macrophage monoculture. ALM: acral lentiginous melanoma, CM: cutaneous metastasis, DM: distant metastasis, F: female, LMM: lentigo maligna melanoma, LNM: lymph node metastasis, M: male, MI: mitosis index, n/a: information not available, NM: nodular melanoma, PT: primary tumor, SSM: superficial spreading melanoma, wt: wild-type [61].

Patient.	MAF Origin	Gender	Age	Primary Melanoma Details				BRAF	LNM	DM	Relative IL-10 Change
				Subtype	Breslow (mm)	Clark	MI	Ulceration			
1	CM	M	90	unclassifiable	5.4	V	14	yes	wt	yes	yes	2.14
2	CM	F	79	SSM	2	IV	n/a	n/a	positive	yes	yes	1.84
2	PT	F	79	SSM	2	IV	n/a	n/a	positive	yes	yes	−0.39
3	CM	F	80	NM	4	III	n/a	yes	wt	n/a	yes	1.99
4	CM	M	73	SSM	0.87	II	6	yes	wt	n/a	yes	0.82
5	CM	M	69	SSM	1	II	n/a	n/a	positive	yes	yes	−0.29
6	PT	M	84	LMM	5.15	IV	22	no	wt	n/a	yes	0.79
7	PT	F	76	NM	4.64	IV	15	no	positive	no	no	−0.27
8	CM	F	66	NM	9	V	n/a	n/a	positive	yes	yes	63.98
9	PT	M	23	unclassifiable	7.51	V	28	no	positive	yes	n/a	1.15
10	CM	M	70	NM	5.2	IV	18	yes	positive	n/a	yes	2.02
11	PT	F	50	SSM	2.92	IV	14	no	positive	n/a	yes	3.48
12	PT	M	56	SSM	1.77	III	4	no	n/a	yes	n/a	−0.18
13	PT	M	85	unclassifiable	10.26	IV	24	yes	positive	no	no	0.91
14	PT	M	74	NM	6.23	IV	18	yes	wt	yes	n/a	−0.09
15	CM	F	62	ALM	9.1	V	12	yes	positive	n/a	yes	0.19
16	CM	F	54	unclassifiable	18.21	V	42	yes	wt	yes	yes	0.15
17	CM	F	62	NM	9	IV	n/a	n/a	wt	yes	yes	1.34
18	CM	M	75	unclassifiable	3.34	IV	14	yes	wt	no	yes	1.12
19	CM	M	72	unclassifiable	2.71	IV	18	no	positive	no	yes	0.12
20	CM	F	52	SSM	10.58	V	28	no	positive	n/a	yes	0.51
21	CM	M	43	SSM	0.953	III	4	yes	positive	yes	yes	0.06
22	CM	F	82	Unknown primary					wt	yes	yes	0.45
23	PT	M	48	unclassifiable	1.75	IV	26-29	yes	wt	n/a	n/a	−0.26
24	PT	F	90	NM	13.24	IV	46	yes	n/a	n/a	n/a	0.85
25	CM	M	41	SSM	0.9	III	6	yes	positive	yes	yes	0.28
26	CM	M	67	SSM	6.18	V	5	yes	positive	yes	yes	0.01
27	PT	M	70	SSM	3.364	IV	3	yes	wt	n/a	n/a	0.39
28	PT	M	51	NM	5.17	IV	16	yes	wt	no	no	1.25
29	PT	M	81	SSM	5.336	IV	6–8	yes	wt	yes	yes	0.87
30	PT	M	74	NM	13.24	V	48	yes	positive	n/a	yes	1.15
31	PT	M	57	unclassifiable	12.3	V	18	yes	positive	yes	yes	0.56
32	CM	F	71	SSM	3.4	IV	12	no	positive	no	yes	0.46

4.4 Prior Exposure to Untreated or BRAF Inhibitor- or Chemotherapy-Treated Melanoma Cells Boosts IL-10-Increasing Ability of MAFs

Going further, we wondered if melanoma cells can influence how MAFs interact with macrophages. To test this, MAFs were incubated with increasing concentrations of conditioned media collected from either SK-MEL-28 or MALME-3M melanoma cell lines,

or cultured, differential adhesion-selected primary melanoma cells. Such exposure to melanoma supernatants augmented the ability of MAFs to increase IL-10 production in THP-1 cells (Figure 4a). Interestingly, this effect was further facilitated when MAFs were cultured in conditioned media derived from BRAF inhibitor- or chemotherapy-treated melanoma cells. When compared to untreated melanoma-conditioned MAFs, the small molecule inhibitors, vemurafenib, dabrafenib, and trametinib with dabrafenib, enhanced the ability of both melanoma cell lines and primary melanoma cells to stimulate MAFs, which ultimately led to an additional increase in THP-1-derived IL-10 secretion. Trametinib treatment of melanoma cells alone was unable to further potentiate the effect of MAFs on THP-1 cells. Finally, we treated melanoma cells with dacarbazine, an alkylating chemotherapeutic agent, and found that drug-treated primary melanoma cells magnified the IL-10 elevating effect of MAFs on THP-1 cells (Figure 4b–d).

DOI:10.14753/SE.2023.2733

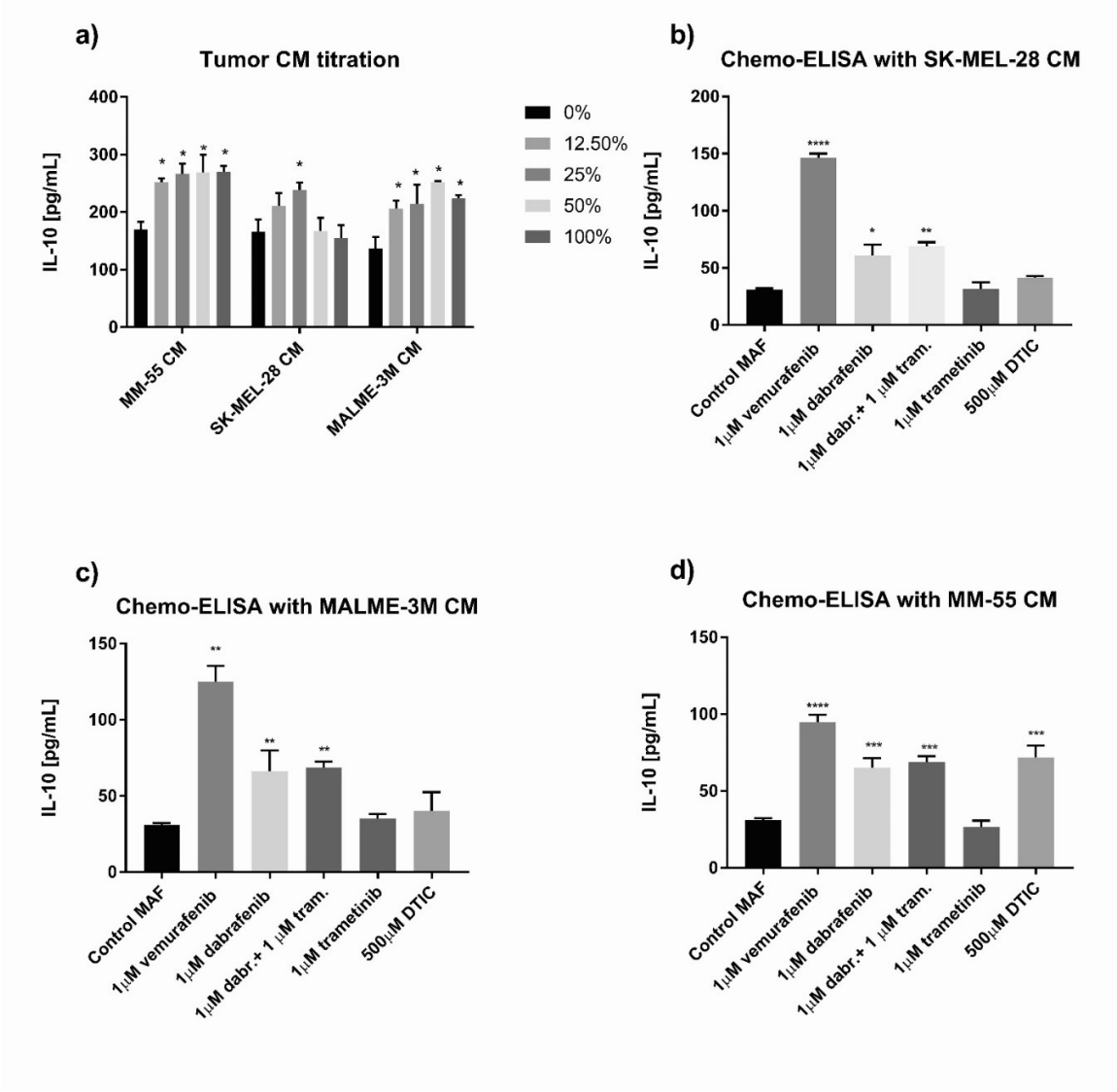

Figure 4. Preincubation of MAFs with conditioned media of melanoma cells. (a) IL-10 abundance in co-cultures of THP-1 macrophages with MAFs that were pre-incubated with different doses of conditioned media derived from MM-55, SK-MEL-28, and MALME-3M melanoma cells, n = 3. (b–d) IL-10 abundance in co-cultures of THP-1 macro-phages with MAFs that were incubated with conditioned media from previously drug-treated MM-55, SK-MEL-28, and MALME-3M melanoma cells, n = 4. dabr. = dabrafenib, DTIC = dacarbazine, tram. = trametinib. Representative data from one of two independent experiments. Error bars represent s.e.m. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, and **** $p < 0.0001$ [61].

DOI:10.14753/SE.2023.2733

4.5 MAFs do not Affect Survival of Melanoma Cells Against BRAF-Inhibitors

Considering that melanoma cells affected the IL-10 increasing ability of MAFs, we wondered if melanoma cells and MAFs would interact and also increase survival of melanoma cells against BRAF-inhibitors. We co-cultured MAFs with BRAF mutated melanoma cell lines (SK-MEL-28 and MALME-3M) and treated the co-culture with vemurafenib and dabrafenib. Our results showed that the AUC of MAF/melanoma co-culture was not greater than that of melanoma monoculture. This indicated that MAFs do not directly increase melanoma survival when treated with BRAF-inhibitors (Figure 5 and table 2)

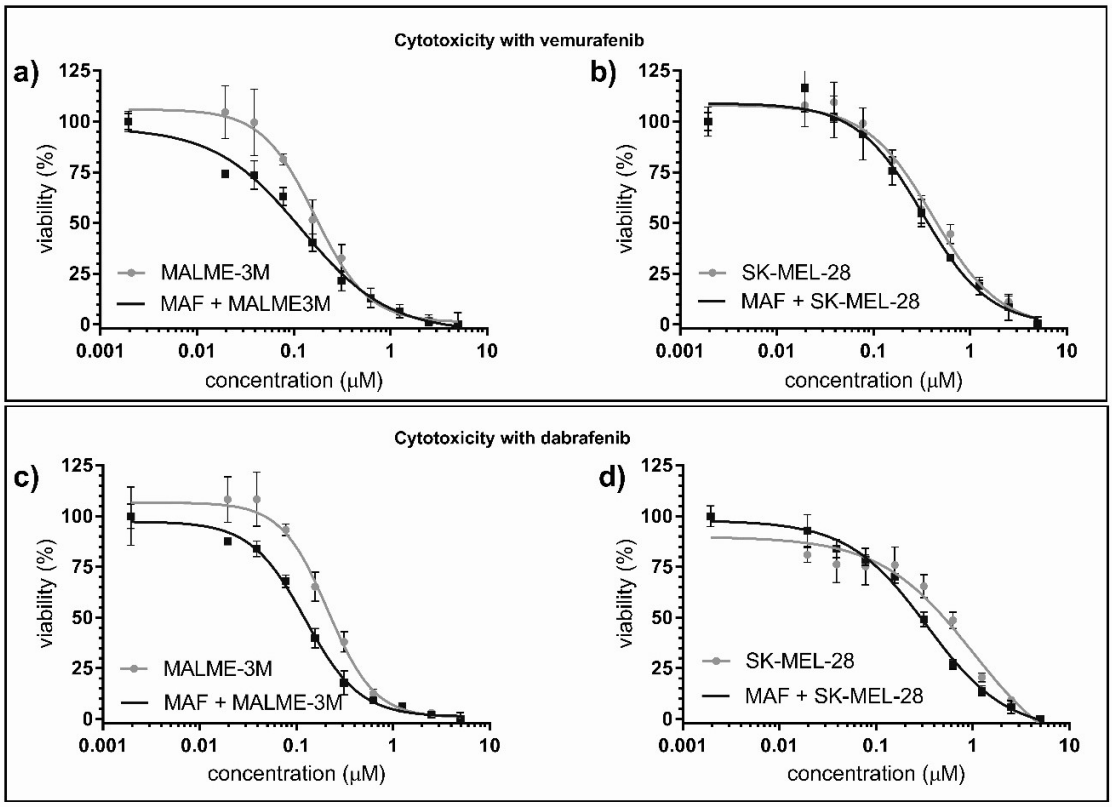

Figure 5. Cytotoxicity of BRAF inhibitor treatment. Viability of MALME-3M and SK-MEL-28 monocultures as well as MAF/melanoma cell line co-cultures when treated with vemurafenib (a and b) and dabrafenib (c and d), n=4. Representative data from one of the three independent experiments.

DOI:10.14753/SE.2023.2733

Table 2. Area under curve of melanoma cell lines and MAF/melanoma co-cultures when treated with either vemurafenib or dabrafenib. Representative data from one of the three independent experiments.

	MALME-3M	MAF + MALME-3M	SK-MEL-28	MAF + SK-MEL-28
vemurafenib	205.8	167.7	247.8	240.5
dabrafenib	218	177.1	215.6	209.7

4.6 Cyclooxygenase (COX) Pathway Plays a Critical Role in MAF-Driven IL-10 Increase

Finally, we set out to explore the molecular mechanisms involved in the immunosuppressive effect of MAFs. MAF monocultures on their own did not produce IL-10. To determine if cell–cell contact with macrophages is needed for the observed IL-10 stimulatory effect, we cultured THP-1 cells with MAFs with or without direct cellular contact. Although the observed IL-10-increase was greater in the direct co-culture setting, treatment of THP-1 cells with MAF-conditioned medium was able to increase IL-10 production as well, suggesting a role for soluble factors (Figure 6). Given the similarity between BMSC-mediated and MAF-derived immunosuppression, we utilized selective pathway inhibitors known to interfere with BMSC immunomodulatory effects. In MAF-THP-1 co-cultures, inhibition of IDO affected both untreated and MAF-exposed THP-1 cells, and therefore a co-culture-specific effect of IDO loss could not be observed (Figure 7a). COX-1 and -2 inhibition abrogated IL-10 increase in THP-1 cells (Figure 7c, d). iNOS inhibition had no effect on MAF-mediated IL-10 elevation (Figures 7b).

DOI:10.14753/SE.2023.2733

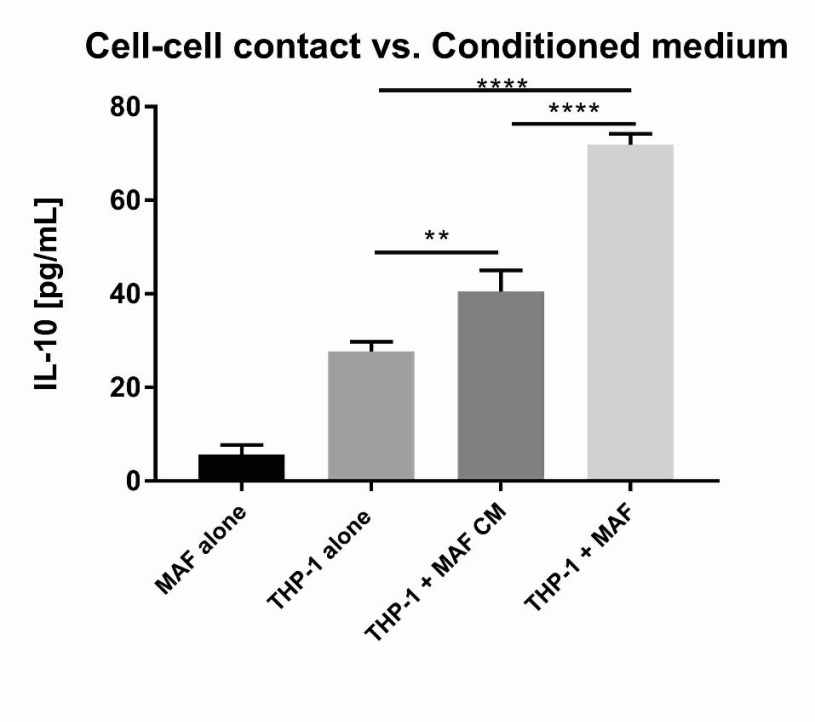

Figure 6. IL-10 concentration in supernatants of MAF monoculture, THP-1 monoculture, MAF-derived conditioned media (MAF CM)-treated THP-1 monoculture, and MAF/THP-1 co-culture, n = 5. Representative data from one of two independent experiments. Error bars represent s.e.m. ** $p < 0.001$ and **** $p < 0.0001$ [61].

DOI:10.14753/SE.2023.2733

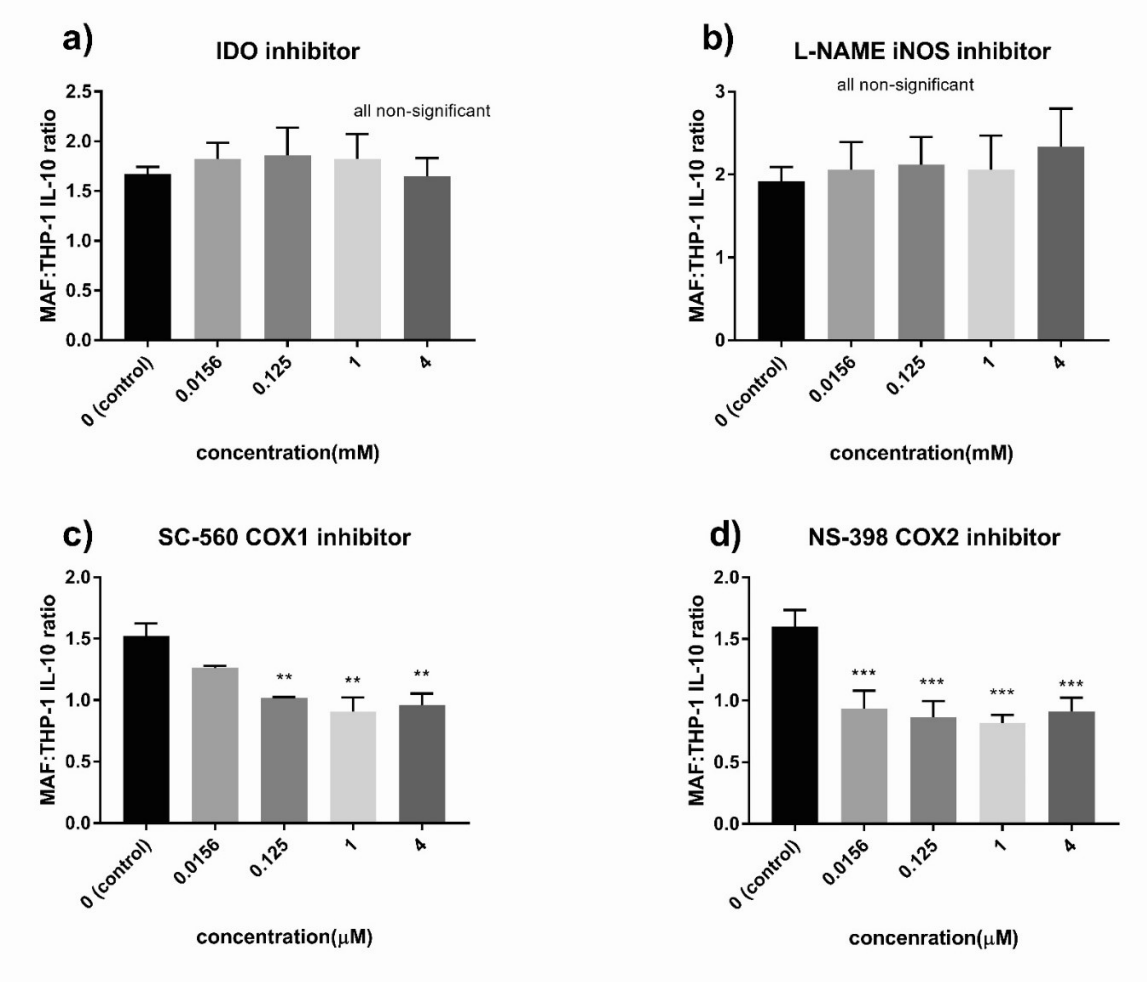

Figure 7. Inhibitors of IL-10 production in MAF/THP-1 macrophages co-culture. Ratio of IL-10 concentration of MAF/THP-1 co-cultures to THP-1 monocultures treated with different concentrations of 1-methyl-d-tryptophan (IDO inhibitor) (a), NG-nitro-L-arginine methyl ester (L-NAME) iNOS inhibitor (b), SC-560 COX1 inhibitor (c), and NS-398 COX2 inhibitor (d), n = 4. Representative data from one of two independent experiments. Error bars represent s.e.m. ** $p < 0.005$ and *** $p < 0.0005$ [61].

DOI:10.14753/SE.2023.2733

5 Discussion

In our experiments, we demonstrated that MAFs possess potent immunoregulatory abilities when cultured with monocyte/macrophages and they are in close contact with these immune cells in tumor stroma.

MAFs are important elements of the melanoma microenvironment [65,66]. They are able to directly influence the growth and metastatic potential of melanoma cells, and mounting evidence suggests that they are also capable of modulating intra-tumoral immune responses by suppressing T cells and NK cells [20,29–31]. In our study, we found that MAF-exposed macrophages change character and increase their production of IL-10, the potent immunosuppressive cytokine.

The M1/M2 paradigm of macrophages was first described a long time ago [67]. M1 macrophages are believed to be pro-inflammatory, promoting anti-cancer immune responses, while M2 macrophages exhibit an immunosuppressive phenotype, dampening intra-tumoral inflammation and thus promoting evasion of anti-cancer immunity [68]. Although the M1/M2 polarity and the corresponding cell surface markers and secreted molecules are well established, a homogenous population of these two phenotypic extremes is rarely seen in vivo. Rather, a heterogeneous mixture of macrophages is found in the tumor microenvironment, representing a continuum between M1 and M2 cells [69,70]. Determining the net immunosuppressive effect of these macrophages is difficult, but the amount of select immunosuppressive molecules made by these cells may be suggestive of their role in evading anti-neoplastic immunity.

One such signature molecule is IL-10, which is considered to be one of the most potent immunosuppressive cytokines [71]. In fact, IL-10 production by tumor-associated macrophages in various cancers has been shown to correlate with disease progression and decreased survival [63,72]. Moreover, intra-tumoral IL-10 expression has been demonstrated to correspond with invasion depth and the metastatic potential of primary melanoma cells, while an increased serum level of IL-10 seems to render poor prognosis in advanced melanoma patients [48,73]. Therefore, we decided to study IL-10 secretion as our primary read-out of macrophage function in the presence of MAFs.

To examine the increase of IL-10 production of macrophages triggered by MAFs, we decided to utilize a modified co-culture system that we previously developed in our previous studies focused on bone marrow-derived stromal cells (BMSCs) – macrophage interactions [52,74] to quantify the immunosuppressive potential of macrophages. The responder cells in this model can be either a macrophage cell line, such as THP-1 cells, or primary macrophages. THP-1 cells are readily available and easy to culture [75], providing a robust system to test our hypothesis. This is why we chose them to use in our experiments. As expected, the presence of MAFs resulted in a marked increase in macrophage IL-10 secretion. This held true for both monocytoid and uncommitted macrophage-type THP-1 cells as well as M1- and M2-polarized THP-1, although in case of M1-like polarized THP-1 cells, the increase was not statistically significant. These data suggest that MAFs are capable of influencing all stages of monocyte/macrophage development. CAFs secrete various chemokines such as MCP-1 and SDF-1 and are able to recruit monocytes to the tumor microenvironment [50]. Once in the cancer stroma, CAFs can directly interact with monocytes and instruct them to adopt a pro-tumorigenic, immunosuppressive phenotype, partly by inducing their IL-10 secretion. After these monocytes have committed to become tumor-associated macrophages, MAFs can continue to influence their behavior and promote IL-10 secretion in their unpolarized M0 and more committed M1 and M2 states as well.

One of the shortcomings of the above model is that MAF-macrophage interactions are studied outside of the context of melanoma. To address this issue, we repeated our experiments using MAFs previously exposed to melanoma. Prior exposure to primary or cell line-derived melanoma cells greatly promoted the MAFs IL-10 increasing ability. Our experiments showed that MAFs when co-cultured with melanoma cells are not capable to increase the tumor survival against the BRAF inhibitor therapy. In our cytotoxicity assay, melanoma monocultures had better viability than those tumor cells co-cultured with MAFs. This suggests that MAFs do not interfere directly with melanoma therapies and need further mechanisms to increase tumor survival. And so, interestingly when MAFs were preconditioned with chemotherapy-treated melanoma cells, the IL-10 increasing ability of

MAFs were enhanced. These observations imply that melanoma cells communicate with MAFs and facilitate their tumor-protective role in steady state and, even more so, under stress. The communication appears to be bidirectional. Once MAFs sense local danger signals and stress-induced melanoma molecules they can confer protection against chemotherapeutic agents and immune recognition via various mechanisms. These may include production of soluble factors such as HGF or neuregulin-1 that protect against chemo-therapeutic drugs [76], or upregulation of programmed death ligands (PD-Ls) via the CXCL5/CXCR2 pathway that facilitate immune evasion [77]. Our results shed light on a possible new protective MAF-initiated pathway, governed by macrophage-derived IL-10. Once IL-10 is secreted, it has complex effect on cancer growth. It has been shown to directly support melanoma proliferation, stimulate angiogenesis, and suppress anti-tumor immune responses [78,79].

The degree of immunosuppression exerted by MAFs may differ greatly in individual patients. Capturing these differences is challenging, but our ex vivo co-culture system may offer a possible tool to predict the immunosuppressive ability of these cells. Our preliminary data show that MAFs derived from thicker melanomas are more immunosuppressive than MAFs obtained from thinner melanomas. This observation is in line with other studies demonstrating increased overall IL-10 expression in thicker melanomas [80,81]. Although our findings are limited by the small number of cases we could examine, if validated by larger studies, our assay may serve as an ex vivo tool to measure the immunosuppressive capacity of MAFs in patients. This could ultimately help predict disease prognosis and potential response to various targeted molecular and immunomodulatory treatments.

The communication between various stromal fibroblast types such as BMSCs and immune cells—mostly T lymphocytes—has been studied extensively. There are several molecular pathways that have been proposed to play an important role in mediating these interactions. The role of cyclooxygenase and nitric oxide pathway has been implicated in BMSC lymphocyte/macrophage interactions in murine models, while the IDO pathway was found to be critical in human BMSC/lymphocyte interactions [82–85]. Similarly, the same

molecules have been implicated before in orchestrating a cancer-supportive microenvironment [86,87].

The COX1 and COX2 enzymes are both capable of making prostaglandins such as PGE2, PGF2, or prostacyclins [88]. COX1 is expressed ubiquitously, while the expression of COX2 is inducible under inflammatory conditions or in cancers [89]. The role of COX2 in melanoma has been suggested by various studies [90–93]. COX2 expression in melanoma cells seems to correlate with invasion depth, and the role of COX2 has been also implicated in tumor angiogenesis, BRAF resistance, and immune evasion during check-point inhibitor therapy [94,95].

IDO is another key immunoregulatory molecule expressed in melanoma [96,97]. Its enzymatic function converts the amino acid tryptophane into kynurenin, which in turn inhibits cytotoxic $CD8^+$ T cells and NK cells and helps recruit immunosuppressive regulatory T cells and myeloid-derived suppressor cells into the tumor microenvironment [98,99]. Intriguingly, it has been recently shown that PGE2 drives the expression of IDO in human melanoma cells, and inhibition of COX2 results in immune destruction of IDO-expressing tumor cells [100].

Last but not least, the iNOS pathway has been recently reported to support melanoma growth via the upregulation of the oncogenic PI3K-AKT pathway, and increased intra-tumoral iNOS activity has also been linked to poor outcomes in melanoma patients [101,102].

During our examinations, we interrogated all three above pathways and found that intact function of cyclooxygenases are critical in the immunomodulatory effect elicited by MAFs using THP-1 cells. Blocking the iNOS and IDO pathways, on the other hand, seemed to have no bearing on the MAF-mediated IL-10 increase of THP-1 macrophages. Although not part of this dissertation, experiments of our team using primary macrophages instead of THP-1 cells showed a critical role of IDO pathway in IL-10 increasing ability of MAFs as well. Although the idea to target CAFs has been around for decades, CAF-specific therapies have not yet led to a breakthrough [103–105]. This is mainly because there are too

many similarities between normal tissue fibroblasts residing in various organs and CAFs, recruited by cancers. An alternative approach could be to identify molecular pathways that are involved in multiple oncogenic processes, including cancer proliferation, angiogenesis, and CAF-mediated support of cancer cells. The more mechanisms we find that depend on a certain unique molecular pathway, the higher the likelihood that antagonizing this master regulatory pathway may be therapeutic as a monotherapy or together with other targeted molecular or immunomodulatory treatments. Our data add an important piece to the puzzle of the complex picture of melanoma biology. The fact that MAF/macrophage interactions are driven by the cyclooxygenase pathway may boost the efforts to repurpose already existing COX inhibitors to treat melanoma patients.

DOI:10.14753/SE.2023.2733

6 Conclusions

To our best knowledge, this is the first study that demonstrates complex interactions between MAFs, macrophages and melanoma cells culminating in increased macrophage-derived IL-10 production.

1. Using combined immunostainings, we demonstrated that FAP-positive MAFs are surrounded by IBA-1-positive macrophages in melanoma stroma, suggesting that these two cell types are indeed in close contact in TME.
2. Utilizing MAF/macrophage co-culture assays, we found that IL-10 secretion in co-cultures was significantly higher than that of macrophage monocultures. IL-10 concentration was dependent on MAF cell count in the co-culture and it was highest at 24 hours of co-culture incubation time. MAFs increased IL-10 production in various phenotypes of THP-1 cells robustly, such as in monocytoid, M0-like macrophage and M2-like macrophage phenotypes.
3. When IL-10 increasing capacity of MAFs from different patient samples were compared to the well-known melanoma clinicopathological factors, we found out that MAFs isolated from thicker melanomas (Breslow depth: >2mm) caused a higher IL-10 output than those from thinner melanomas.
4. Conditioned media derived from melanoma cells enhanced the IL-10 increasing ability of MAFs, which was further facilitated when melanoma cells were pretreated with BRAF/MEK inhibitors and chemotherapy.
5. Incubation of BRAF mutated melanoma cells with MAFs did not result in a better viability of tumor cells against treatment with BRAF inhibitors.
6. Inhibitors of COX1 and COX2 enzymes hampered the cross-talk between MAFs and THP-1 macrophages resulting in a decrease of IL-10 concentration in co-cultures. Inhibitors of IDO and iNOS pathways did not affect the cross-talk and accordingly the IL-10 concentration of co-cultures did not change in comparison to the macrophage mono-cultures.
7. In our experiments, MAFs were shown to play an important role in regulating macrophage functions, promoting a pro-tumorigenic, IL-10-rich environment.

DOI:10.14753/SE.2023.2733

MAFs in the presence of macrophages may help us to better understand the role of stromal microenvironment in fostering tumor-immune privilege, and new data can ultimately lead to the development of novel prognostic tools and innovative therapies.

DOI:10.14753/SE.2023.2733

7 Summary

Melanoma is the deadliest skin cancer. Although the overall prognosis of melanoma became better in the past decade with the new targeted and immunotherapies, the prognosis of metastatic melanoma remained poor. Extensive research has been done focusing not only on the melanoma tumor cells but also on the other elements of tumor microenvironment. MAFs are integral parts of tumor microenvironment, providing a protective network for melanoma cells.

The aim of this thesis was first to elucidate the interactions between MAFs, melanoma cells and macrophages, second to investigate the immunosuppressive effects of MAFs in this context.

Using immunohistochemistry, we showed that MAFs and macrophages are in intimate contact within the tumor stroma. We then demonstrated that MAFs alone do not secrete IL-10, a potent immunosuppressive cytokine, but they are indeed powerful inducers of IL-10 production in various macrophage types in vitro. We found out that MAFs could not increase the viability of melanoma cells directly when MAF/melanoma co-cultures were treated with BRAF inhibitors, but IL-10 increasing capability of MAFs is greatly augmented by the presence of treatment-naïve and chemotherapy-treated melanoma cells. MAFs derived from thick melanomas (Breslow thickness >2mm) appear to be more immunosuppressive than those isolated from thinner melanomas. The IL-10 increasing effect of MAFs is mediated, at least in part, by cyclooxygenases, as inhibiting the cyclooxygenase 1 and 2 enzymes hampered the IL-10 increasing ability of MAFs in MAF-macrophage co-cultures.

The results showed in this thesis indicate that MAF-induced IL-10 production in macrophages may contribute to melanoma aggressiveness, and targeting the cyclooxygenase pathway may abolish MAF–macrophage interactions and decrease levels of immunosuppressive IL-10. Further research is needed to investigate other elements of cross-talk between MAFs and cells of immune system.

8 References

1. Heppt MV, Wang JX, Hristova DM, Wei Z, Li L, Evans B, Beqiri M, Zaman S, Zhang J, Irmler M, Berking C, Besch R, Beckers J, Rauscher FJ, Sturm RA, Fisher DE, Herlyn M, Fukunaga-Kalabis M. MSX1-Induced Neural Crest-Like Reprogramming Promotes Melanoma Progression. J Invest Dermatol. 2018 Jan 1;138(1):141–149.

2. Bailey CM, Morrison JA, Kulesa PM. Melanoma revives an embryonic migration program to promote plasticity and invasion. Pigment Cell Melanoma Res. 2012 Sep;25(5):573–583.

3. Schadendorf D, Fisher DE, Garbe C, Gershenwald JE, Grob JJ, Halpern A, Herlyn M, Marchetti MA, McArthur G, Ribas A, Roesch A, Hauschild A. Melanoma. Nat Rev Dis Primer. 2015 Apr 23;1(1):1–20.

4. Cancer today [Internet]. [cited 2020 Oct 19]. Available from: http://gco.iarc.fr/today/home

5. Melanoma of the Skin - Cancer Stat Facts [Internet]. SEER. [cited 2020 Oct 19]. Available from: https://seer.cancer.gov/statfacts/html/melan.html

6. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011 Mar 4;144(5):646–674.

7. Quezada SA, Peggs KS, Simpson TR, Allison JP. Shifting the equilibrium in cancer immunoediting: from tumor tolerance to eradication. Immunol Rev. 2011 May;241(1):104–118.

8. Koikawa K, Kibe S, Suizu F, Sekino N, Kim N, Manz TD, Pinch BJ, Akshinthala D, Verma A, Gaglia G, Nezu Y, Ke S, Qiu C, Ohuchida K, Oda Y, Lee TH, Wegiel B, Clohessy JG, London N, Santagata S, Wulf GM, Hidalgo M, Muthuswamy SK, Nakamura M, Gray NS, Zhou XZ, Lu KP. Targeting Pin1 renders pancreatic cancer

eradicable by synergizing with immunochemotherapy. Cell. 2021 Sep 2;184(18):4753-4771.e27.

9. Kubo N, Araki K, Kuwano H, Shirabe K. Cancer-associated fibroblasts in hepatocellular carcinoma. World J Gastroenterol. 2016 Aug 14;22(30):6841–6850.

10. Yuan Y, Jiang YC, Sun CK, Chen QM. Role of the tumor microenvironment in tumor progression and the clinical applications (Review). Oncol Rep. 2016 May;35(5):2499–2515.

11. Hu B, Wu Z, Jin H, Hashimoto N, Liu T, Phan SH. CCAAT/enhancer-binding protein beta isoforms and the regulation of alpha-smooth muscle actin gene expression by IL-1 beta. J Immunol Baltim Md 1950. 2004 Oct 1;173(7):4661–4668.

12. Shiga K, Hara M, Nagasaki T, Sato T, Takahashi H, Takeyama H. Cancer-Associated Fibroblasts: Their Characteristics and Their Roles in Tumor Growth. Cancers. 2015 Dec 11;7(4):2443–2458.

13. Cornil I, Theodorescu D, Man S, Herlyn M, Jambrosic J, Kerbel RS. Fibroblast cell interactions with human melanoma cells affect tumor cell growth as a function of tumor progression. Proc Natl Acad Sci U S A. 1991 Jul 15;88(14):6028–6032.

14. Jobe NP, Rösel D, Dvořánková B, Kodet O, Lacina L, Mateu R, Smetana K, Brábek J. Simultaneous blocking of IL-6 and IL-8 is sufficient to fully inhibit CAF-induced human melanoma cell invasiveness. Histochem Cell Biol. 2016 Aug 1;146(2):205–217.

15. 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 J Int Soc Matrix Biol. 2018 Dec;74:52–61.

16. Zhou L, Yang K, Wickett RR, Kadekaro AL, Zhang Y. Targeted deactivation of cancer-associated fibroblasts by β-catenin ablation suppresses melanoma growth. Tumour Biol J Int Soc Oncodevelopmental Biol Med. 2016 Oct;37(10):14235–14248.

17. Straussman R, Morikawa T, Shee K, Barzily-Rokni M, Qian ZR, Du J, Davis A, Mongare MM, Gould J, Frederick DT, Cooper ZA, Chapman PB, Solit DB, Ribas A, Lo RS, Flaherty KT, Ogino S, Wargo JA, Golub TR. Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. Nature. 2012 Jul 26;487(7408):500–504.

18. Capparelli C, Rosenbaum S, Berger AC, Aplin AE. Fibroblast-derived neuregulin 1 promotes compensatory ErbB3 receptor signaling in mutant BRAF melanoma. J Biol Chem. 2015 Oct 2;290(40):24267–24277.

19. Fedorenko IV, Wargo JA, Flaherty KT, Messina JL, Smalley KSM. BRAF Inhibition Generates a Host-Tumor Niche that Mediates Therapeutic Escape. J Invest Dermatol. 2015 Dec;135(12):3115–3124.

20. 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. 2017 Mar 21;8(12):19780–19794.

21. Monteran L, Erez N. The Dark Side of Fibroblasts: Cancer-Associated Fibroblasts as Mediators of Immunosuppression in the Tumor Microenvironment. Front Immunol [Internet]. 2019 Aug 2 [cited 2020 Oct 20];10. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6688105/

22. Mhaidly R, Mechta-Grigoriou F. Fibroblast heterogeneity in tumor micro-environment: Role in immunosuppression and new therapies. Semin Immunol. 2020 Apr;48:101417.

23. Mace TA, Ameen Z, Collins A, Wojcik S, Mair M, Young GS, Fuchs JR, Eubank TD, Frankel WL, Bekaii-Saab T, Bloomston M, Lesinski GB. Pancreatic cancer-associated stellate cells promote differentiation of myeloid-derived suppressor cells in a STAT3-dependent manner. Cancer Res. 2013 May 15;73(10):3007–3018.

24. Costa A, Kieffer Y, Scholer-Dahirel A, Pelon F, Bourachot B, Cardon M, Sirven P, Magagna I, Fuhrmann L, Bernard C, Bonneau C, Kondratova M, Kuperstein I, Zinovyev A, Givel AM, Parrini MC, Soumelis V, Vincent-Salomon A, Mechta-Grigoriou F. Fibroblast Heterogeneity and Immunosuppressive Environment in Human Breast Cancer. Cancer Cell. 2018 Mar 12;33(3):463-479.e10.

25. Kuen J, Darowski D, Kluge T, Majety M. Pancreatic cancer cell/fibroblast co-culture induces M2 like macrophages that influence therapeutic response in a 3D model. PloS One. 2017;12(7):e0182039.

26. Comito G, Giannoni E, Segura CP, Barcellos-de-Souza P, Raspollini MR, Baroni G, Lanciotti M, Serni S, Chiarugi P. Cancer-associated fibroblasts and M2-polarized macrophages synergize during prostate carcinoma progression. Oncogene. 2014 May;33(19):2423–2431.

27. Freeman P, Mielgo A. Cancer-Associated Fibroblast Mediated Inhibition of CD8+ Cytotoxic T Cell Accumulation in Tumours: Mechanisms and Therapeutic Opportunities. Cancers. 2020 Sep 21;12(9):E2687.

28. Li T, Yang Y, Hua X, Wang G, Liu W, Jia C, Tai Y, Zhang Q, Chen G. Hepatocellular carcinoma-associated fibroblasts trigger NK cell dysfunction via PGE2 and IDO. Cancer Lett. 2012 May 28;318(2):154–161.

29. Balsamo M, Scordamaglia F, Pietra G, Manzini C, Cantoni C, Boitano M, Queirolo P, Vermi W, Facchetti F, Moretta A, Moretta L, Mingari MC, Vitale M. Melanoma-associated fibroblasts modulate NK cell phenotype and antitumor cytotoxicity. Proc Natl Acad Sci. 2009 Dec 8;106(49):20847–20852.

30. Érsek B, Silló P, Cakir U, Molnár V, Bencsik A, Mayer B, Mezey E, Kárpáti S, Pós Z, Németh K. Melanoma-associated fibroblasts impair CD8+ T cell function and modify expression of immune checkpoint regulators via increased arginase activity. Cell Mol Life Sci CMLS. 2020 Apr 23;

31. Ziani L, Buart S, Chouaib S, Thiery J. Hypoxia increases melanoma-associated fibroblasts immunosuppressive potential and inhibitory effect on T cell-mediated cytotoxicity. Oncoimmunology. 2021;10(1):1950953.

32. Sica A, Erreni M, Allavena P, Porta C. Macrophage polarization in pathology. Cell Mol Life Sci CMLS. 2015 Nov;72(21):4111–4126.

33. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol. 2005 Dec;5(12):953–964.

34. Bashir S, Sharma Y, Elahi A, Khan F. Macrophage polarization: the link between inflammation and related diseases. Inflamm Res Off J Eur Histamine Res Soc Al. 2016 Jan;65(1):1–11.

35. Arvanitakis K, Koletsa T, Mitroulis I, Germanidis G. Tumor-Associated Macrophages in Hepatocellular Carcinoma Pathogenesis, Prognosis and Therapy. Cancers. 2022 Jan 4;14(1):226.

36. Xiao M, He J, Yin L, Chen X, Zu X, Shen Y. Tumor-Associated Macrophages: Critical Players in Drug Resistance of Breast Cancer. Front Immunol. 2021;12:799428.

37. Wang X, Wu Y, Gu J, Xu J. Tumor-associated macrophages in lung carcinoma: From mechanism to therapy. Pathol Res Pract. 2021 Dec 18;229:153747.

38. Liu H, Yang L, Qi M, Zhang J. NFAT1 enhances the effects of tumor-associated macrophages on promoting malignant melanoma growth and metastasis. Biosci Rep. 2018 Dec 21;38(6):BSR20181604.

39. Sato T, Terai M, Tamura Y, Alexeev V, Mastrangelo MJ, Selvan SR. Interleukin 10 in the tumor microenvironment: a target for anticancer immunotherapy. Immunol Res. 2011 Dec 1;51(2):170–182.

40. Mannino MH, Zhu Z, Xiao H, Bai Q, Wakefield MR, Fang Y. The paradoxical role of IL-10 in immunity and cancer. Cancer Lett. 2015 Oct 28;367(2):103–107.

41. Seo N, Hayakawa S, Tokura Y. Mechanisms of immune privilege for tumor cells by regulatory cytokines produced by innate and acquired immune cells. Semin Cancer Biol. 2002 Ağustos;12(4):291–300.

42. Grazia Roncarolo M, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K, Levings MK. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. Immunol Rev. 2006;212(1):28–50.

43. Pisa P, Halapi E, Pisa EK, Gerdin E, Hising C, Bucht A, Gerdin B, Kiessling R. Selective expression of interleukin 10, interferon gamma, and granulocyte-macrophage colony-stimulating factor in ovarian cancer biopsies. Proc Natl Acad Sci U S A. 1992 Aug 15;89(16):7708–7712.

44. Venetsanakos E, Beckman I, Bradley J, Skinner JM. High incidence of interleukin 10 mRNA but not interleukin 2 mRNA detected in human breast tumours. Br J Cancer. 1997;75(12):1826–1830.

45. Knoefel B, Nuske K, Steiner T, Junker K, Kosmehl H, Rebstock K, Reinhold D, Junker U. Renal cell carcinomas produce IL-6, IL-10, IL-11, and TGF-beta 1 in primary cultures and modulate T lymphocyte blast transformation. J Interferon Cytokine Res Off J Int Soc Interferon Cytokine Res. 1997 Feb;17(2):95–102.

46. Huang M, Wang J, Lee P, Sharma S, Mao JT, Meissner H, Uyemura K, Modlin R, Wollman J, Dubinett SM. Human non-small cell lung cancer cells express a type 2 cytokine pattern. Cancer Res. 1995 Sep 1;55(17):3847–3853.

47. Kim J, Modlin RL, Moy RL, Dubinett SM, McHugh T, Nickoloff BJ, Uyemura K. IL-10 production in cutaneous basal and squamous cell carcinomas. A mechanism for evading the local T cell immune response. J Immunol Baltim Md 1950. 1995 Aug 15;155(4):2240–2247.

48. Dummer W, Becker JC, Schwaaf A, Leverkus M, Moll T, Bröcker EB. Elevated serum levels of interleukin-10 in patients with metastatic malignant melanoma. Melanoma Res. 1995 Feb;5(1):67–68.

49. Mazur A, Holthoff E, Vadali S, Kelly T, Post SR. Cleavage of Type I Collagen by Fibroblast Activation Protein-α Enhances Class A Scavenger Receptor Mediated Macrophage Adhesion. PloS One. 2016;11(3):e0150287.

50. Gok Yavuz B, Gunaydin G, Gedik ME, Kosemehmetoglu K, Karakoc D, Ozgur F, Guc D. Cancer associated fibroblasts sculpt tumour microenvironment by recruiting monocytes and inducing immunosuppressive PD-1+ TAMs. Sci Rep. 2019 Feb 28;9(1):3172.

51. Zhang Q, Chai S, Wang W, Wan C, Zhang F, Li Y, Wang F. Macrophages activate mesenchymal stem cells to acquire cancer-associated fibroblast-like features resulting in gastric epithelial cell lesions and malignant transformation in vitro. Oncol Lett. 2019 Jan;17(1):747–756.

52. Németh K, Leelahavanichkul A, Yuen PST, Mayer B, Parmelee A, Doi K, Robey PG, Leelahavanichkul K, Koller BH, Brown JM, Hu X, Jelinek I, Star RA, Mezey E. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. Nat Med. 2009 Jan;15(1):42–49.

53. Borriello L, Nakata R, Sheard MA, Fernandez GE, Sposto R, Malvar J, Blavier L, Shimada H, Asgharzadeh S, Seeger RC, DeClerck YA. Cancer-Associated

Fibroblasts Share Characteristics and Protumorigenic Activity with Mesenchymal Stromal Cells. Cancer Res. 2017 Sep 15;77(18):5142–5157.

54. Raaijmakers MIG, Widmer DS, Maudrich M, Koch T, Langer A, Flace A, Schnyder C, Dummer R, Levesque MP. A new live-cell biobank workflow efficiently recovers heterogeneous melanoma cells from native biopsies. Exp Dermatol. 2015 May;24(5):377–380.

55. Genin M, Clement F, Fattaccioli A, Raes M, Michiels C. M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide. BMC Cancer. 2015 Aug 8;15(1):577.

56. Rádai Z, Windt T, Nagy V, Füredi A, Kiss NZ, Ranđelović I, Tóvári J, Keglevich G, Szakács G, Tóth S. Synthesis and anticancer cytotoxicity with structural context of an α-hydroxyphosphonate based compound library derived from substituted benzaldehydes. New J Chem. 2019 Sep 9;43(35):14028–14035.

57. Windt T, Tóth S, Patik I, Sessler J, Kucsma N, Szepesi Á, Zdrazil B, Özvegy-Laczka C, Szakács G. Identification of anticancer OATP2B1 substrates by an in vitro triple-fluorescence-based cytotoxicity screen. Arch Toxicol. 2019 Apr;93(4):953–964.

58. Jackson MV, Morrison TJ, Doherty DF, McAuley DF, Matthay MA, Kissenpfennig A, O'Kane CM, Krasnodembskaya AD. Mitochondrial Transfer via Tunneling Nanotubes is an Important Mechanism by Which Mesenchymal Stem Cells Enhance Macrophage Phagocytosis in the In Vitro and In Vivo Models of ARDS. Stem Cells Dayt Ohio. 2016 Aug;34(8):2210–2223.

59. Maximov V, Chen Z, Wei Y, Robinson MH, Herting CJ, Shanmugam NS, Rudneva VA, Goldsmith KC, MacDonald TJ, Northcott PA, Hambardzumyan D, Kenney AM. Tumour-associated macrophages exhibit anti-tumoural properties in Sonic Hedgehog medulloblastoma. Nat Commun. 2019 Jun 3;10(1):2410.

60. Puré E, Blomberg R. Pro-tumorigenic roles of fibroblast activation protein in cancer: back to the basics. Oncogene. 2018 Aug;37(32):4343–4357.

61. Çakır U, Hajdara A, Széky B, Mayer B, Kárpáti S, Mezey É, Silló P, Szakács G, Füredi A, Pós Z, Érsek B, Sárdy M, Németh K. Mesenchymal-Stromal Cell-like Melanoma-Associated Fibroblasts Increase IL-10 Production by Macrophages in a Cyclooxygenase/Indoleamine 2,3-Dioxygenase-Dependent Manner. Cancers. 2021 Dec 7;13(24):6173.

62. Essa AAM, Yamazaki M, Maruyama S, Abé T, Babkair H, Raghib AM, Megahed EMED, Cheng J, Saku T. Tumour-associated macrophages are recruited and differentiated in the neoplastic stroma of oral squamous cell carcinoma. Pathology (Phila). 2016 Apr;48(3):219–227.

63. Takahashi H, Sakakura K, Kudo T, Toyoda M, Kaira K, Oyama T, Chikamatsu K. Cancer-associated fibroblasts promote an immunosuppressive microenvironment through the induction and accumulation of protumoral macrophages. Oncotarget. 2017 Jan 31;8(5):8633–8647.

64. Fernandez-Garcia B, Eiro N, Miranda MA, Cid S, González LO, Domínguez F, Vizoso FJ. Prognostic significance of inflammatory factors expression by stroma from breast carcinomas. Carcinogenesis. 2016 Aug;37(8):768–776.

65. Romano V, Belviso I, Venuta A, Ruocco MR, Masone S, Aliotta F, Fiume G, Montagnani S, Avagliano A, Arcucci A. Influence of Tumor Microenvironment and Fibroblast Population Plasticity on Melanoma Growth, Therapy Resistance and Immunoescape. Int J Mol Sci. 2021 May 17;22(10):5283.

66. Somasundaram R, Herlyn M, Wagner SN. The role of tumor microenvironment in melanoma therapy resistance. Melanoma Manag. 2016 Mar;3(1):23–32.

67. Mantovani A, Sica A, Locati M. Macrophage polarization comes of age. Immunity. 2005 Oct;23(4):344–346.

68. Sica A, Larghi P, Mancino A, Rubino L, Porta C, Totaro MG, Rimoldi M, Biswas SK, Allavena P, Mantovani A. Macrophage polarization in tumour progression. Semin Cancer Biol. 2008 Oct;18(5):349–355.

69. Italiani P, Boraschi D. From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation. Front Immunol [Internet]. 2014 Oct 17 [cited 2020 Oct 19];5. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4201108/

70. Boutilier AJ, Elsawa SF. Macrophage Polarization States in the Tumor Microenvironment. Int J Mol Sci. 2021 Jun 29;22(13):6995.

71. Itakura E, Huang RR, Wen DR, Paul E, Wünsch PH, Cochran AJ. IL-10 expression by primary tumor cells correlates with melanoma progression from radial to vertical growth phase and development of metastatic competence. Mod Pathol Off J U S Can Acad Pathol Inc. 2011 Jun;24(6):801–809.

72. Chen L, Shi Y, Zhu X, Guo W, Zhang M, Che Y, Tang L, Yang X, You Q, Liu Z. IL-10 secreted by cancer-associated macrophages regulates proliferation and invasion in gastric cancer cells via c-Met/STAT3 signaling. Oncol Rep. 2019 Aug;42(2):595–604.

73. Nemunaitis J, Fong T, Shabe P, Martineau D, Ando D. Comparison of serum interleukin-10 (IL-10) levels between normal volunteers and patients with advanced melanoma. Cancer Invest. 2001;19(3):239–247.

74. McClain Caldwell I, Hogden C, Nemeth K, Boyajian M, Krepuska M, Szombath G, MacDonald S, Abshari M, Moss J, Vitale-Cross L, Fontana JR, Mezey E. Bone Marrow-Derived Mesenchymal Stromal Cells (MSCs) Modulate the Inflammatory Character of Alveolar Macrophages from Sarcoidosis Patients. J Clin Med. 2020 Jan 19;9(1):278.

75. Auwerx J. The human leukemia cell line, THP-1: a multifacetted model for the study of monocyte-macrophage differentiation. Experientia. 1991 Jan 15;47(1):22–31.

76. Cheng H, Terai M, Kageyama K, Ozaki S, McCue PA, Sato T, Aplin AE. Paracrine Effect of NRG1 and HGF Drives Resistance to MEK Inhibitors in Metastatic Uveal Melanoma. Cancer Res. 2015 Jul 1;75(13):2737–2748.

77. Li Z, Zhou J, Zhang J, Li S, Wang H, Du J. Cancer-associated fibroblasts promote PD-L1 expression in mice cancer cells via secreting CXCL5. Int J Cancer. 2019 Oct 1;145(7):1946–1957.

78. García-Hernández ML, Hernández-Pando R, Gariglio P, Berumen J. Interleukin-10 promotes B16-melanoma growth by inhibition of macrophage functions and induction of tumour and vascular cell proliferation. Immunology. 2002 Feb;105(2):231–243.

79. Wiguna AP, Walden P. Role of IL-10 and TGF-β in melanoma. Exp Dermatol. 2015 Mar;24(3):209–214.

80. Redondo P, Sánchez-Carpintero I, Bauzá A, Idoate M, Solano T, Mihm MC. Immunologic escape and angiogenesis in human malignant melanoma. J Am Acad Dermatol. 2003 Aug;49(2):255–263.

81. Howell WM, Turner SJ, Bateman AC, Theaker JM. IL-10 promoter polymorphisms influence tumour development in cutaneous malignant melanoma. Genes Immun. 2001 Feb;2(1):25–31.

82. Hegyi B, Kudlik G, Monostori E, Uher F. Activated T-cells and pro-inflammatory cytokines differentially regulate prostaglandin E2 secretion by mesenchymal stem cells. Biochem Biophys Res Commun. 2012 Mar 9;419(2):215–220.

83. English K, Barry FP, Field-Corbett CP, Mahon BP. IFN-gamma and TNF-alpha differentially regulate immunomodulation by murine mesenchymal stem cells. Immunol Lett. 2007 Jun 15;110(2):91–100.

84. Meisel R, Zibert A, Laryea M, Göbel U, Däubener W, Dilloo D. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. Blood. 2004 Jun 15;103(12):4619–4621.

85. Hinden L, Shainer R, Almogi-Hazan O, Or R. Ex Vivo Induced Regulatory Human/Murine Mesenchymal Stem Cells as Immune Modulators. Stem Cells Dayt Ohio. 2015 Jul;33(7):2256–2267.

86. Gasparri AM, Jachetti E, Colombo B, Sacchi A, Curnis F, Rizzardi GP, Traversari C, Bellone M, Corti A. Critical role of indoleamine 2,3-dioxygenase in tumor resistance to repeated treatments with targeted IFNgamma. Mol Cancer Ther. 2008 Dec;7(12):3859–3866.

87. Stolina M, Sharma S, Lin Y, Dohadwala M, Gardner B, Luo J, Zhu L, Kronenberg M, Miller PW, Portanova J, Lee JC, Dubinett SM. Specific inhibition of cyclooxygenase 2 restores antitumor reactivity by altering the balance of IL-10 and IL-12 synthesis. J Immunol Baltim Md 1950. 2000 Jan 1;164(1):361–370.

88. Vane JR, Bakhle YS, Botting RM. Cyclooxygenases 1 and 2. Annu Rev Pharmacol Toxicol. 1998;38:97–120.

89. Liu B, Qu L, Yan S. Cyclooxygenase-2 promotes tumor growth and suppresses tumor immunity. Cancer Cell Int. 2015;15:106.

90. Wang SJ, Khullar K, Kim S, Yegya-Raman N, Malhotra J, Groisberg R, Crayton SH, Silk AW, Nosher JL, Gentile MA, Mehnert JM, Jabbour SK. Effect of cyclo-oxygenase inhibitor use during checkpoint blockade immunotherapy in patients with metastatic melanoma and non-small cell lung cancer. J Immunother Cancer. 2020 Oct;8(2):e000889.

91. Vergani E, Dugo M, Cossa M, Frigerio S, Di Guardo L, Gallino G, Mattavelli I, Vergani B, Lalli L, Tamborini E, Valeri B, Gargiuli C, Shahaj E, Ferrarini M,

Ferrero E, Gomez Lira M, Huber V, Vecchio MD, Sensi M, Leone BE, Santinami M, Rivoltini L, Rodolfo M, Vallacchi V. miR-146a-5p impairs melanoma resistance to kinase inhibitors by targeting COX2 and regulating NFkB-mediated inflammatory mediators. Cell Commun Signal CCS. 2020 Sep 23;18(1):156.

92. Jafarian AH, Mohamadian Roshan N, Gharib M, Moshirahmadi V, Tasbandi A, Ayatollahi AA, Ayatollahi H. Evaluation of Cyclooxygenase-2 Expression in Association with Clinical-Pathological Factors in Malignant Melanoma. Iran J Pathol. 2019;14(2):96–103.

93. Kim SH, Roszik J, Cho SN, Ogata D, Milton DR, Peng W, Menter DG, Ekmekcioglu S, Grimm EA. The COX2 Effector Microsomal PGE2 Synthase 1 is a Regulator of Immunosuppression in Cutaneous Melanoma. Clin Cancer Res Off J Am Assoc Cancer Res. 2019 Mar 1;25(5):1650–1663.

94. Tudor DV, Bâldea I, Lupu M, Kacso T, Kutasi E, Hopârtean A, Stretea R, Gabriela Filip A. COX-2 as a potential biomarker and therapeutic target in melanoma. Cancer Biol Med. 2020 Feb 15;17(1):20–31.

95. Gipsyianti N, Aziz A, Hernowo BS, Usman HA. High Expression of COX-2 Associated with the Depth of Invasion on Acral Melanoma by Increasing TGF-β1. Clin Cosmet Investig Dermatol. 2021;14:209–216.

96. Rubel F, Kern JS, Technau-Hafsi K, Uhrich S, Thoma K, Häcker G, von Bubnoff N, Meiss F, von Bubnoff D. Indoleamine 2,3-Dioxygenase Expression in Primary Cutaneous Melanoma Correlates with Breslow Thickness and Is of Significant Prognostic Value for Progression-Free Survival. J Invest Dermatol. 2018 Mar;138(3):679–687.

97. de Lecea MV, Palomares T, Al Kassam D, Cavia M, Geh JLC, de Llano P, Muñiz P, Armesto D, Martinez-Indart L, Alonso-Varona A. Indoleamine 2,3 dioxygenase as a

prognostic and follow-up marker in melanoma. A comparative study with LDH and S100B. J Eur Acad Dermatol Venereol JEADV. 2017 Apr;31(4):636–642.

98. Prendergast GC, Malachowski WJ, Mondal A, Scherle P, Muller AJ. Indoleamine 2,3-Dioxygenase and Its Therapeutic Inhibition in Cancer. Int Rev Cell Mol Biol. 2018;336:175–203.

99. Brody JR, Costantino CL, Berger AC, Sato T, Lisanti MP, Yeo CJ, Emmons RV, Witkiewicz AK. Expression of indoleamine 2,3-dioxygenase in metastatic malignant melanoma recruits regulatory T cells to avoid immune detection and affects survival. Cell Cycle Georget Tex. 2009 Jun 15;8(12):1930–1934.

100. Hennequart M, Pilotte L, Cane S, Hoffmann D, Stroobant V, Plaen ED, Van den Eynde B. Constitutive IDO1 Expression in Human Tumors Is Driven by Cyclooxygenase-2 and Mediates Intrinsic Immune Resistance. Cancer Immunol Res. 2017 Aug;5(8):695–709.

101. Ding Z, Ogata D, Roszik J, Qin Y, Kim SH, Tetzlaff MT, Lazar AJ, Davies MA, Ekmekcioglu S, Grimm EA. iNOS Associates With Poor Survival in Melanoma: A Role for Nitric Oxide in the PI3K-AKT Pathway Stimulation and PTEN S-Nitrosylation. Front Oncol. 2021;11:631766.

102. Ekmekcioglu S, Ellerhorst J, Smid CM, Prieto VG, Munsell M, Buzaid AC, Grimm EA. Inducible nitric oxide synthase and nitrotyrosine in human metastatic melanoma tumors correlate with poor survival. Clin Cancer Res Off J Am Assoc Cancer Res. 2000 Dec;6(12):4768–4775.

103. De P, Aske J, Dey N. Cancer-Associated Fibroblast Functions as a Road-Block in Cancer Therapy. Cancers. 2021 Oct 19;13(20):5246.

104. Maia A, Wiemann S. Cancer-Associated Fibroblasts: Implications for Cancer Therapy. Cancers. 2021 Jul 14;13(14):3526.

DOI:10.14753/SE.2023.2733

105. Belhabib I, Zaghdoudi S, Lac C, Bousquet C, Jean C. Extracellular Matrices and Cancer-Associated Fibroblasts: Targets for Cancer Diagnosis and Therapy? Cancers. 2021 Jul 11;13(14):3466.

9 Bibliography of candidate's publications

Thesis-related publications:

1. **Çakır U***, Hajdara A*, Széky B*, Mayer B, Kárpáti S, Mezey É, Silló P, Szakács G, Füredi A, Pós Z, Érsek B, Sárdy M and Németh K. Mesenchymal-Stromal Cell-like Melanoma-Associated Fibroblasts Increase IL-10 Production by Macrophages in a Cyclooxygenase/Indoleamine 2,3-Dioxygenase-Dependent Manner. Cancers (Basel). 2021 Dec 7;13(24):6173.
   *shared first authorship
   IF: 6.639
2. Érsek B, Silló P, **Cakir U**, Molnár V, Bencsik A, Mayer B, Mezey E, Kárpáti S, Pós Z, Német K. Melanoma-associated fibroblasts impair CD8+ T cell function and modify expression of immune checkpoint regulators via increased arginase activity. Cellular and Molecular Life Sciences. 2020 Apr 23; 78(2) pp 661-673.
   IF: 9.261

Other publications:

1. Gil J, Kim Y, Szeitz B, Doma V, **Çakır U**, Almeida NP de, Hagemeijer YP, Guryev V, Johansson JG, Sharma Y, Parada IP, Horvath Z, Guedes J de S, Monnerat G, Carneiro GRA, Nogueira FC, Lee B, Oskolas H, Kuroli E, Hársing J, Sugihara Y, Kuras M, Appelqvist R, Wieslander E, Domont GB, Baldetorp B, Hong R, Huszty G, Vizkeleti L, Tímár J, Fenyö D, Betancourt LH, Jakobsson J, Malm J, Sanchez A, Szász AM, Horvatovich P, Rezeli M, Kárpáti S, Marko-Varga G. Proteogenomics Reveals how Metastatic Melanoma Modulates the Immune System to Allow Immune Evasion. 2021 Apr p. 2021.04.10.439245. Available from: https://www.biorxiv.org/content/10.1101/2021.04.10.439245v1
2. Betancourt LH, Gil J, Kim Y, Doma V, **Çakır U**, Sanchez A, Murillo JR, Kuras M, Parada IP, Sugihara Y, Appelqvist R, Wieslander E, Welinder C, Velasquez E, de

Almeida NP, Woldmar N, Marko-Varga M, Pawłowski K, Eriksson J, Szeitz B, Baldetorp B, Ingvar C, Olsson H, Lundgren L, Lindberg H, Oskolas H, Lee B, Berge E, Sjögren M, Eriksson C, Kim D, Kwon HJ, Knudsen B, Rezeli M, Hong R, Horvatovich P, Miliotis T, Nishimura T, Kato H, Steinfelder E, Oppermann M, Miller K, Florindi F, Zhou Q, Domont GB, Pizzatti L, Nogueira FCS, Horvath P, Szadai L, Tímár J, Kárpáti S, Szász AM, Malm J, Fenyö D, Ekedahl H, Németh IB, Marko-Varga G. The human melanoma proteome atlas—Defining the molecular pathology. Clinical and Translational Medicine. 2021;11(7):e473.

IF: 11.492

3. Betancourt LH, Gil J, Sanchez A, Doma V, Kuras M, Murillo JR, Velasquez E, **Çakır U**, Kim Y, Sugihara Y, Parada IP, Szeitz B, Appelqvist R, Wieslander E, Welinder C, de Almeida NP, Woldmar N, Marko-Varga M, Eriksson J, Pawłowski K, Baldetorp B, Ingvar C, Olsson H, Lundgren L, Lindberg H, Oskolas H, Lee B, Berge E, Sjögren M, Eriksson C, Kim D, Kwon HJ, Knudsen B, Rezeli M, Malm J, Hong R, Horvath P, Szász AM, Tímár J, Kárpáti S, Horvatovich P, Miliotis T, Nishimura T, Kato H, Steinfelder E, Oppermann M, Miller K, Florindi F, Zhou Q, Domont GB, Pizzatti L, Nogueira FCS, Szadai L, Németh IB, Ekedahl H, Fenyö D, Marko-Varga G. The Human Melanoma Proteome Atlas—Complementing the melanoma transcriptome. Clinical and Translational Medicine. 2021;11(7):e451.

   IF: 11.492

4. Baranyai F, Czirbesz K, **Cakir U,** Haller Á, Pónyai K. Successful treatment of acne scars. Bőrgyógyászati és Venerológiai Szemle. 2022;98.1. 12-23

DOI:10.14753/SE.2023.2733

10 Acknowledgments

First and foremost, I would like to thank my supervisors Dr. Krisztián Németh and Prof. Dr. Miklós Sárdy for their years of continuous support and immense patience throughout my research. I would like to express my gratitude to Prof. Dr. Miklós Sárdy, who believed in my capabilities and made my PhD. study possible at the Department of Dermatology, Venereology and Dermatooncology, and also later helped to start my residency in dermatology.

I am very grateful for guidance of Dr. Krisztián Németh throughout my PhD. years, who even from overseas, helped me day and night with countless hours of video calls and constant support to improve my research with unique ideas.

I am very grateful for my mentors in lab, Dr. Pálma Silló and Dr. Balázs Mayer who supported and taught me many procedures we used in our stem cell lab and who were always available whenever I needed help.

I owe my gratitude to Prof. Dr. Sarolta Kárpáti, who always helped with her expertise in dermatology.

I would like to thank my colleagues in Stem Cell Laboratory, Anna Hajdara and Balázs Széky. Especially Anna's help was so significant without her contribution my research would not be possible.

I would like to thank dr. Éva Mezey from the Adult Stem Cell Section of National Institutes of Dental and Craniofacial Research in National Institutes of Health who helped with immunostaining experiments.

I am grateful to Dr. Barbara Érsek and Dr. Zoltán Pós from the Department of Genetics, Cell and Immunobiology, Semmelweis University and to Dr. Gergely Szakács and Dr. András Füredi as well as Szilárd Tóth and Veronika Nagy from Drug Resistance Research Group, Institute of Enzymology, Eötvös Lóránd Research Network for their helps in experiments.

DOI:10.14753/SE.2023.2733

I would like to thank my dermatooncologist colleagues Prof. dr. Péter Holló, dr. Daniella Kuzmanovszki, dr. Béla Tóth, dr. Veronika Tóth, dr. József Szakonyi for providing patient-related clinical data, and also I would like to thank my dermatopathologist colleagues dr. Judit Hársing, dr. Enikő Kuroli, dr. Anita Mohos for making histopathological properties of samples I used in my research available. Furthermore, I owe my gratitude to dr. Gyula Bottlik, who helped with sample collection in the operation room.

I am thankful for the support of all my colleagues in Department of Dermatology, Venereology and Dermatooncology, and special thanks to colleagues in Genetics Lab.

I would like to thank Prof. Dr. György Markó-Varga and colleagues from European Cancer Moonshot Lund Center of Lund University.

Furthermore, I owe my gratitude to the Hungarian National Research, Development and Innovation Office, Semmelweis University Dean's Office, National Institutes of Dental and Craniofacial Research group and Nékám Association for funding my research.

Last but not least, grateful thanks to my family and friends for the endless encouragement and help throughout the years of my PhD. studies.