Microphthalmia-Associated Transcription Factor (MITF) Regulates Immune Cell Migration into Melanoma

Gabriela M Wiedemann*,†, Celina Aithal*,†, Angelina Kraechan*,‡, Constanze Heise*,‡, Bruno L Cadilha*,‡, Jin Zhang*,‡, Peter Duewell*,‡, Robert Ballotti*,‡, Stefan Endres*‡ and Sebastian Kobold*,‡

*Center of Integrated Protein Science Munich (CIPS-M) and Division of Clinical Pharmacology, Klinikum der Universität München, Lindwurmstrasse 2a, 80337 Munich, Germany; †Department of Medicine II, Klinikum rechts der Isar, Technische Universität München, Germany; ‡INSERM U1065 (Team 1), C3M, Biology and Pathologies of Melanocytes, 151, route Saint Antoine de Ginestière, BP2 3194, 06204 Nice Cedex3

Abstract

Microphthalmia-associated transcription factor (MITF) is a key transcription factor in melanoma development and progression. MITF amplification and downregulation have been observed in a significant proportion of melanoma patients and correlate with clinical outcomes. Here, we have investigated the effect of MITF on melanoma chemokine expression and immune cell attraction. In B16F10 melanoma cells, MITF knockdown reduced expression of CXCL10, with concomitantly decreased attraction of immune cells and accelerated tumor outgrowth. Conversely, overexpression of MITF in YUMM1.1 melanoma cells also led to an increased immune cell attraction in vitro. Subcutaneous YUMM1.1 melanomas overexpressing MITF however showed a reduced immune infiltration of lymphocytes and an increased tumor growth. In human melanoma cell lines, silencing of MITF enhanced chemokine production and immune cell attraction, while overexpression of MITF led to lower immune cell attraction. In summary, our results show that MITF regulates chemokine expression in murine and in human melanoma cells, and affects in vivo immune cell attraction and tumor growth. These results reveal a functional relationship between MITF and immune cell infiltration, which may be exploited for cancer therapy.

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Introduction

Microphthalmia-associated transcription factor (MITF) is a key transcriptional regulator of the melanocyte cell lineage. It is expressed in 80% of human melanomas and plays an important role in melanoma development and progression [1,2]. MITF is known to regulate a diverse range of transcriptional targets including genes involved in cell cycle arrest, DNA repair, proliferation, survival, and apoptosis as well as cell differentiation [3]. Amplification of MITF is found in 15% to 20% of human metastatic melanomas and has been linked to poor survival [4]. Evidence for the role of changes in MITF levels in melanoma is contradictory. High expression of MITF was found in melanoma relapse after combined BRAF and MEK inhibitor therapy [5]. However, resistance to targeted therapy has also been associated with a decreased expression of MITF [6]. Low levels of MITF are associated with increased invasiveness of melanomas but also with cell senescence, whereas high levels result in differentiation [7–13]. These findings highlight a central regulatory role of MITF in melanoma cell phenotypic versatility and further underline the importance of understanding its dynamic regulation.

Address all correspondence to: Sebastian Kobold, MD, Division of Clinical Pharmacology, Klinikum der Universität München, Lindwurmstraße 2a, 80337 München. E-mail: Sebastian.kobold@med.uni-muenchen.de

1 G. M. W. and C. A. contributed equally to this work. 2 Member of the German Center of Lung Research. 3 Equipe labellisée ARC 2015.

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In the past decade, immunotherapy using checkpoint blocking antibodies has changed the treatment of advanced and metastasized melanoma patients [14]. Their effectiveness demonstrates the importance of the immune system in melanoma therapy. However, melanomas in a significant number of patients either do not respond to checkpoint inhibiting antibodies at all or relapse after initial tumor regression. Primary but also secondary treatment failure may result from a lack of effector T cells at the tumor site and is associated with a bad prognosis [15]. Tumors with low immune cell infiltration are frequently referred to as “cold tumors” as opposed to the immune cell-rich “hot tumors” typically responding well to checkpoint blockade. The origins of these different phenotypes are still poorly understood. There is a need for new therapeutic strategies, which convert poorly infiltrated tumors into “hot tumors” [16].

So far, little is known about the effects of MITF on melanoma immune cell infiltration. It has recently been shown that MITFlow melanomas display an increased response to exogenous TNF resulting in higher infiltration by CD14+ myeloid cells [17]. In addition, in melanoma cells exhibiting a state of senescence due to stable MITF knockdown, an increased expression of the chemokine CCL2 was described favoring proinvasive capacities of melanoma cells in an autocrine manner [12]. These studies suggest that MITF expression levels may affect the melanoma immune landscape. However, no detailed analyses of chemokine expression and immune cell infiltration in melanomas with different MITF expression levels have been performed so far.

In the present work, we investigated the effects of MITF knockdown as well as MITF overexpression in different murine and human melanoma models on chemokine expression and immune cell infiltration, in vitro and in vivo. We demonstrate that MITF downregulation and MITF upregulation result in changes in the chemokine expression profile on both RNA and protein levels. The differential chemokine expression patterns lead to altered immune cell migration towards tumor cells in vitro and correlated with accelerated tumor outgrowth in vivo in both conditions. Thus, our data suggest a role for MITF in regulating tumor immune cell infiltration.

**Material and Methods**

**Mice, Cell Lines, and Animal Experiments**

C57BL/6 mice were purchased from Janvier (St. Berthevin, France) or Charles River (Wilmington, MA) and were 5 to 12 weeks of age. All animal studies were approved by the local regulatory agency (Regierung von Oberbayern). The human melanoma cell lines WM8 and WM35 and the murine B16F10 melanoma cell line were described previously [18–20]. The murine YUMM1.1 cell line [21] was kindly provided by Dr. Rosenberg (Yale University, USA). Cell lines were cultured in complete DMEM or RPMI medium (PAA Laboratories) and were routinely tested for mycoplasma contamination by MycoAlert Mycoplasma Detection Kit (LONZA). For in vitro tumor models, syngeneic tumor cells were injected subcutaneously into the flank of C57BL/6 mice. Mice were sacrificed after 10 to 30 days as indicated. For isolation of tumor-infiltrating lymphocytes, tumors were mechanically disrupted, incubated with 1 mg/ml collagenase and 0.05 mg/mL DNase (both Sigma Aldrich), and passed through a cell strainer. Single-cell suspensions were directly analyzed or layered on a gradient of 44% Percoll (Biochrome, Berlin, Germany) and 67% Percoll prior to centrifugation at 800xg for 30 minutes.

**Flow Cytometry**

Multicolor flow cytometry was performed using a BD FACS Canto II or BD LSR Fortessa (BD Bioscience, Germany). Flow cytometry antibodies were purchased from Biolegend (San Diego, CA). Anti-human CD8 was purchased from ThermoFisher Scientific (Waltham, MA). The following antibodies were used: anti-human CD3 (OKT3), anti-human CD4 (OKT4), anti-human CD8 (OKT8), anti-human CD19 (HIB19), anti-human CD56 (5.H11), anti-human CD11c (B15), anti-human CD14 (63D3), anti-mouse CD3 (17A2), anti-mouse CD4 (GK1.5), anti-mouse FOXP3 (MF-14), anti-mouse CD8 (52-6.7), anti-mouse NK1.1 (PK136), anti-mouse CD11c (N418), anti-mouse CD11b (M1/70), and Zombie Aqua Fixable Viability Kit.

**siRNA Transfection**

siRNA against human and murine MITF has been previously published [22,23]. For human siRNA knockdown of MITF, the MITF-specific sequence 5′-CUUGAUAUCCGAUACC-d (TT)-3′ and the control sequence 5′-CGU AGC CGG AAU ACU UCG A-3′ (LuciferaseGL2) were used. For murine siRNA knockdown, the sequence 5′-GGUGAUAUCCGAUCAAG-3′ (siMITF) and the control sequence 5′-UUCUCCGgAACgUGU- CACgUTT-3′ (siControl) were used. For transfection, a single pulse of 50 nM siRNA was administered to the cells at 50% to 70% confluency by transfection with 5 μl of Lipofectamine RNAiMAX in opti-MEM medium (both Invitrogen).

**Chemokine ELISA**

Chemokine protein levels in tumor cell supernatants were determined by R&D DuoSet ELISA for CCL2, CCL5, and CXCL10 (Mouse CCL2/JE/MCP-1, Mouse CCL5/RANTES, Mouse CXCL10/IP-10/CRG-2 DuoSet ELISA, R&D Systems, Minneapolis, MN). Cell free culture supernatants of YUMM1.1 melanoma cells were collected after 48 hours of culture. Culture medium of B16F10 melanoma cells was changed 48 hours after MITF siRNA transfection, and cell free supernatants were collected after another 48 hours of culture and analyzed according to the manufacturer’s protocol. Absorbance was measured at 470 nm using the Mithras-Reader (MikroWin program, version 4.41).

**Chemokine Protein Arrays**

Chemokine protein levels in tumor cell supernatants or tumor lysates were determined by R&D Proteome Profiler Human/ Mouse Chemokine Array Kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s protocol. For lysates, tumors were frozen in liquid nitrogen and mechanically pulverized. Organ powder was resolved in Bio-Rad Cell Lysis Buffer (Bio-Rad, Hercules, CA) and centrifuged at 13,000xg for 30 minutes. Supernatant was collected and stored at –80°C. Protein levels in organ lysates were quantified by Bradford protein assay (Protein Assay Kit, Bio-Rad, Hercules, CA).

**Western Blot**

For MITF protein levels, cells were lysed after 48 hours of culture using RIPA-Buffer (50 mM Tris/Cl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-DOC, 0.1% Protease Inhibitor, pH 7.4). Protein concentration was quantified by Bradford protein assay (Protein Assay Kit, BioRad, Hercules, CA). The lysates were run on a 10% polyacrylamide gel. MITF was detected using anti-MITF (clone...
| Gene            | Sequence L | Accession Code | Position L | Sequence R | Accession Code | Position R |
|-----------------|------------|----------------|------------|------------|----------------|------------|
| CCL1 murine     | L: cccctgaagttatatccagtgtta | NM_011329.3 | L: 195-217 | R: gcagctttctctacctttgttca | R: 268-290 |
| CCL2 murine     | L: ctcaccaggtgtgtga | NM_011333.3 | L: 139-156 | R: gatcatcttgctggtgaatgagt | R: 192-214 |
| CCL3 murine     | L: tgcccttgctgttcttctct | NM_011337.2 | L: 119-138 | R: gtggaatcttccggctgtag | R: 212-231 |
| CCL4 murine     | L: ggcctctctctcctcttgct | NM_013652.2 | L: 98-117  | R: ggagggtcagagcccatt | R: 154-171 |
| CCL5 murine     | L: tgcagaggactctgagacagc | NM_013653.3 | L: 3-23    | R: gagtggtgtccgagccata | R: 133-151 |
| CCL6 murine     | L: gcccctggccttgctctctctct | NM_009139.3 | L: 175-195 | R: gcaggtgactggagccttat | R: 238-257 |
| CCL7 murine     | L: gcctgctctcccctctctct | NM_013654.3 | L: 92-111  | R: ttgacatagcagcatgtggat | R: 162-182 |
| CCL8 murine     | L: ctcaccaggtgtgtga | NM_021443.3 | L: 72-91   | R: gcaggtgactggagccttat | R: 129-148 |
| CCL9 murine     | L: ccccaggcttcagataatca | NM_011338.2 | L: 230-247 | R: cccatgtgaaacatttcaatttc | R: 299-321 |
| CCL11 murine    | L: gcctgctctcccctctctct | NM_011330.3 | L: 140-158 | R: cgcacagtgacggtgtgata | R: 214-231 |
| CCL12 murine    | L: ccaccaggtgtgtga | NM_011331.2 | L: 100-120 | R: gcctgctctcccctctctct | R: 175-194 |
| CCL13 murine    | L: gcctgctctcccctctctct | NM_010779.2 | L: 61-80   | R: ggtaggaggggagggagggagggagt | R: 121-140 |
| CCL17 murine    | L: cctggaggagggagggagt | NM_011332.3 | L: 84-103  | R: ccagggtgcctggaggagggagt | R: 60 |
| CCL19 murine    | L: ccaaccaggtgtgtga | NM_011888.2 | L: 157-176 | R: gcctgctctcccctctctct | R: 164-183 |
| CCL20 murine    | L: accgggagggagggagggagt | NM_016960.2 | L: 256-285 | R: gcctgctctcccctctctct | R: 299-318 |
| CCL21a murine   | L: ctcaccaggtgtgtga | NM_011124.4 | L: 428-445 | R: gcctgctctcccctctctct | R: 501-519 |
| CCL22 murine    | L: cctggaggagggagggagt | NM_009137.2 | L: 72-91   | R: gcctgctctcccctctctct | R: 144-163 |
| CCL24 murine    | L: ctcaccaggtgtgtga | NM_019777.4 | L: 235-252 | R: gcctgctctcccctctctct | R: 291-309 |
| CCL25 murine    | L: ctcaccaggtgtgtga | NM_009138.3 | L: 496-515 | R: gcctgctctcccctctctct | R: 563-582 |
| CCL26 murine    | L: ctcaccaggtgtgtga | NM_001013412.2 | L: 167-186 | R: gcctgctctcccctctctct | R: 231-250 |
| CCL27a murine   | L: ctcaccaggtgtgtga | NM_001048179.1 | L: 89-106 | R: gcctgctctcccctctctct | R: 161-181 |
| CCL28 murine    | L: ctcaccaggtgtgtga | NM_020279.3 | L: 208-226 | R: gcctgctctcccctctctct | R: 231-250 |
| CXCL1 murine    | L: cctggaggagggagggagt | NM_008176.3 | L: 39-58   | R: gcctgctctcccctctctct | R: 150-167 |
| CXCL2 murine    | L: ctcaccaggtgtgtga | NM_009140.2 | L: 308-353 | R: gcctgctctcccctctctct | R: 379-398 |
| CXCL3 murine    | L: ctcaccaggtgtgtga | NM_203320.2 | L: 317-336 | R: gcctgctctcccctctctct | R: 404-426 |
| CXCL4 murine    | L: ctcaccaggtgtgtga | NM_009932.4 | L: 303-322 | R: gcctgctctcccctctctct | R: 376-395 |
| CXCL5 murine    | L: ctcaccaggtgtgtga | NM_009141.2 | L: 73-92   | R: gcctgctctcccctctctct | R: 141-159 |
| CXCL7 murine    | L: ctcaccaggtgtgtga | NM_023785.2 | L: 129-148 | R: gcctgctctcccctctctct | R: 204-222 |
| CXCL9 murine    | L: ctcaccaggtgtgtga | NM_008599.4 | L: 72-91   | R: gcctgctctcccctctctct | R: 127-146 |
| CXCL10 murine   | L: ctcaccaggtgtgtga | NM_021274.1 | L: 53-70   | R: gcctgctctcccctctctct | R: 146-163 |
| CXCL11 murine   | L: ctcaccaggtgtgtga | NM_019494.1 | L: 55-74   | R: gcctgctctcccctctctct | R: 125-146 |
| CXCL12 murine   | L: ctcaccaggtgtgtga | NM_021704.3 | L: 278-297 | R: gcctgctctcccctctctct | R: 328-347 |
| CXCL13 murine   | L: ctcaccaggtgtgtga | NM_018866.2 | L: 34-51   | R: gcctgctctcccctctctct | R: 92-110 |
| CXCL14 murine   | L: ctcaccaggtgtgtga | NM_019568.2 | L: 209-226 | R: gcctgctctcccctctctct | R: 265-283 |
| CXCL15 murine   | L: ctcaccaggtgtgtga | NM_011339.2 | L: 43-60   | R: gcctgctctcccctctctct | R: 106-128 |
| CXCL16 murine   | L: ctcaccaggtgtgtga | NM_023158.6 | L: 801-823 | R: gcctgctctcccctctctct | R: 858-877 |
| CXCL17 murine   | L: ctcaccaggtgtgtga | NM_153576.2 | L: 135-154 | R: gcctgctctcccctctctct | R: 206-225 |
| Gene | Sequence | Accession Code | Position |
|------|----------|----------------|----------|
| XCL1 murine | L: agacttctctcctgactttcct | NM_008510.1 | L: 24-46 |
|        | R: gacttcagtccccacacctt | | R: 79-98 |
| CXCL1 murine | L: cactctgatgctgatgatac | NM_009142.3 | L: 229-249 |
|        | R: cagaagcgtctgtgctgtg | | R: 287-306 |
| HPRT murine | L: ccctcctcactcctggctctt | NM_013556.2 | L: 105-123 |
|        | R: aacctggttcatcatcgcta | | R: 175-195 |
| CCL1 human | L: tgctgctgctgctgctgctg | NM_002981.2 | L: 103-121 |
|        | R: cgagagagagagagagagagag | | R: 168-167 |
| CCL2 human | L: agctctcgccctctctctct | NM_002982.3 | L: 79-96 |
|        | R: gggcttgaggtctgctgctg | | R: 153-171 |
| CCL3 human | L: tgagctgctgctgctgctg | NM_002983.2 | L: 154-173 |
|        | R: cagctgctgctgctgctg | | R: 211-228 |
| CCL3L1 human | L: cgctgctgctgctgctgctg | NM_01001437.3 | L: 135-154 |
|        | R: tgtcgggaggtgtagctga | | R: 192-210 |
| CCL3L3 human | L: cgctgctgctgctgctgctg | NM_002984.2 | L: 125-144 |
|        | R: tgtcgggaggtgtagctga | | R: 182-200 |
| CCL5 human | L: cgctgctgctgctgctgctg | NM_002985.2 | L: 200-220 |
|        | R: cagctgctgctgctgctg | | R: 254-272 |
| CCL7 human | L: cactctgctgctgctgctg | NM_002986.2 | L: 78-97 |
|        | R: gggcttgaggtctgctgctg | | R: 165-188 |
| CCL8 human | L: cactctgctgctgctgctg | NM_002987.2 | L: 509-527 |
|        | R: ggttgccaggtctgctgctg | | R: 584-603 |
| CCL13 human | L: cgctgctgctgctgctgctg | NM_002988.2 | L: 67-89 |
|        | R: cagctgctgctgctgctg | | R: 148-166 |
| CCL14 human | L: cgctgctgctgctgctgctg | NM_032962.4 | L: 263-283 |
|        | R: cagctgctgctgctgctg | | R: 341-360 |
| CCL15 human | L: cgctgctgctgctgctgctg | NM_032965.5 | L: 567-585 |
|        | R: cagctgctgctgctgctg | | R: 638-660 |
| CCL16 human | L: cgctgctgctgctgctgctg | NM_004590.3 | L: 95-114 |
|        | R: cagctgctgctgctgctg | | R: 151-172 |
| CCL17 human | L: cgctgctgctgctgctgctg | NM_002989.2 | L: 48-68 |
|        | R: cagctgctgctgctgctg | | R: 136-153 |
| CCL18 human | L: cgctgctgctgctgctgctg | NM_002988.3 | L: 114-131 |
|        | R: cagctgctgctgctgctg | | R: 167-188 |
| CCL19 human | L: cgctgctgctgctgctgctg | NM_006274.2 | L: 161-179 |
|        | R: cagctgctgctgctgctg | | R: 235-253 |
| CCL20 human (var1) | L: cagctgctgctgctgctgctg | NM_004591.2 | L: 71-92 |
|        | R: cagctgctgctgctgctg | | R: 143-162 |
| CCL21 human | L: cgctgctgctgctgctgctg | NM_002989.2 | L: 73-94 |
|        | R: cagctgctgctgctgctg | | R: 158-175 |
| CCL22 human | L: cgctgctgctgctgctgctg | NM_002990.4 | L: 178-199 |
|        | R: cagctgctgctgctgctg | | R: 253-254 |
| CCL23 human (var1) | L: cagctgctgctgctgctgctg | NM_145898.3 | L: 62-81 |
|        | R: cagctgctgctgctgctg | | R: 148-170 |
| CCL25 human (var1) | L: cagctgctgctgctgctgctg | NM_005624.3 | L: 378-397 |
|        | R: cagctgctgctgctgctg | | R: 457-479 |
| CCL27 human | L: cagctgctgctgctgctgctg | NM_006664.2 | L: 105-123 |
|        | R: cagctgctgctgctgctg | | R: 149-168 |
| CCL28 human | L: cagctgctgctgctgctgctg | NM_148672.2 | L: 228-247 |
|        | R: cagctgctgctgctgctg | | R: 271-290 |
|        | | | R: 296-313 |
| Comp. Factor D human | L: cagctgctgctgctgctgctg | NM_001928.2 | L: 384-401 |
|        | R: cagctgctgctgctgctg | | R: 340-359 |
| CCL1 human | L: cagctgctgctgctgctgctg | NM_001151.2 | L: 425-444 |
|        | R: cagctgctgctgctgctg | | R: 431-452 |
| CCL2 human | L: cagctgctgctgctgctgctg | NM_002089.3 | L: 506-525 |
|        | R: cagctgctgctgctgctg | | R: 444-469 |
| CCL3 human | L: cagctgctgctgctgctgctg | NM_002090.2 | L: 537-554 |
|        | R: cagctgctgctgctgctg | | R: 340-358 |
| CCL4 human | L: cagctgctgctgctgctgctg | NM_002619.3 | L: 388-407 |
|        | R: cagctgctgctgctgctg | | R: 185-203 |
| CCL5 human | L: cagctgctgctgctgctgctg | NM_002994.4 | L: 233-250 |
|        | R: cagctgctgctgctgctg | | R: 310-329 |
| CCL6 human | L: cagctgctgctgctgctgctg | NM_002993.3 | L: 170-187 |
|        | R: cagctgctgctgctgctg | | R: 237-263 |
| CCL7 human | L: cagctgctgctgctgctgctg | NM_002704.3 | L: 183-203 |
|        | R: cagctgctgctgctgctg | | R: 232-255 |
| CCL9 human | L: cagctgctgctgctgctgctg | NM_002416.1 | L: 363-385 |
|        | R: cagctgctgctgctgctg | | R: 469-494 |
| CCL10 human | L: cagctgctgctgctgctgctg | NM_001565.3 | L: 214-232 |
|        | R: cagctgctgctgctgctg | | R: 272-291 |
| CCL11 human | L: cagctgctgctgctgctgctg | NM_005409.4 | L: 249-268 |
|        | R: cagctgctgctgctgctg | | R: 304-323 |
TABLE 1 (continued)

| Gene          | Sequence              | Accession Code | Position |
|---------------|-----------------------|----------------|----------|
| CXCL13 human  | L: `cctcccttcaggccca` | NM_006419.2    | L: 97-116 |
|               | R: `tcacagttgaagtcctca` |                | R: 149-172 |
| CXCL14 human  | L: `aaatctggaagaacgcaaa` | NM_004887.4    | L: 622-641 |
|               | R: `tggagctggacagcaac` |                | R: 688-707 |
| CXCL16 human  | L: `tcctctggtcagcaggaag` | NM_022059.2    | L: 1255-1275 |
|               | R: `caggttatagacgceca` |                | R: 1399-1331 |
| CXCL17 human  | L: `accggacagctctcaag` | NM_198477.2    | L: 317-334 |
|               | R: `gggctctcaggaaccaatct` |               | R: 375-394 |
| gp10 human    | L: `aggggcttctcggcaac` | NM_002184.3    | L: 1097-1116 |
|               | R: `gaagagacgaggggagtg` |                | R: 1146-1165 |
| il-16 human   | L: `ggaggtctcgcacgcaag` | NM_004513.5    | L: 1865-1882 |
|               | R: `ccacacagctggaatgme` |                | R: 1960-1978 |
| XCL1 human    | L: `actctctgctagacatgc` | NM_002995.2    | L: 132-151 |
|               | R: `tgaggtactctgcacac` |                | R: 226-248 |
| XCL2 human    | L: `actctctgctagacatgc` | NM_003175.3    | L: 1-20 |
|               | R: `tgaggtactctgcacac` |                | R: 92-114 |
| MITF human (var5) | L: `aggggcttctcggcaac` | NM_198158.2    | L: 681-702 |
|               | R: `tgaggtactctgcacac` |                | R: 752-775 |
| GAPDH human (var1) | L: `agccacatcgctcagacac` | NM_002046.5    | L: 169-187 |
|               | R: `ggcctctgacagcaac` |                | R: 216-234 |

D5, ThermoFisher Scientific, 1:1000) and secondary mouse monoclonal IgG HRP-conjugated antibody (clone HA007, R&D Systems, 1:2000). β-Actin mouse monoclonal IgG HRP-conjugated antibody (clone C4, Santa Cruz Biotechnology, Santa Cruz, CA; 1:3000) was used as a loading control. Chemiluminescence was measured using ChemiDoc Imaging Systems (BioRad). Densitometry was performed with Image Lab Software (BioRad).

Semi-quantitative Real-Time PCR

Chemokine mRNA in tumor cell lines and tumor lysates was quantified by real-time PCR. RNA from single cell suspensions or tumor lysates was extracted using Trizol Reagent (Life Technologies, Carlsbad, CA) and quantified with the NanoDrop instrument (ThermoFisher Scientific, Waltham, MA) with ChemiDoc Imaging Systems (BioRad). Densitometry was performed with Image Lab Software (BioRad).

Transduction of MITF

All constructs were generated by overlap extension PCR and recombinant expression cloning into the retroviral pMPT71 vector, as follows: The retroviral vector pMPT7 (kindly provided by C. Baum, Hannover) was used for transfection of the ecotropic packaging cell line Plat-E for transduction of murine cell line YUMM1.1 and the packaging cell line Plat-A for the human cell lines. Transduction protocols have been described in detail before [24]. In brief, the packaging cell lines Plat-E or Plat-A were transfected with an MITF-linker-GFP construct, where the linker 2A-sequence was noncleaving through deletion of two amino acids. This allowed to confirm nuclear expression of MITF by confocal microscopy. The produced retrovirus was used to transduce YUMM1.1-, WM8-, and WM35-cells. Transduction efficacy for MITF was controlled by qRT-PCR. The human MITF-M transcript variant 5 (Plasmid #38131, Addgene, Cambridge, MA) was used for transduction of both murine and human cell lines.

Statistics

Statistical analysis was performed with GraphPad Prism Software. Significance was analyzed with unpaired Student’s t test or, for tumor growth, with two-way ANOVA with Bonferroni correction. Error bars indicate SEM.

Results

MITF Knockdown and Immune Cell Infiltration in Murine B16F10 Melanoma

MITF knockdown has been shown to affect secretion of defined cytokines and chemokines from melanoma cells [12,17]. We performed siRNA-mediated knockdown of MITF on B16F10 melanoma cells (Figure 1A), which highly express MITF at baseline, and analyzed mRNA expression of C-C and C-X-C motif chemokines (Supplementary Figure 1A). MITF knockdown was confirmed with Western blot (Supplementary Figure 1B). Chemokine mRNA levels changed in B16F10 cells upon transfection with siRNA targeting MITF (siMITF) as compared to a siRNA control (siControl). CCL17, CCL25, and CXCL4 were upregulated in MITF knockdown B16F10 cells, whereas CCL5 and CXCL10, highly expressed at
baseline levels, were significantly downregulated both on mRNA (Supplementary Figure 1A) and on protein level (Figure 1B). We next analyzed the effects of MITF knockdown on immune cell migration towards melanoma cells. In vitro, migration of various immune cell subtypes towards supernatants of siMITF-transfected B16F10 cells was reduced as compared to siControl-transfected cells (Figure 1C). In fact, reduction of CD8+ and CD4+ T cells, NK cells, B cells, and CD11c+ dendritic cells (DCs) was most pronounced. In vivo, siMITF-transfected B16F10 cells showed an accelerated growth rate as compared to control-transfected B16F10 cells (Figure 1D). Quantification of tumor-infiltrating immune cells recapitulated in vitro observations, showing lower recruitment of immune cells to the tumor site; overall numbers of CD45+ cells as well as numbers of CD3+ T cells and CD11b+ myeloid cells in MITF knockdown tumors were reduced (Figure 1E). The number of DC and NK cells remained unchanged (Supplementary Figure 1C). Our in vitro results were further confirmed by analyzing protein levels of intratumoral chemokines; CXCL10 was reduced in siMITF-transfected tumors. In addition, CCL6, CCL8, CCL9/10, CCL12, CCL21, CCL22, CCL27, CCL28, CXCL1, CXCL9, CXCL11, and CX3CL1 were strongly reduced in siMITF-transfected tumors as compared to controls (Supplementary Figure 1D).

**MITF Overexpression in YUMM1.1 Melanoma Cells and In Vitro Immune Cell Migration**

We next examined YUMM1.1 melanoma cells, a cell line derived from a genetically engineered mouse model characterized by BRAF activation as well as PTEN and Cdkn2a inactivation, which is considered to mimic characteristics of human melanoma [21]. YUMM1.1 cells express very low levels of MITF at baseline, which make them a suitable tool for MITF overexpression studies. We transduced YUMM1.1 cells with MITF (or GFP as a control) and confirmed overexpression of MITF by qRT-PCR (Figure 2A) and

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**Figure 1.** MITF knockdown in B16F10 melanoma results in altered chemokine levels and immune cell attraction. (A) B16F10 cells were transfected with siControl or MITF siRNA. Bars display MITF copies per HPRT as determined by quantitative RT-PCR. (B) Levels of CCL5 and CXCL10 in supernatants of transfected B16F10 cells were analyzed after 48 hours of culture by chemokine ELISA. (C) A total of 10⁶ C57BL/6 splenocytes were allowed to migrate for 6 hours towards supernatants or RPMI 0.5% BSA and analyzed by flow cytometry. Dots indicate migrated cells per well. (D, E) C57BL/6 mice were injected with 2.5 × 10⁵ B16F10 cells transfected with siControl (n = 8) or MITF siRNA (n = 8). (D) Tumor growth over time is displayed. Tumors were harvested on days 11 or 15, and immune cells were analyzed by flow cytometry. (E) Cells per mg tumor are displayed. Panels A to C are representative of three independent experiments. Panels D and E are pooled from two independent experiments.
Western Blot (Supplementary Figure 2B). When analyzing chemokine expression in YUMM1.1 cells, we found that MITF overexpression had no significant influence on CCL2 expression (Supplementary Figure 2C) but particularly led to upregulated CCL5 and CXCL10 expression on mRNA (Supplementary Figure 2A) and protein levels (Figure 2B) as compared to control cells. This was paralleled by increased immune cell migration, specifically, of the lymphocyte populations such as CD8+ and CD4+ T cells, B cells, and NK cells, which was induced by supernatants of MITF-overexpressing YUMM1.1 cells (Figure 2C). No difference in migration was seen for DC and other CD11b+ myeloid cells (Supplementary Figure 2D).

**MITF Overexpression and Tumor Growth in YUMM1.1 Melanomas**

Next, we injected MITF-transduced YUMM1.1 cells subcutaneously into mice. Surprisingly, MITF-transduced YUMM1.1 tumors showed accelerated growth as compared to control-transduced cells

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**Figure 2.** MITF overexpression in YUMM1.1 cells changes chemokine expression and immune cell attraction. (A) Relative MITF mRNA expression of MITF-transduced YUMM1.1 cells. (B) ELISA assay for chemokine detection in supernatants of transduced YUMM1.1 cells was analyzed after 48 hours of culture. (C) A total of 10^6 C57BL/6 splenocytes were allowed to migrate for 6 hours towards YUMM1.1 supernatants or migration medium and analyzed by flow cytometry. Dots indicate migrated cells per well. Three independently collected supernatants were used. (D, E) C57BL/6 mice were injected with 2 × 10^6 YUMM melanoma cells transduced with MITF (n = 10) or with GFP (n = 9). (D) Tumor growth over time. Tumors were harvested on days 22 or 27 and analyzed by flow cytometry. (E) Cells per mg tumor. Data are pooled from two independent experiments. Panel B is representative of three independent experiments.
When analyzing tumor infiltrating immune cells, we observed that the in vivo phenotype closely resembled the phenotype we had observed in MITF knockdown B16F10 tumors. The number of total CD45+ cells as well as of CD8+ T cells and CD4+ T helper cells was decreased, while the numbers of CD11b+ myeloid cells and NK cells remained unchanged (Figure 2E and Supplementary Figure 2). In contrast to the B16 model, numbers of B220+ B cells and CD11c+ DC were also decreased in MITF-transduced YUMM1.1

Figure 3. MITF knockdown in human melanoma cell lines WM8 and WM35. (A, B) Human WM8 (A) or WM35 (B) melanoma cells were transfected with control or with MITF siRNA. Knockdown efficiency was confirmed by q-RT-PCR. Copies of MITF per GAPDH are displayed. A total of 10^6 human PBMCs were transferred into the upper well of a Transwell migration system; lower wells were loaded with WM8 or WM35 supernatants or migration medium. Cells were left to migrate for 6 hours, and migrated cells were analyzed by flow cytometry. Dots indicate migrated cells per well. Data are representative of at least three independent experiments.

Figure 4. MITF overexpression in human melanoma cell lines WM8 and WM35. (A, B) Relative MITF mRNA expression of MITF-transduced WM8 (A) or WM35 (B) melanoma cells. MITF expression was confirmed by qRT-PCR. A total of 10^6 human PBMCs were transferred into the upper well of a Transwell migration system; lower wells were loaded with WM8 or WM35 supernatants or migration medium. Cells were left to migrate for 6 hours, and migrated cells were analyzed by flow cytometry. Dots indicate migrated cells per well. Data are representative of three independent experiments. (C) Chemokine mRNA per GAPDH in MITF-transduced (MITF) and MITF knockdown (siMITF) WM8 and WM35 cells relative to control condition (siC, siControl; UT, untransduced). Data are pooled from three independent experiments.
tumors (Figure 2E). When analyzing intratumoral chemokine levels, we found that mRNA expression of most chemokines in MITF-transduced tumors remained unchanged or was reduced when compared to control tumors (Supplementary Figure 2F). This included CCL5 and CXCL10, which we had found upregulated in MITF-transduced YUMM1.1 cells in vitro (Supplementary Figure 2F).

MITF in Human Melanoma Cells and Immune Cell Migration

We next sought to determine the role of MITF expression levels in human melanoma cells. We investigated WM8 and WM35 melanoma cell lines, both known to express intermediate baseline levels of MITF. This allows performing knockdown and overexpression studies within the same cell line. MITF knockdown was performed with siRNA and was confirmed by qPCR (Figure 3, A and B) and Western Blot (Supplementary Figure 3, A and B). MITF knockdown resulted in an increased migration of peripheral blood mononuclear cells (PBMC), more specifically of CD56+ NK cells, towards tumor cell supernatants of both WM8 and WM35 cells (Figure 3, A and B). For WM8 cells, an increased migration of CD14+ monocyes towards tumor cell supernatant was observed (Figure 3A). There were no consistent changes in the migration of T or B cells towards MITF-silenced supernatants of WM8 or WM35 cells, with increased migration of CD4+ T cells in only 1 out of 3 experiments (Supplementary Figure 3, C and D). MITF overexpression in WM8 and WM35 cells was confirmed by qPCR (Figure 4, A and B) and Western Blot (Supplementary Figure 4, A and B). MITF overexpression resulted in reduced migration of CD14+ myeloid cells and CD15+ cells towards tumor supernatants (Figure 4, A and B). When analyzing chemokine expression levels in WM8 and WM35 cells upon MITF siRNA knockdown or MITF overexpression, we found a comparable expression pattern in both tumor cell lines; MITF knockdown resulted in increased expression of CCL2; CXCL1; and, in WM8 cells only, of CXCL2, whereas levels of CCL15 and CCL19 were reduced in both cell lines. Vice versa, MITF overexpression mostly decreased expression of chemokines, e.g., CCL18, CXCL14, and CXCL16 (Figure 4C).

Discussion

We have analyzed the influence of MITF on melanoma cell chemokine expression and immune cell attraction. Unexpectedly, MITF was found to have opposing effects on chemokine expression in murine and human melanoma cells: In the murine cell lines, MITF knockdown resulted in reduced immune cell attraction, while MITF overexpression consistently resulted in increased immune cell migration. The exact opposite was observed in the human melanoma cell lines analyzed. We assume that this may be due to differences in genetic backgrounds, driver mutations of the melanoma cell lines, and diverging underlying mutations rather than to species-specific differences. Gene expression differences between murine and human melanoma cell lines have been described before [25]. For example, it is known that loss of PTEN, as for the YUMM1.1 cell line, mediates an immunosuppressive phenotype associated with decreased T cell infiltration [26]. Moreover, BRAF mutations have previously been reported to influence MITF transcription [27]. A more extensive analysis of different human and murine melanoma cell lines combined with an in-depth study of genomic mutations and their interactions with MITF will be necessary to answer the observed differences of human and murine melanoma cell lines.

Chemokine expression patterns and immune cell subtypes attracted to the tumor cells also differed between the murine and human melanoma models: murine cell lines mostly attracted lymphoid cell types, whereas human cell lines preferentially attracted CD14+ monocytic cells. This is in line with previous reports, according to which B16F10 tumors are marked by a dominantly lymphoid cell infiltration, whereas human melanomas induce migration of myeloid cells [17,28]. Also in pancreatic cancer, different immune cell infiltrates in mice and humans have been reported: murine models are dominated by inhibitory myeloid cell infiltrates, while in humans, pancreatic adenocarcinomas T cells are highly prevalent [29]. Despite many conserved mechanisms and pathways, differences exist between the human and the murine immune system, including surface receptor expression, chemokine expression, and functional aspects of immune cell populations [30].

Interestingly, only few chemokines were expressed on mRNA level in the two murine cell lines studied. Of those, only CXCL10 was considerably reduced in MITF-deficient B16F10 cells. Strikingly, CXCL10 and CCL5 were upregulated in MITF-transduced YUMM1.1 melanoma cells. These chemokines are known to recruit T and NK cells to tumors via CCR5 and CXCR3. In CXCR3-KO mice, B16F10 tumors have decreased infiltration with CD8+ T cells and reduced survival of mice [31–33]. Intratumoral CCL5 has also been reported to play a role in melanoma progression, with melanoma cell-intrinsic CCR5 expression [34,35]. It would be interesting to further dissect the role of MITF-regulated expression of these chemokines in melanoma.

In the human melanoma cell lines investigated in this study, MITF siRNA knockdown led to an increased migration of CD14+ monocytic cells, whereas overexpression of MITF showed opposing effects. These findings are in line with a recent study reporting the recruitment of CD14+ cells into MITFlow human melanomas [17]. In this study, murine MITFlow cell lines showed a high infiltration with CD14+ myeloid cells linked to a high expression of CCL2, CCL5, and CXCL10. In contrast to our study however, MITFlow cell lines were directly compared to MITFhigh cell lines, which did not allow for assessing acute changes of MITF levels within the same cell line. In our human melanoma cells, chemokine arrays showed an induction of CCL2 upon MITF knockdown, which is in line with current studies describing the CCL2-MITF axis as a component of the senescence-related secretome [12,17]. We also observed increased levels of CXCL1 in MITF-knockdown human melanoma cells. CXCL1 is known to be critically involved in melanoma development, angiogenesis, and growth [36,37]. MITF has been demonstrated to bind the CXCL1 promoter region in chromatin immunoprecipitation assays [38].

In our in vivo models, we observed reduced numbers of T cells and myeloid cells in MITF-knockdown B16F10 tumors concurrent with higher tumor growth. Surprisingly, MITF-transduced YUMM1.1 cells also showed accelerated tumor growth, and we found decreased intratumoral immune cell numbers. This is in contrast to the observed increase in immune cell attraction to MITF-transduced YUMM1.1 cells in vitro. When analyzing intratumoral chemokine levels, we found these diverging from in vitro tumor cell chemokine expression: overall chemokine levels were lower in both MITF-knockdown and MITF-transduced tumors. The opposing findings in vitro and in vivo for the YUMM1.1 cell line may be attributed to the
tumor environment where, in contrast to cell culture, stromal and immune cells contribute to chemokine production. While we can clearly demonstrate in vitro how changes in MITF levels affect chemokine levels in and immune cell attraction to melanoma cells, the complexity of the tumor microenvironment in vivo seems to overcome the in vitro effects, at least in the YUMM1.1 model. Several explanations are possible for the discrepancy of our in vitro and in vivo observations: tumor-associated immune cells might contribute to a high extent to intratumoral chemokine levels and thereby influence the tumor microenvironment; interactions with the tumor microenvironment as well as the high growth rate might affect the biology of YUMM1.1 tumor cells in vivo, modifying chemokine secretion; or immune cell-mediated selective pressure might lead to the domination of MITF low tumor cells in vivo. These results shed further light on the discrepancies in the consequences of different MITF levels for patient’s prognosis. While higher or lower levels of MITF might have opposing consequences on the melanoma cells themselves, the impact on the environment will differ as well, thus potentially compensating or reverting function of MITF levels on disease outcome. Our data clearly show that changes in MITF levels influence the tumor microenvironment by suppressing immune cell migration to or accumulation at the tumor site and, thus, leading to an increased tumor growth. This is of importance since BRAF inhibitors, which are currently used for melanoma treatment, influence intratumoral MITF levels [39].

It is hard to define the exact role for MITF in shaping the immunological tumor microenvironment because of differing interactions with melanoma driver mutations in different melanoma cell lines (such as BRAF mutations, which are highly prevalent in human melanomas but only present in the YUMM1.1 cell line). Moreover, the kinetics of dynamic effects, as compared to stably decreased levels of MITF, may play a role in our knockdown and overexpression experiments. In our studies, we have both observed divergences between in vitro and in vivo findings as well as divergences between human and murine melanoma cell lines. We believe that melanomas of different origin, endowed with diverging underlying mutations and variable MITF expression at steady state, will react differently to transient changes in MITF levels. A detailed study of a higher number of different melanomas and their interactions with the immune system will be necessary to depict effects of MITF levels in different contexts. Nevertheless, our results show that MITF clearly influences chemokine expression and immune cell attraction in different murine and human melanomas. Since the emergence of checkpoint blockade has revolutionized melanoma therapy in the past years [14,40] it has become more and more important to understanding the factors which regulate melanoma immunogenicity and immune cell infiltration. Our study provides a first step to understanding the factors which regulate melanoma immunogenicity and immune cell infiltration. Our study provides a first step to understanding how changes in MITF levels affect chemokine levels in vivo and influence the interaction of melanoma cells with immune cells and might provide a basis for further therapeutic interventions in combination with targeted immunotherapies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2018.10.014.

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Conflict of Interest Statement

The authors declare that no potential conflicts of interest exist.

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