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

PhD thesis

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Budapest
2022

1. Introduction

Melanoma is a highly aggressive melanocyte-derived skin cancer. Although overall melanoma survival rates are increasing with the new immuno- and targeted therapies, survival rates for metastatic melanomas remain low. This can be not only due to tumor cells but also other cells in the tumor microenvironment (TME). Melanoma TME consists of various cell types, such as melanoma-associated fibroblasts (MAFs) and tumor-associated macrophages (TAMs). MAFs as an important part of TME possess many abilities to support tumor growth, metastasis formation, and even drug resistance. They are also important regulators of the anti-tumor immune response. Studies showed that MAFs modulate NK cell phenotype and anti-tumor cytotoxicity and also impair cytotoxic activity of $CD8^+$ T cells.

TAMs are present in large amounts in the stroma of many cancers and are known to play an important role in tumor development. Macrophages secrete several immunomodulatory cytokines. One such cytokine is interleukin-10 which promotes immune escape and progression of tumors via inhibiting the pro-inflammatory/anti-tumor milieu.

My research focused on the immunosuppressive effects of MAFs, and examined the effect of MAFs on macrophage-derived IL-10 production.

2. Objectives

The objectives of my research were to investigate the interactions between MAFs and macrophages and examine if MAFs are able to modulate macrophage-driven IL-10 secretion. In order to achieve this objective, we examined if:

2.1. MAFs were in close contact with macrophages in the melanoma microenvironment,

2.2. MAFs caused an increase of IL-10 production of macrophages,

2.3. The increase of IL-10 production was affected by MAFs cell count, macrophage phenotype and co-culture incubation time,

2.4. There is a correlation between IL-10 increasing ability of MAFs and clinicopathological factors of melanoma,

2.5. The IL-10 increasing ability of MAFs could be enhanced when pretreated with conditioned media from BRAF inhibitor- and chemotherapy treated melanoma,

2.6. Melanoma tumor cells had improved chemoresistance against BRAF inhibitors when co-cultured with MAFs,

2.7. 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, blood specimens from healthy donors and 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 monocytes were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin (P/S), and 1% l-glutamine. BRAF mutated melanoma cells isolated from the excised tumors (MM-55) as well as BRAF mutated human malignant melanoma cell lines SK-MEL-28 and MALME-3M were maintained in standard Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% P/S, and 1% L-glutamine. MAFs were propagated in MAF medium (DMEM supplemented with 20% FBS, 1% 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 (Érsek et al., 2020). 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. After 48 h, conditioned media (CM) derived from MAFs was collected.

3.3. Immunostaining of Melanoma Samples for FAP and Iba-1

Paraffin embedded tissue sections were cut onto positively charged slides, baked overnight in a 65 °C oven, deparaffinized, and then antigen retrieval was performed. The sections were then blocked with BSA to inhibit non-specific binding of the antibodies. First, the tumor stroma was labelled using antibody to FAP at 4 °C overnight, followed by 1 h incubation with a rabbit IgG VisUCyte HRP polymer, and then an Alexa-594 conjugated Tyramide . Following a second microwave session, the second primary antibody, Iba-1, was applied to the sections similarly as before. 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. (Genin et al., 2015). First, THP-1 monocytes differentiated into M0-like macrophages by 24 h incubation with phorbol 12-myristate 13-acetate, followed by 24 h incubation in fresh RPMI 1650 medium. M0-like macrophages were polarized into M1-like macrophages by 24 h incubation with interferon-γ (IFN-γ) and lipopolysaccharide (LPS). Macrophage M2-like polarization was achieved by 72 h incubation with interleukin 4 and interleukin 13.

3.5. Cell Culture Assays

For MAF-macrophage co-culture assays, MAFs and THP-1 macrophages of various phenotypes were incubated for 24 h. To enhance cytokine production, cells were stimulated with LPS for an additional 18 h. Lastly, the plates were centrifuged, and supernatants were collected .

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 and incubated as described above.

3.6. Cytotoxicity assay

MAFs 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 microplate reader at 485 nm excitation and 510 nm emission wavelengths for GFP.

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 cultured in basal medium (BM) for 48 h, later 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. 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. Subsequently, cells were incubated in fresh culture medium for another 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 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 twice or three times and in triplicate/quadruplicate.

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 (Jackson et al., 2016). 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.

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

To examine if MAFs increase IL-10 secretion in monocytes/macrophages, 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. 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. 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 macrophages 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.

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 and histological parameters of melanoma patients. 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. 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.

4.4. Prior Exposure to Untreated or BRAF Inhibitor- or Chemotherapy-Treated Melanoma Cells Boosted 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. 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.

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 (area under curve) of MAF/melanoma co-culture viability was not bigger than that of melanoma mono-culture. This indicated that MAFs do not increase directly melanoma survival when treated with BRAF-inhibitors.

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. 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. Cyclooxigenase-1 and -2 inhibition abrogated IL-10 increase in THP-1 cells. iNOS inhibition had no effect on MAF-mediated IL-10 elevation.

5. 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 the 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 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 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 monocultures.
7. In our experiments, MAFs were shown to play an important role in regulating macrophage functions, promoting a pro-tumorigenic, IL-10-rich environment. 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.

6. Bibliography of candidate's publications

Thesis-related publications:

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