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ISOLATION, CHARACTERIZATION AND FUNCTIONAL EXAMINATION OF MELANOMA ASSOCIATED FIBROBLASTS

PhD thesis

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List of Abbreviations

α-SMA alpha-smooth muscle actin

BEC (S-(2-Boronoethyl)-L-cysteine) hydrochloride

BM Basal medium

BMC bone-marrow derived cell

BTLA B- and T-lymphocyte attenuator

CAF cancer associated fibroblast

CM conditioned media

CTL Cytotoxic T lymphocyte

DF normal dermal fibroblast

DiD (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt)

DiL (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate)

DMEM Dulbecco's Modified Eagle Medium

ECM extracellular matrix

EGF epidermal growth factor

FGF fibroblast growth factor

FAP fibroblast-activating protein

FBS fetal bovine serum

FSP-1 fibroblast-specific protein 1

gzmB granzyme B

HGF hepatocyte growth factor

HVEM herpesvirus entry mediator

IC isotype control

ICR immune checkpoint receptor

IDO Indoleamine-2,3-dioxygenase

IL Interleukin

IFN-γ Interferon gamma

LAG-3 Lymphocyte-activation gene 3

MAF Melanoma associated fibroblast

NO Nitric oxide

NOS Nitric oxide synthase

POSTN Periostin

PD-1 Programmed cell death protein 1

PD-L1 Programmed death-ligand 1

PDPN Podoplanin

PDGFRα Platelet-derived growth factor receptor alpha

PDGFRβ Platelet-derived growth factor receptor beta

PGE2 Prostaglandin E2

Runx3 Runt-related transcription factor 3

TCR T-cell receptor

TGF-β Transforming growth factor beta

TIGIT T cell immunoreceptor with Ig and ITIM domains

TIL Tumor-infiltrating lymphocytes

TIM-3 T cell immunoglobulin and mucin-domain containing-3

TNFα Tumor necrosis factor alpha

VEGF Vascular endothelial-derived growth factor

VISTA V-domain Ig suppressor of T cell activation

1. Introduction (with the scientific background and the relevant literature)

1.1 Tumor microenvironment: definition, cell types and their function

Cancer is a complex unorganized tissue with a dynamic structure. The tumor stroma provides a continually changing environment with pro-, and antitumorigenic effects[1]. It consists of variety of cells, such as immune cells, fibroblasts, endothelial cells, pericytes, adipocytes, and also signaling molecules and extracellular matrix[2].
Communication between the cells of the tumor microenvironment and the cancer cells through soluble factors, exosomes and cell fusion are in dynamic evolution. Transcriptional reprogramming, transition of stromal cells and the changes in the composition in response to oncogenic signals are essential in tumor initiation, growth, progression, metastases and therapeutic resistance[3][4].
The stroma derived cytokines, chemokines and exosomes promote tumor malignancy through local and systemic signals. Largely dependent on these secreted factors bone-marrow derived cells (BMCs) are mobilized to home the tumor and promote progression, invasion, metastasis and therapy resistance. Tumor-activated BMCs also prepare pre-metastatic niche at a distance[5]. Myeloid-derived suppressor cells suppress immun responses against the tumor antigens. Some of the tumor signals act on immune cells and polarize them toward antitumorigenic (Th1) or protumorigenic (Th2) phenotypes[6]. Recruited macrophages increase angiogeneseis, intravasation and invasion of tumor cells. Nonimmun mesenchymal cells respond not only to oncogenic signals, but also communicate with each other providing an evolving environment and contributing to tumor heterogeneity[7].

1.2 Cancer fibroblasts

Cancer associated fibroblasts (CAFs) are a heterogenous cell population of activated fibroblasts associated with cancer cells at all stages of tumor progression with potent effects on tumorigenesis[8].

They have been called carcinoma-associated fibroblasts[9], cancer associated myofibroblasts[10], peritumoral fibroblasts, reactive stromal fibroblasts and tumour-associated fibroblasts since the 1970s[11].

They are elongated, blast-like cells with spindle-shaped morphology, with an increased proliferation rate, enhanced collagen production and migratory behaviour in vitro with the ability to adhere to plastic. CAFs have a similar phenotype with extraordinary plasticity to fibroblasts associated with inflammatory conditions or wound healing[11]. As a sign of their activation they express myofibroblast markers, such as the prototypical marker alpha-smooth muscle actin (α-SMA), fibroblast-activating protein (FAP) and vimentin. They also express the fibroblast marker fibroblast-specific protein 1 (FSP-1), and other markers such as neuronglial antigen-2, Thy-1 (CD90), tenascin (TN)-C, periostin (POSTN), paladin, podoplanin (PDPN), platelet-derived growth factor receptor alpha (PDGFRα) and platelet-derived growth factor receptor beta (PDGFRβ). Currently there is *no exclusive marker* that defines CAFs. They may express all of these markers, or only a subset depending the sources and the different stages of activation[12].

The presence of mesenchymal markers, such as α-SMA, vimentin and absence of epithelial (cytokeratin, E-cadherin) and endothelial (CD31) markers have been used to define CAFs[13].

CAFs secrete a variety of cytokines (eg, interleukin (IL)-1, IL-6 and IL-8), chemokines (eg, CXCL12, CXCL14, and CCL7), and growth factors (ie, vascular endothelial-derived growth factor (VEGF), transforming growth factor beta (TGF-β), hepatocyte growth factor (HGF), epidermal growth factor (EGF), or fibroblast growth factor (FGF)). As a major source of extracellular matrix (ECM) proteins, they secrete collagens and proteoglycans (ie, fibronectin, laminin, TN). CAFs are capable to remodel the stroma through increased secretion of matrix metalloproteinases. [11][12] [14]

This CAF phenotype can persist in vitro, in cell culture without any further oncogenic stimuli[15].

Stromal fibroblasts in cancers are recruited from different sources. Depending on the cell of origin, on site-specific properties and on the prevalent microenvironment they display

heterogenous subpopulations.

Activation/differentiation of CAFs is mainly mediated by TGF-β1. Other growth factors (ie, PDGF, EGF, FGF), cytokines (ie. IL-6) and even epigenetic regulators (ie, miRNAs and oxidative stress) also may take part in this process[11][16][17][18].

The most immediate source of CAFs are local stromal fibroblasts and tissue-resident fibroblast precursor cells.

Differentiated cells within the tumor, such as epithelial cells, endothelial cells, smooth muscle cells, stellate cells, pericytes and adipocytes may transdifferentiate to give rise to CAFs.

BMCs are another important cell type, which not only contribute to CAF population by itself, but also attract local fibroblasts to differentiate into CAFs[5][19][20].

1.3 Melanoma associated fibroblasts

CAF can be found in virtually all solid tumors; in some of them, they are present in very high numbers predominating the stroma (e.g. ovarian cancer), while in others they are less abundant (e.g. melanoma). In melanoma, it is well documented that CAF (melanoma associated fibroblasts, MAF) are rather capable suppressors of NK cell activity via multiple ways, including PGE2-dependent supression[21][22][23], and it seems that this phenomenon is probably not restricted to melanoma [24][25]. Interestingly, it has been shown that activated effector CD8+ T cells display the prostaglandin receptors involved in MAF-mediated NK cell suppression. Also, CD8+ T cells exhibit decreased interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα) and IL-2 production and impaired survival upon exposure to PGE2 [26]. However, it has not yet been analyzed whether MAF could influence CD8+ CTL responses via PGE2, via the release of immunosuppressive cytokines, metabolic reprogramming, immune checkpoint modulation, or other ways of immunosuppression. Considering that i) CD8+ T cells play

a pivotal role in melanoma rejection, ii) state-of-the art melanoma therapies may achieve partial to complete patient responses by mobilizing CTLs, and iii) factors contributing to therapy failure remain elusive, in this study, we sought to analyze possible parallels between MAF action on NK cells and CTLs, focusing on the latter[27][28].

2. Objectives

A growing body of evidence suggests that the cancer microenvironment plays a critical role in anti-tumor immunity during cancer development and immunotherapies targeting check-point blockade. Although melanomas are one of the most promising targets for these therapies, studies looking at interactions between melanoma-associated fibroblasts (MAFs) and CD8+ T cells have not been described.

The aim of our study was to

I. Develop a proper isolation technique for CAFs from surgically resected primery melanomas or cutan metastasis,

II. Isolate normal dermal fibroblasts (DFs) from normal skin of the same patient.

III. Analyze the effect of MAFs on CD8+ T cell activity, and

IV. Clarify their mechanism of action.

3. *Results*

3.1 Isolation and characterization of MAFs

Skin samples were collected from thirteen melanoma patients. Fibroblasts were isolated from excised cutaneous melanomas (melanoma-associated fibroblasts, MAF) or the healthy distal edge of excised tissue (normal dermal fibroblasts, DF) by enzymatic digestion and differential adhesion[29][30]. Primary cultures of fibroblasts were extensively characterized by flow cytometry to verify their identity and exclude samples contaminated with melanoma cells. A three-marker panel for melanoma specific (melanA and gp100) and CAF markers (fibroblast-activation protein or FAP) was used (**Fig1**). Further experiments excluded MAF cultures showing >3-4% melanA or gp100 positivity (**Fig1,** marked with asterisks); two such cultures were found out of sixteen.
DFs used as controls were obtained from non-tumor intact margins (tissue >1cm from the tumor margin) using the same method. MAF and DF in the same patient were successfully isolated in nine cases. These autologous matched primary cell pairs were used for all subsequent analyses. Cells were used for five passages or less and maintained in standard Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 1% penicillin-streptomycin (P/S) and 1% L-glutamine.[27]

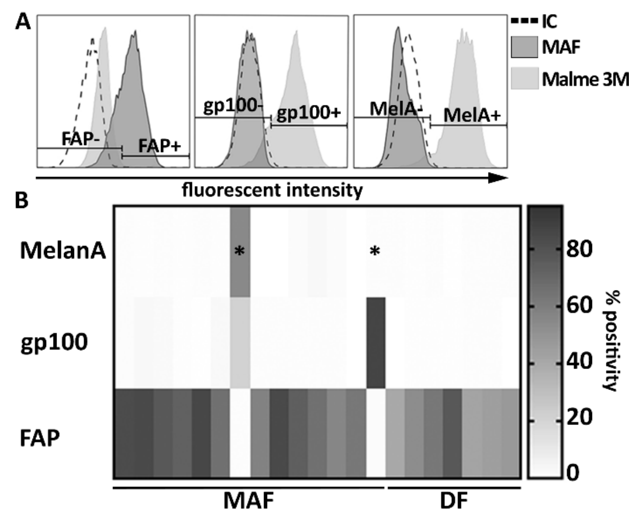

Fig1 Flow cytometric characterization of primary fibroblast cultures from excised melanoma specimens

(A) Representative flow cytometry plots showing typical staining of validated melanoma-free MAF cultures and Malme 3M melanoma cells used as reference. Specific staining for FAP-, gp100- and Melan-A antibodies is shown compared to isotype control (IC) (B) A heat plot summarizes expression patterns of all three markers, as observed on isolated MAF (n=19) and DF samples (n=11). Two of the fifteen primary MAF cultures marked with an asterisk were identified as melanoma contamination and censored. Pooled data from six independent measurements.

3.2 Early events in CD8+ T cell activation are compromised by MAF supernatants

We first tested whether the early steps of CD8+ T cell activation are affected by soluble factors released by MAF. To this end, we examined whether MAF-conditioned media (CM) affects the expression of the early T cell activation marker CD69 or the cytotoxic degranulation marker CD107a. We found that CD8+ T cells activated by anti-CD3/28 in the presence of MAF-CM displayed less CD69 than CD8+ T cells activated in the presence of DF-CM, as the frequency of CD69+ cells was reduced. Likewise, the ratio of CD69(+) vs. CD69(-) cells decreased significantly in the MAF-CM vs DF-CM groups (**Fig2A**). However, no significant differences in cell surface CD107a levels were found (not shown). [27]

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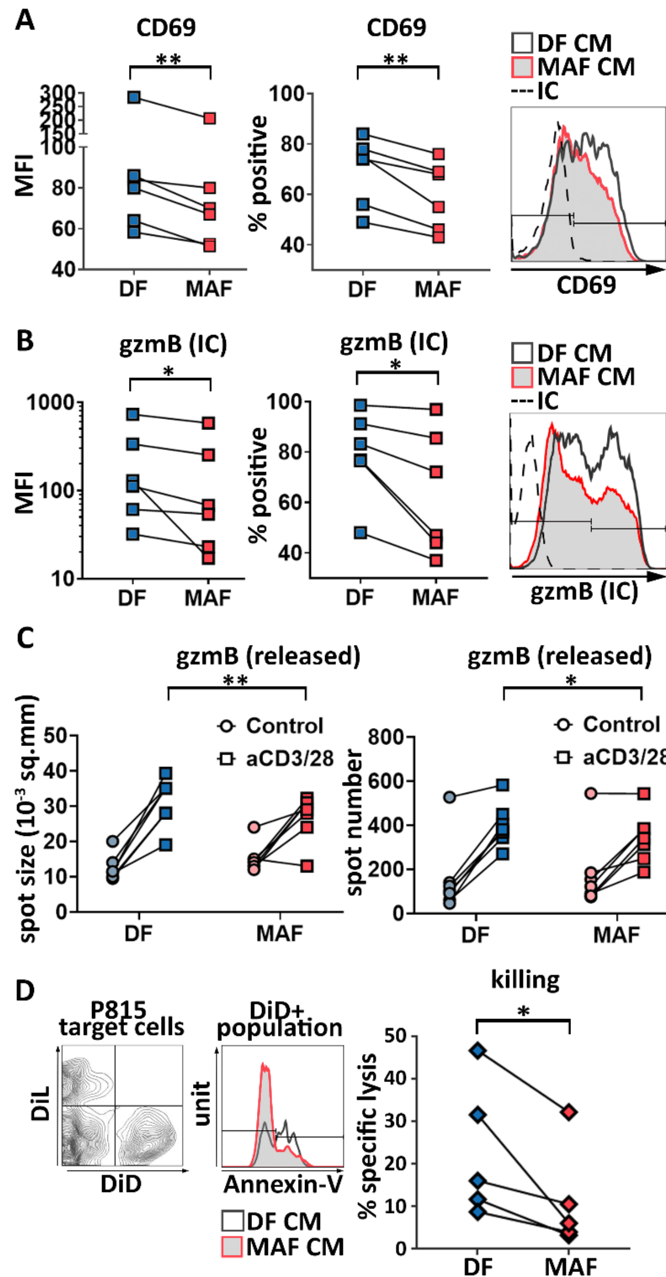

Fig2 MAF-CM interferes with CD8+ T cell activation, intracellular granzyme B production, granzyme B release, and in vitro killing

(A) CD69 expression was examined after exposure of CD8+ T cells from healthy blood donors to MAF- or DF-derived conditioned media and subsequent activation with anti-CD3/CD28. Mean fluorescence intensity (MFI) was compared by paired t-test (p=0.0053; n=6) and the percentage of CD69-positive cells analyzed by paired t-test (p=0.0058; n=6) (results of two independent experiments) and representative staining. (B) Representative flow cytometry data showing intracellular (IC) gzmB staining of split

CD8+ T cell cultures activated by aCD3/28 after exposure to MAF-CM or DF-CM. Pairwise analysis of mean fluorescence intensity and percentage of positive cells are shown. (paired t test p=0.0346, p=0.0327, n=6 respectively; three independent experiments). (C) Comparative analysis of gzmB release (spot area) and gzmB-releasing cell count (spot count) in control and aCD3/28-treated CTLs after exposure to MAF-CM and DF-CM using ELISPOT assay. (Two Way RM ANOVA, p=0.0409, p=0.0050, respectively, n=7, one experiment) (D) Redirected killing assays were performed using P815 cells DID-prestained and preloaded with aCD3/28 (specific lysis target) and their DIL-stained but untreated counterparts (non-specific lysis controls). Lysed P815 cells were identified using annexin V-staining and data were analyzed by ratio paired t-test (p=0.0223, n=5) (two independent experiments). Representative staining and data analysis are shown.

3.3 MAF supernatants selectively interfere with the production and release of CD8+ T cell effector molecules

We next examined whether MAF-derived soluble factors could modulate the expression or killing activity of cytotoxic T cell effector molecules. We tested activation-induced IFN-γ and granzyme B (gzmB) production by intracellular flow cytometry. There were no significant differences between MAF- and DF-directed CD8+ T cells in terms of IFN-γ production. However, intracellular gzmB levels were significantly reduced in the presence of MAF-CM in CD8+ T cells compared to DF-CM-treated cells. Similar to CD69, both gzmB production and the frequency of gzmB-positive cells (**Fig2B**) were reduced. Over the dose range studied, the effect of MAF-CM on gzmB production by CTL was comparable to that of in vitro exposure to TGF-β, with or without fibroblast-conditioned medium. Subsequent analysis of gzmB and IFN-γ release in MAF-exposed CTLs by ELISPOT confirmed these results. ELISPOT confirmed that both the number of aCD3/28-activated gzmB secreting CD8+ T cells and the amount of gzmB they released were reduced by pre-incubaton with MAF-CM (**Fig2C**). However, consistent with flow data, IFN-γ release was not affected by MAF.[27]

3.4 MAF-derived soluble factors interfere with CD8+ T cell-mediated killing

Since the production and release of gzmB correlates with the killing ability of cytotoxic T cells, the in vitro cytotoxic effector function of CD8+ T cells activated in the presence of MAF-CM was also analyzed. Standard redirected killing assays were performed using P815 cells as targets [31][32][33][34][35], either preloaded with anti-CD3/28 antibodies and labeled with DiD (target for specific killing) or left untreated and labeled with DiL (to assess aspecific killing of bystander non-target cells). We observed that the MAF CM-treated CD8+ T cell group induced significantly less cell death than the DF CM-treated group (**Fig2D**), consistent with the observed attenuation of gzmB production.[27]

3.5 MAF disrupts T-cell signaling

Recognizing that impaired T cell function may result from a lack of adequate co-stimulation or other forms of defective signaling, we next analyzed whether MAF supernatants affected T cell signaling required for full T cell activation and differentiation. In a preliminary study, we examined early (CD3ζ-chain activation, Tyr142-P), and downstream of T-cell signaling events (NF-κB activation, Ser536-P) as well as a key transcription factor associated with late CD8+ T cell differentiation (Runt-related transcription factor 3 or Runx3). MAF inhibited NF-κB phosphorylation, whereas CD3ζ and Runx3 were unaffected (**Fig3A-C**). Based on this observation, we next used phosphokinase protein arrays to further understand aberrant T cell signaling downstream of the TCR. Although most TCR-related kinase pathways remained functional, inhibition of NF-κB activation was accompanied by increased ERK1/2 phosphorylation in MAF-exposed CD8+ T cells (**Fig3D**). Taken together, these data suggest an imbalance between aCD3-mediated TCR signaling and aCD28-provided co-stimulation in MAF CM-exposed T cells.[27]

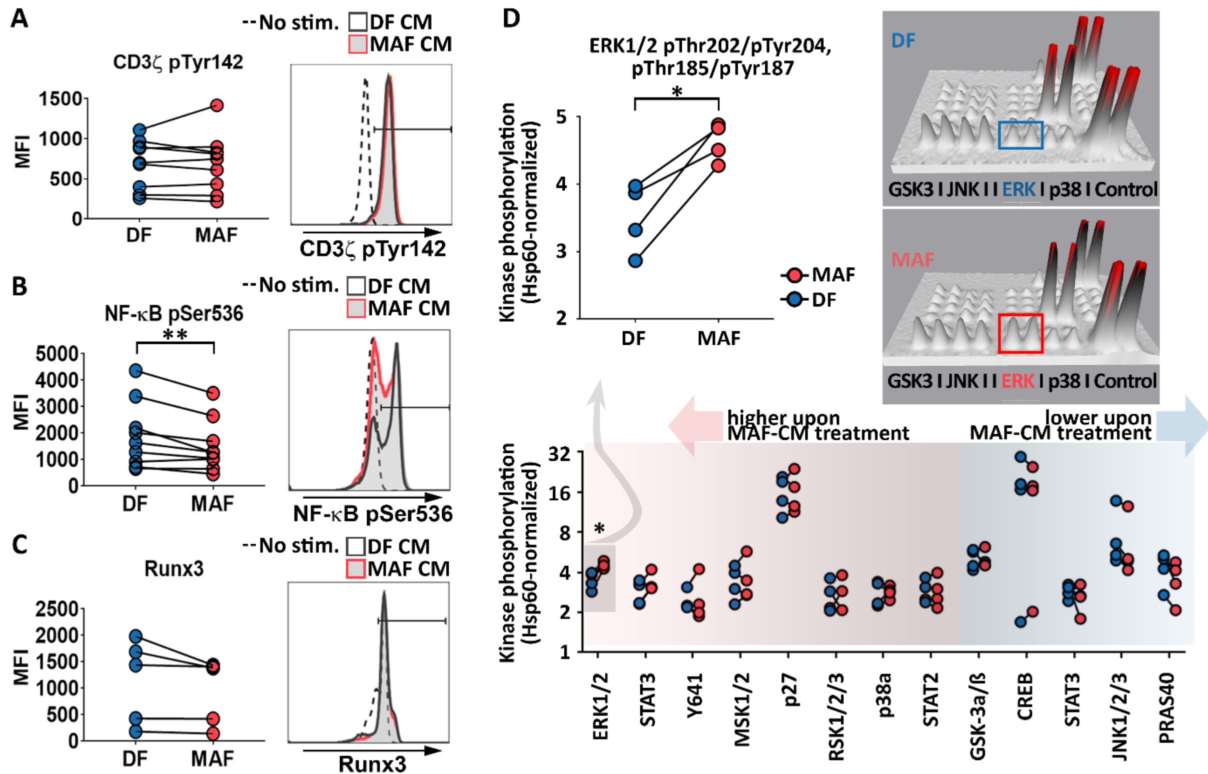

Fig3 MAF-CM modulates CTL phosphorylation cascades downstream of TCR

(A) Analysis of CD3ζ-chain activation (n=9) (B) NF-κB activation (n=9) and (C) Runx3 levels (n=5) in unstimulated and DF-CM or MAF-CM-exposed CD8+T cells after aCD3/28 activation. CD3ζ-chain and NF-κB phosphorylation were assessed 10 min post activation; Runx3 levels 48 h post activation. Representative data are shown to the right (Ratio paired t-test p=0.0093 for NF-κB; results from two independent experiments). (D) Screening analysis of 43 additional phosphokinases in DF- and MAF-CM-treated CD8+ T cells using a phosphokinase array (n=4). Shown are the phosphorylation patterns of 13 typical CD8+ T cell signaling proteins detected by the array 10 minutes after T cell activation (bottom). ERK1/2 phosphorylation data are magnified (top left, paired t-test p=0.0147). Representative 3D densitometry data showing differences in ERK1/2 activation (top right). Each phosphorylated protein is represented by a pair of spikes; the volume of spike corresponds to the intensity of protein phosphorylation. Results obtained from one experiment.

3.6 MAF supernatant modifies CD8+ T cell immune checkpoint receptor expression

Immune checkpoint receptors (ICR) are known to regulate the activity of CD8+ T cells during antitumor immune responses. Therefore, we wanted to investigate whether MAF affects the synthesis of selected ICRs in CD8+ T cells. Expression pattern of PD-1 (Programmed cell death protein 1), TIM-3 (T cell immunoglobulin and mucin-domain containing-3), LAG-3 (Lymphocyte-activation gene 3), TIGIT (T cell immunoreceptor with Ig and ITIM domains) and BTLA (B- and T-lymphocyte attenuator) was analyzed after treatment with MAF and DF-CM. We found increased expression of TIGIT and BTLA in CD45RO+ non-naïve/memory cytotoxic T cells after exposure to MAF-CM compared to DF-CM (**Fig4**). In contrast to DF-CM, the expression of PD-1, TIM-3, or LAG-3 on CD8+ T cells was not affected by MAF-CM, regardless of aCD3/28 activation or naïve/memory status.[27]

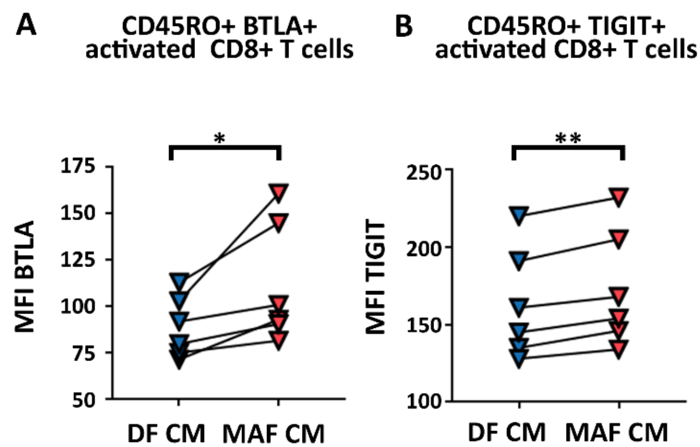

Fig4 MAF-CM-treatment activated non-naïve CD8+ T cells increases BTLA and TIGIT checkpoint receptor expression

BTLA and TIGIT expression on activated non-naïve/memory CD45RO+ CD8+ T cells after exposure to DF- and MAF-conditioned media; paired t-test (BTLA: p=0.0353 and TIGIT: p=0.0050, n=6, data from one experiment.

3.7 MAF exhibits a skewed profile of immune checkpoint modulators

The expression of CAF-ICR ligands and the possible role of these molecules in CAF-mediated immune regulation have been controversial. To analyze the question whether MAF might affect T-cell ICR signaling, the cell surface expression of several ICR ligands was compared between MAF and DF. We tested for the presence and expression of Herpesvirus entry mediator (HVEM), Galectin-3, Galectin-9, Programmed death-ligand 1 (PD-L1), CD155 (poliovirus receptor) and V-domain Ig suppressor of T cell activation (VISTA). We found that the levels of the negative CTL regulators VISTA and HVEM were increased in MAF compared to DF. (**Fig5**). [27]

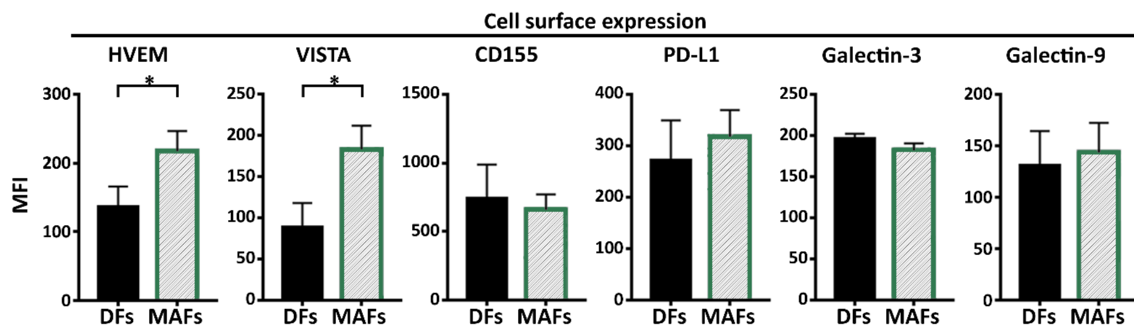

Fig5 MAF maintains increased expression of immune checkpoint regulators HVEM and VISTA

Comparative analysis of cell surface herpesvirus entry mediator (HVEM), galectin-3, galectin-9, programmed death-ligand 1 (PD-L1), CD155 (poliovirus receptor) and V-domain Ig suppressor of T cell activation (VISTA) expression (mean fluorescence intensity) on MAFs and DFs analyzed by flow cytometry (n=6 Mann-Whitney U test BTLA p=0.029 and HVEM p=0.0311). Data from three independent experiments.

3.8 MAFs show increased L-arginase activity and secrete increased levels of CXCL12

Next, we sought to explore the molecular pathways involved in MAF CD8+ T cell interactions. Candidate molecules were selected based on previous reports, including

immunomodulatory factors synthetized by CAFs. Secreted TGF-β [36], IL-6, CXCL12 [37] and PGE2 [21] were analyzed in MAF-CM and DF-CM by ELISA. L-arginine catabolism by intracellular L-arginase [38] was analyzed in cell lysates using a specific L-arginase activity assay. L-tryptophan catabolism by indolamine-2,3-dioxygenase [39] was assessed by measuring kynurenine in CM. L-arginase activity was detected in both MAF and DF, and it was greatly increased in MAF compared to DF cells (**Fig6A)**. To support this finding, we next analyzed NO production by intracellular DAF-FM staining in a similar fashion, comparing MAFs and DFs. L-arginase and nitric oxide synthase (NOS) actively compete for free L-arginine as their common substrate, and it has been repeatedly shown that an increase in arginase activity induces uncoupling of NOS, leading to the reduction of NO production [26]. In fact, we found that MAF produced significantly less NO than DF (**Fig6B**), suggesting that L-arginase upregulation and subsequent L-arginine depletion in MAF reached critical levels sufficient to interfere with normal NO production in these cells. IL-6, and IDO activity were detected, but neither of them showed any significant difference between MAFs and DFs (**Fig6C,D**). No detectable levels of TGF-β and PGE2 were found in MAF- or DF-CM (not shown), consistent with our finding that the presence or absence of MAF- or DF-CM did not alter the impact of external TGF-β on CTLs gzmB production. Interestingly, increased levels of secreted CXCL12 were detected in MAF supernatants, consistent with other reports describing increased CXCL12 production by CAFs in human and murine carcinomas [37][40] (**Fig6E**).[27]

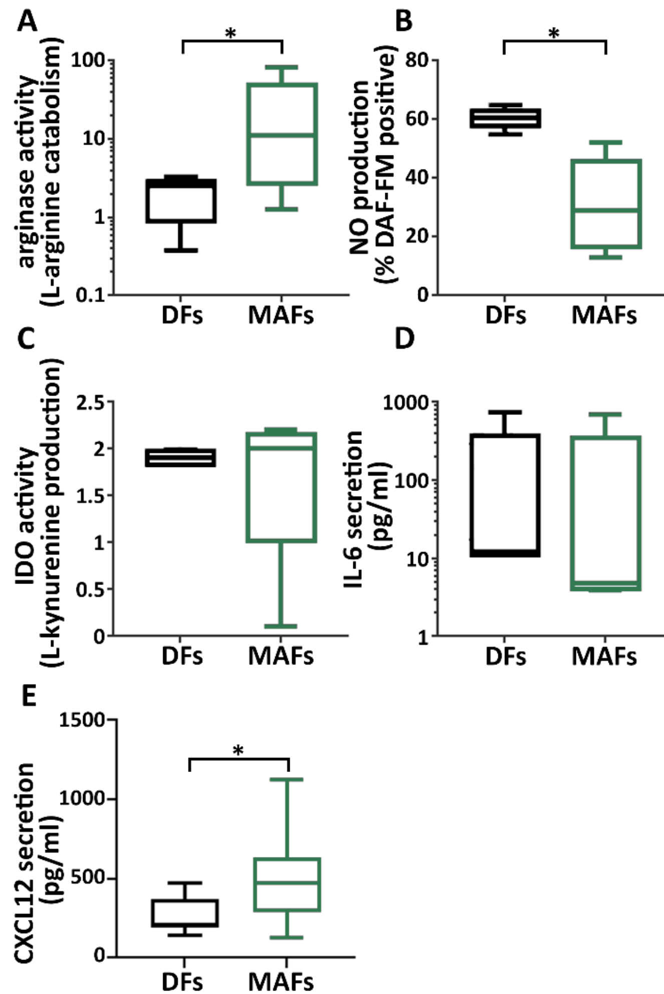

Fig6 Increased arginase activity and apparent NOS uncoupling in MAF cells

(A) Comparison of intracellular arginase activity in MAF and DF cell lysates derived from the same patients. Results shown have been normalized to total protein content (min to max; p=0.0186, n=5, ratio paired t-test) (data from two independent experiments). (B) MAFs and DFs from the same patient were stained with DAF-FM and assessed for the abundance of NO producing cells (min to max; p=0.0113, n=5, paired t-test, data from two independent experiments). There were also significant differences in the amount of produced NO (MFI) per cell (p=0.0021, data not shown). (C, D) IL-IDO activity/L-kynurenine production, IL-6 and CXCL12 release (paired t-test, p=0.0178, n=8, one experiment) in MAFs and DFs were assessed of by ELISA.

3.9 MAF-derived L-arginase induces increased expression of TIGIT and BTLA in CD8+ T cells

To test whether the increased L-arginase activity in MAF is responsible for the MAF-induced suppression of CD8+ T cells, additional batches of MAF-CM and DF-CM were prepared both in the presence and absence of BEC hydrochloride, a potent and selective inhibitor of both L-arginase 1 and 2. Next, the stability of any previously observed T cell-related dysfunctions was reassessed with and without L-arginase inhibition. These analyses demonstrate that MAF-suppressed CD69 and gzmB expression, and MAF-reduced CTL-mediated killing cannot be recovered by abrogating L-arginase activity in MAF. However, the increased expression of BTLA and TIGIT by MAF-CM could be neutralized by selective inhibition of L-arginase **(Fig7A)**. To confirm these findings, we transfected MAF with a mammalian expression vector encoding full-length human arginase 2 under the control of a CMV promoter and examined its effect on both BTLA and TIGIT. We found that forced expression of arginase in MAF further increased BTLA and TIGIT on CD8+ T cells (**Fig7B**). Taken together, these data suggest that MAF-mediated enhanced expression of BTLA and TIGIT on CD8+ T cells is an L-arginase-dependent phenomenon.[27]

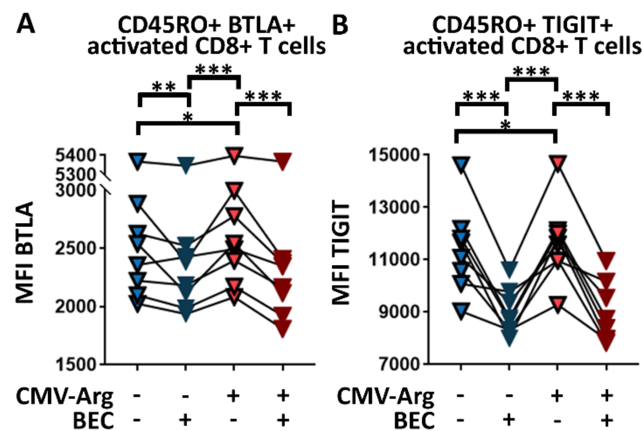

Fig7 Increased MAF arginase activity is responsible for aberrant ICR expression on CTLs

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*Comparative analysis of ICR expression on activated non-naïve/memory CTLs exposed to conditioned media collected from MAF maintained in the presence and absence of BEC hydrochloride, a selective arginase inhibitor, and transgenic arginase overexpression (CMV-Arg).BTLA (A) and TIGIT (B) expression of activated CD45RO+ CD8+ T cells is shown under the influence of BEC and CMV-Arg. (Two-Way RM ANOVA, * $p<0.05$, ** $p<0.01$ *** $p<0.001$, $n=8$). Data from two independent experiments.*

4. Discussion

Our data suggest that soluble factors released by MAFs affect various aspects of cytotoxic T cell function. Here, we show that *ex vivo* MAFs can create an environment that affects nearly all stages of T cell activation, from early post-activation events to terminal differentiation and effector function. All observed effects were invariably detrimental to CD8+ T cell activity. This raises the question of whether MAF can contribute to the complex, multifaceted attack of CTLs by cancer cells in vivo, leading to TIL dysfunction in various advanced solid tumors, including melanoma.

This study provides multiple lines of evidence that FAP+ melanoma-associated fibroblasts exhibit an immunosuppressive, tumor-promoting phenotype, consistent with observations made by others in colon cancer [41] and pancreatic cancer [37]. MAF-derived soluble factors compared to healthy DF significantly downregulated the expression of surface CD69 on activated CD8+ T cells, suggesting that fundamental destruction of T cells occurs even in the earliest stages of T cell activation. MAF-derived factors reduce granzyme B production and release, but do not alter IFNγ production, secretion, or cytotoxic degranulation by CD8+ T cells, implying impaired selectivity for some components of the T-cell effector machinery in nearby MAFs. Consistent with this finding, we showed in redirected killing assays that MAFs also inhibited the ex vivo killing ability of CD8+ T cells, which may be related to the production of attenuated granzyme B. However, it is important to note that experimental killing assays [42], including the redirected killing assays used here [43][44][32] measuring *ex vivo* killing capacity, should be interpreted with caution, as their results do not necessarily reflect actual antitumor killing activity in vivo. Nonetheless, these results suggest that MAFs not only interfere with the T cell activation process, but may also disrupt the efficient elimination of target cells (i.e. cancer cells) in the tumor parenchyma.

In addition, we found selective dysregulation of certain immune checkpoint regulators on CD8+ T cells by MAFs. Soluble MAF factors significantly increased the proportion of TIGIT and BTLA positive CD8+ T cells, especially in non-naïve/memory CD45RO+ T cells. This observation is interesting because several ICRs, including BTLA [33] and TIGIT [45] are potent negative regulators of T-cell activity and are being actively explored as promising targets for next generation immunotherapies. Monoclonal

antibodies against BTLA are the subject of preclinical studies, while TIGIT blockade is in Phase I clinical trials (NCT03119428). Furthermore, CD45RO+ T cell activity is critical for rejection of various solid tumors [46][47][48], including checkpoint blockade therapy-induced remission in melanoma. Several lines of evidence suggest that the frequency of circulating CD45RO+ T cells [49], especially treatment-induced systemic [50][51] and intratumoral [52] expansion of the CD45RO+ subset, is important for the success of checkpoint blockade. Interestingly, the changes observed in CD8+ T cell ICR expression (higher BTLA levels) were accompanied by complementary changes in MAF-derived ICR ligands (increased HVEM expression). This raises the question whether MAFs can further inhibit CTLs by activating the HVEM-BTLA ICR signaling axis if direct contact occurs between the two cell types [53].

In conjunction with the above results, potential differences were searched between the MAF and DF cells that could shed light on the mechanisms responsible for the apparent dysfunction of MAF-exposed CD8+ T cells. We found that the intracellular signaling cascade of CD8+ T cells is affected by chronic exposure to soluble MAF mediators. Increased ERK1/2 and decreased NF-κB phosphorylation suggest that MAF reprograms two important T-cell signaling pathways, possibly the RAS guanyl-releasing protein 1 – ERK1/2 pathway and the protein kinase Cθ – NF-κB pathway. Mature naïve T cells activated by aCD3 (stimulation) and aCD28 (costimulation) similarly depend on both pathways of TCR/CD3 signaling [54]. However, it has been well documented that sufficient CD28-induced co-stimulation depends on the protein kinase Cθ-NF-κB pathway [55][56][57][58]. Since both full T cell maturation and the exertion of effector function are dependent on appropriate costimulation, these data suggest that the phenotypic changes observed in effector function of CD8+ T cells exposed to MAF may originate in attenuated T cell costimulation.

Furthermore, we found that arginase activity was greatly increased in MAF cells, leading to uncoupling of NOS and a significant perturbation of steady-state NO production. L-arginine deficiency induces T cell suppression by: i) CD3ζ downregulation [59], ii) G0/G1 cell cycle arrest due to failure of cyclin D3 protein synthesis and cdk4 activation [60], iii) increased phosphorylation of eIF2 and iv) global suppression of protein synthesis in affected T cells [61] v) interference with lytic function in situ [62]. To date, no direct evidence has been provided that CAF induces T cell suppression via arginase, although

clinical data clearly indicate that ARG2 overexpression by CAF predisposes to decreased overall survival in pancreatic cancer [38] and ARG2 production by CAF has been observed in unrelated solid tumors, too [63]. It has been suggested that cancer-associated fibroblasts may also exploit arginase to interfere with T cell activity [64][65][66], however, to our best knowledge, this is the first study substantiating this hypothesis on the example of melanoma-associated fibroblasts.

We found that selective blockade of arginase activity by the potent, small-molecule inhibitor, BEC hydrochloride neutralized increased TIGIT and BTLA expression on CD45RO+ CD8+ T cells 48h after activation, whereas arginase transfection has the opposite effect. Taken together, these data suggest that aberrant ICR expression on CTLs in the presence of MAF-CM is an arginase-mediated phenomenon. Interestingly, a recent publication also suggested that BTLA may be arginase-dependent, as BTLA mRNA expression in activated CD8+ T cells in the absence of arginase 2 decreased 48h after activation [67]. Notably, BEC hydrochloride maintained its potency even after increasing MAF arginase expression by stable transfection. This is interesting because arginase inhibitors, either alone or in combination with checkpoint inhibitors, are currently being tested as antineoplastic agents in various solid malignancies (NCT02903914). These clinical studies are consistent with preclinical data suggesting that arginase blockade may increase the efficacy of the PD-1/PD-L1 blockade [68][69]. Thus, our data may support the notion that arginase inhibition may be a tool to make some solid tumors more susceptible to multiple forms of ICR blockade.

Last but not least, we have shown that MAFs secrete more CXCL12 compared to autologous DF cells. High levels of CXCL12 may have chemorepellent effects and may be involved in the exclusion of CD8+ T cells from solid tumors [70][37]. Although multiple lines of evidence suggest that increased CAF-secreted CXCL12 is tumor-promoting [40], more studies are needed to explore to exact role of MAF-derived CXCL12 in melanoma biology.

A particularly interesting aspect of our observations is the remarkable robustness of the CTL-suppressive MAF phenotype. In all experiments, MAF was maintained in the absence of melanoma cells for several weeks before being used for CD8+ T cell assays. Our data clearly show that despite extensive *in vitro* cultivation, MAFs do not revert to a rather benign DF phenotype. The CTL-suppressive MAF phenotype remained stable even

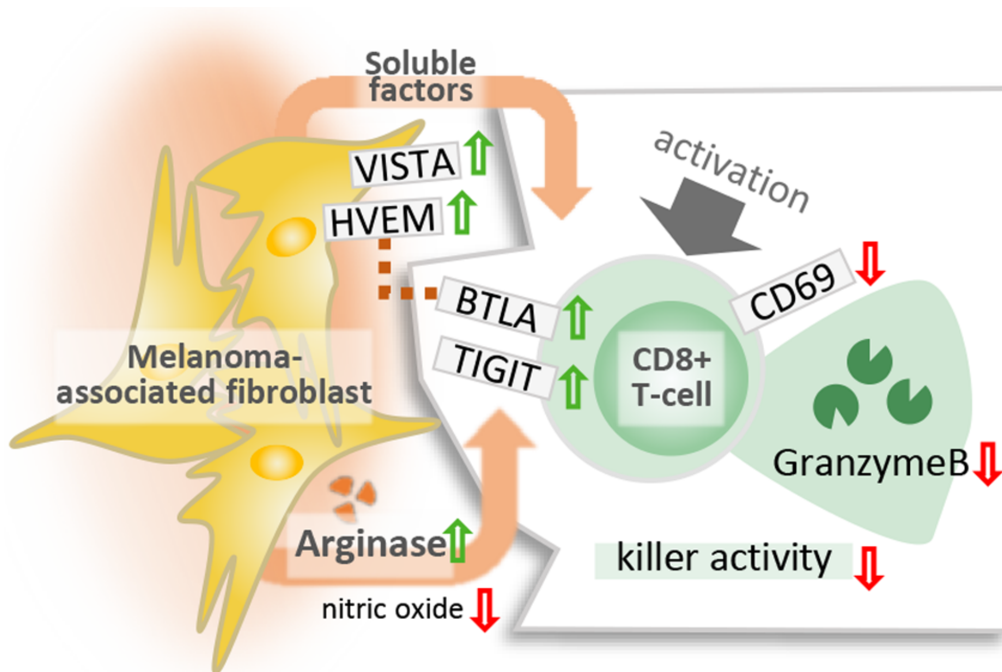
after isolation from the cancer microenvironment. This observation supports a recent view by many leading authors in the field that at least some of the changes that facilitate the transition from DF to CAF may be due to highly stable epigenetic modifications [71][72][73][74]. That is, certain parameters of the MAF phenotype may emerge after a priming event, leading to long-term consequences, rather than being maintained by cancer cells through continuously secreted cytokines or similar mechanisms [64][75]. Although the exact mechanism of this tumor-induced fibroblast transformation is unclear, several studies suggest that direct cell-cell contact as well as cell-derived soluble factors such as TGF-β and LIF may play an important role [76][71] in the this process.[27]

5. Conclusions

In this study we report that human MAFs inhibit various CD8+ cytotoxic T cells functions *in vitro* including granzyme B production and redirected cancer cell killing. This suppressive effect correlates with an increased expression of T cell receptors such as TIGIT and BTLA, and a simultaneous increase in MAF-derived activators of these receptors (VISTA and HVEM). A comprehensive analysis of MAF-derived soluble factors and cell lysates revealed a marked increase in arginase/nitric oxide ratios in these cells compared to normal dermal fibroblasts. Inhibition of the arginase/nitric oxide pathway in MAFs abolished the TIGIT/BTLA-increasing effect of MAFs on T cells.

To the best of our knowledge, this is the first study that demonstrates MAF-mediated CD8+ cytotoxic cell suppression and suggests a direct involvement of MAF-derived arginase in regulating costimulatory molecule expression in T cells. We think that our data add an important piece to the puzzle in an exciting era when next generation immune check-point inhibitors, such as antibodies targeting TIGIT or BTLA, are being developed and molecular therapies targeting arginase are being tested in clinical trials.

6. Summary



This study shows that MAFs suppress CD8+ T cell activity and demonstrates that arginase plays a key role in this phenomenon. MAFs and DFs were isolated from melanoma biopsies as Melan-A-/gp100-/FAP+ stromal cells. CD8+ T cells from healthy blood donors were activated in the presence of MAF- and DF-conditioned media and markers related to CTL activation, cytotoxic degranulation, immune checkpoint regulation and in vitro killing activity were evaluated by flow cytometry, ELISPOT and redirected killing assays, respectively. Soluble mediators responsible for MAF-mediated effects have been identified by ELISA, flow cytometry, knock-in experiments and inhibitor assays. Activated/non-naïve CTLs exposed to MAF-CM displayed dysregulated ERK1/2 and NF-κB signaling, impaired CD69 and granzyme B expression, impeded killing activity, and upregulated expression of the negative immune checkpoint receptors TIGIT and BTLA. In addition, MAFs compared to DFs displayed increased amounts of VISTA and HVEM, the ligand of BTLA on T cells, increased endogenous L-arginase activity and CXCL12 release. Transgenic arginase overexpression continued to increase, while selective arginase inhibition neutralized MAF-induced TIGIT and BTLA expression on CTLs. Our data suggest that MAF disrupts intracellular CTL signaling

through soluble mediators, causes CTL anergy and alters immune checkpoint receptor availability via L-arginine depletion[27].

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8. Bibliography of the candidate's publications

Publications related to the dissertation

Érsek Barbara[1], Silló Pálma[1], Ugur Cakir, Molnár Viktor, Bencsik András, Mayer Balázs, Mezey Eva, Kárpáti Sarolta, Pós Zoltán, Németh Krisztián
Melanoma-associated fibroblasts impair CD8+ T cell function and modify expression of immune checkpoint regulators via increased arginase activity

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