M-CSF and GM-CSF Receptor Signaling Differentially Regulate Monocyte Maturation and Macrophage Polarization in the Tumor Microenvironment

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Abstract

Tumors contain a heterogeneous myeloid fraction comprised of discrete MHC-IIhi and MHC-IIlo tumor-associated macrophage (TAM) subpopulations that originate from Ly6Chi monocytes. However, the mechanisms regulating the abundance and phenotype of distinct TAM subsets remain unknown. Here, we investigated the role of macrophage colony-stimulating factor (M-CSF) in TAM differentiation and polarization in different mouse tumor models. We demonstrate that treatment of tumor-bearing mice with a blocking anti-M-CSF monoclonal antibody resulted in a reduction of mature TAMs due to impaired recruitment, extravasation, proliferation, and maturation of their Ly6Chi monocyte precursors. M-CSF signaling blockade shifted the MHC-IIlo/MHC-IIhi TAM balance in favor of the latter as observed by the preferential differentiation of Ly6C hi monocytes into MHC-II hi TAMs. In addition, the genetic and functional signatures of MHC-IIhi TAMs were downregulated upon M-CSF receptor blockade, indicating that M-CSFR signaling shapes the MHC-IIlo TAM phenotype. Conversely, granulocyte macrophage (GM)-CSFR had no effect on the mononuclear tumor infiltrate or relative abundance of TAM subsets. However, GM-CSF signaling played an important role in fine-tuning the MHC-IIlo phenotype. Overall, our data uncover the multifaceted and opposing roles of M-CSFR and GM-CSFR signaling in governing the phenotype of macrophage subsets in tumors, and provide new insight into the mechanism of action underlying M-CSFR blockade. Cancer Res; 76(1): 35–42. ©2015 AACR.

Introduction

Tumors are often highly infiltrated with inflammatory cells such as tumor-associated macrophages (TAM). TAMs are now considered to promote tumor growth in multiple ways, including induction of angiogenesis, remodeling of the extracellular matrix, stimulation of cancer cell proliferation and metastasis, and inhibition of adaptive immunity, the clinical significance of which is evidenced by the correlation between high TAM density and worse outcome in most cancer types.

Ly6C hi monocytes differentiate into distinct TAM subsets with specialized functions and a different intratumoral localization: normoxic M1-like MHC-II hi and hypoxic M2-like MHC-II hi TAM (3, 4). As hypoxia is not the main driver of TAM infiltration and differentiation (4), we considered myelopoietic growth factor receptor signaling as governor of TAM heterogeneity. In this respect, M-CSF–driven macrophage activation leads to the expression of a substantial part of the M2 transcriptome, while granulocyte macrophage colony-stimulating factor (GM-CSF) induces a M1 phenotype (5, 6). As such, blocking M-CSFR (CSF1R, CD115) signaling in myometrial macrophages stimulated the occurrence of an M1-like MHC-II hi population at the expense of M2-like MHC-II lo macrophages in the pregnant mouse uterus (7). M-CSFR signaling was recently also shown to be critical for TAM differentiation and shaping the M1/M2 TAM phenotype (8–11). However, which aspects of the monocyte-to-TAM differentiation pathway are influenced by M-CSFR and whether GM-CSF also skews the TAM phenotype remain largely unexplored (12).

Using mouse tumors of different histologic origin, we show here, that Ly6C hi monocytes depend on M-CSFR signaling for their recruitment and extravasation to tumors and differentiation to mature TAM. M-CSFR signaling governs the phenotype of M2-like MHC-II lo TAM, and its blockade resulted in a preferential differentiation of monocytes to M1-like MHC-II hi TAM. Although GM-CSFR receptor (GM-CSFR, CSF-2R, or CD115) signaling is not determining monocyte recruitment and differentiation, it fine tunes the molecular profile of MHC-II hi TAM, hence uncovering...
the differential role played by M-CSFR and GM-CSFR signaling in macrophage differentiation and polarization in tumors.

Materials and Methods

Detailed Materials and Methods can be found in the Supplementary Information.

Mice, cell lines, and tumor models

Female C57BL/6 mice and ubiquitin-GFP mice (C57BL/6) were purchased from Janvier and Jackson, respectively. Cx3cr1gfp/Cx3cr1gfp (C57BL/6), GM-CSFR(–/−) (Csf2rb−/−) (C57BL/6), Il4ra−/−(C57BL/6), and MMTV-PyMT (FVB) mice were respectively provided by Frank Tacke (Aachen University, Germany), Melanie Greter (University of Zurich, Germany), Frank Brombacher (University of Cape Town, South Africa), and Massiliano Mazzone (KULeuven, Belgium). Procedures followed the guidelines of the Belgian Council for Laboratory Animal Science. C57BL/6 or Balb/c mice were injected subcutaneously with 3 × 10^6 3LL-R Lewis lung carcinoma or TS/A breast carcinoma cells, respectively.

Tumor preparation, flow cytometry, and cell sorting

Tumor single-cell suspensions were prepared as described (3). Antibodies for cell surface staining are listed in Supplementary Table S1. To prevent aspecific binding, TAMs were preincubated with rat anti-mouse CD16/CD32 (2.4G2, BD Biosciences). Delta fluorescence intensity (ΔMFII) was calculated as: (MFII staining) – (MFII isotype staining). FACs data were acquired using a BD FACS Canto II (BD Biosciences) and analyzed using FlowJo (Tree Star, Inc.). To purify monocytes or TAM, cells were sorted using a BD FACS Aria II (BD Biosciences) from 4 to 8 pooled tumors or pooled blood from 6 to 8 mice.

Anti-M-CSFR antibody administration

Anti-M-CSFR antibody (300 µg per intravenous injection; clone AFS98) or isotype control antibody [anti-human CD44 clone Hermes-1 (rat IgG2a)] producing hybridomas were originally provided by Miriam Merad and antibodies were prepared in-house. Treatment schedules of mice are listed in the legend of each figure. Briefly, mice were either treated every two days starting from tumor inoculation or received one shot of antibody before sacrifice depending on the experiment.

Measurement of IL34, M-CSF, GM-CSF, and CCL2 production

Concentrations of cytokines and chemokines were measured by ELISA following the manufacturer's protocol (R&D Systems; see Supplementary Information).

Adoptive bone marrow and monocyte transfers

Blood and bone marrow Ly6C<sup>hi</sup>GFP<sup>+</sup> monocytes were sorted from ubiquitin-GFP mice. A total of 10<sup>6</sup> cells were intravenously injected in 3LL-R tumor-bearing mice. The fate of the GFP<sup>+</sup> progeny was determined 48 hours later. For bone marrow adoptive transfers, 10<sup>7</sup> bone marrow cells from ubiquitin-GFP mice were injected 4 hours before checking GFP<sup>+</sup>Ly6C<sup>hi</sup> monocyte presence in blood and tumor.

Extravasation of monocytes

Mice were intravenously injected 1 minute before sacrifice with 1 µg rat anti-CD45/Pecy7 (30-F11; eBioscience) or rat IgG2b isotype control/PECy7 (eB149/10H5; eBioscience) as was described before (7, 13).

Intravital imaging

Intravital imaging was performed following a previously reported protocol (14) with minor modifications (Supplementary Information).

In vitro phagocytosis assay

For in vitro latex uptake, freshly isolated tumor single-cell suspensions were cultured for 40 minutes at 4°C (control) or 37°C, in the presence of yellow–green latex microspheres (Polysciences; 0.5 µm) diluted 1:5,000. Latex uptake by tumor CD11b<sup>+</sup>Ly6G<sup>−</sup> cells was assessed via flow cytometry.

Bromodeoxyuridine labeling

Tumor-bearing mice were given an intraperitoneal injection of 1 mg bromodeoxyuridine (BrdUrd) 4 hours before sacrifice. Tumors were collected and BrdUrd intracellular staining was performed following the manufacturer's instructions (BD Biosciences; Supplementary Information).

RNA extraction, cDNA preparation, and quantitative real-time PCR

These experiments were performed as described before (3). RNA was extracted using TRIzol (Invitrogen) and was reverse transcribed with oligo(dT) and SuperScript II RT (Invitrogen). Quantitative real-time PCR was performed in an iCycler, with iQ SYBR Green Supermix (Bio-Rad). Primer sequences are listed in Supplementary Table S2. PCR cycles consisted of 1° 94°C, 45° 55°C, 1° 72°C. Gene expression was normalized using ribosomal protein S12 (Mrps12) as a housekeeping gene.

Statistical analysis

Significance was determined by the Student t test or ANOVA followed by a post test using GraphPad Prism 6.0 software. A P value < 0.05 was considered statistically significant. All graphs show mean ± SEM.

Results

M-CSFR signaling blockade impairs the extravasation of tumor-infiltrating Ly6C<sup>hi</sup> monocytes

Treatment with blocking anti-M-CSFR monoclonal antibody (mAb) AFS98, but not an isotype control mAb, caused a significant reduction of the CD11b<sup>+</sup>Ly6G<sup>−</sup>SiglecF<sup>+</sup> infiltrate in subcutaneous 3LL-R lung carcinoma tumors (Fig. 1A, gating strategy in Supplementary Fig. S1A), subcutaneous TS/A mammary carcinoma tumors, and transgenic MMTV-PyMT mammary carcinomas (Supplementary Fig. S1B).

To assess whether anti-M-CSFR treatment impaired the extravasation of monocytes into 3LL-R tumors, we injected anti-CD45/PECy7 intravenously to label all intravascular hematopoietic cells (7, 13) and one minute later (a time span that is too short for labeled cells to extravasate) we quantified labeled versus unlabeled Ly6C<sup>hi</sup> monocytes in excised tumors. Significantly, more Ly6C<sup>hi</sup> monocytes remained in the tumor vasculature upon M-CSFR blockade (Supplementary Fig. S1C). These findings were corroborated via intravital microscopy of anti-M-CSFR–treated tumors in CX3CR1<sup>gfp</sup>/C0 mice (Fig. 1B). Reduced monocyte recruitment and extravasation could be secondary to an anti-M-CSFR–mediated...
M-CSF and GM-CSF Receptor in Determining the TAM Phenotype

Figure 1.
M-CSF regulates the behavior of tumor-infiltrating monocytes. A, the absolute number of CD11b⁺ Ly6G⁻ SiglecF⁻ cells was determined in 3LL-R tumor single-cell suspensions after treatment of tumor-bearing mice with either isotype antibody or α-M-CSFR antibody every two days from day 0 postinjection until sacrifice (day 14 postinjection). Results are representative of three independent experiments with n ≥ 4. B, representative intravital images of isotype-treated and α-M-CSFR-treated 3LL-R tumors. 3LL-R tumor-bearing CX3CR1-GFP⁺/⁻ reporter mice received one shot of isotype antibody or α-M-CSFR antibody just before imaging the tumor (green, CX3CR1⁺ cells; red, labeled vessels). The percentage of CX3CR1⁺ cells in the parenchyma or in the tumor blood vessels was analyzed. Two fields were analyzed per animal, with two animals per group. C, influence of M-CSFR blockade on monocyte recruitment to the tumor. One million GFP⁺ bone marrow cells were adoptively transferred to 11-day-old 3LL-R tumor-bearing mice. GFP⁺ bone marrow cells were either treated with α-M-CSFR antibody for 30 minutes and washed before transfer (α-M-CSFR-treated cells) or mice were injected with α-M-CSFR antibody 12 hours before GFP⁺ bone marrow transfer (α-M-CSFR-treated recipients). Four hours after GFP⁺ bone marrow transfer, mice were sacrificed and tumors were collected. Graphs show the percentage of GFP⁺Ly6C⁶0 monocytes within the total amount of GFP⁺ cells that were found back in the tumor. Results are representative of three independent experiments with n ≥ 4. D, effect of M-CSFR blockade on monocyte proliferation. 11-day-old 3LL-R tumor-bearing mice were given sequentially: (i) α-M-CSFR antibody or isotype antibody intravenously 12 hours before sacrifice, (ii) one BrdUrd shot (or left untreated as control) 4 hours before sacrifice, and (iii) CD45-PECy7 or isotype PeCy7 antibody 1 minute before sacrifice. BrdUrd incorporation was measured using intracellular flow cytometry. The graph shows the percentage of BrdUrd⁺ cells within the Ly6C⁶0 peripheral blood monocytes or Ly6C⁶0 tumor-associated monocytes. The latter was divided in a CD45-PECy7⁻ unlabeled population (i.e., tumor mass monocytes). Results are representative of three independent experiments with n ≥ 4. All experiments, graphs show mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

decrease in local CCL2 production, as was demonstrated before in other tissues (7). However, when GFP⁺ bone marrow cells were transferred intravenously to tumor-bearing mice pretreated with anti-M-CSFR, the recruitment of GFP⁺Ly6C⁶0 monocytes into tumors was not significantly reduced compared with control mAb-pretreated mice, arguing against major chemoattractant alterations caused by anti-M-CSFR (Fig. 1C). Accordingly, CCL2 protein levels in the tumor were not influenced by M-CSFR blockade (Supplementary Fig. S1D).

To assess whether M-CSFR signaling blockade has monocyte-intrinsic effects, GFP⁺ bone marrow cells were pretreated with anti-M-CSFR in vitro before transfer into tumor-bearing mice. Significantly less anti-M-CSFR–bound GFP⁺Ly6C⁶0 monocytes could be traced back in the tumor (Fig. 1C). Importantly, their numbers did not differ in peripheral blood, excluding monocyte elimination in the circulation as underlying mechanism for their reduced tumor penetration (Supplementary Fig. S1E). Notably, the expression of adhesion molecules known to be involved in monocyte extravasation (15) were not affected by anti-M-CSFR (Supplementary Fig. S1F). Hence, M-CSFR blockade lowers monocyte recruitment and extravasation to tumors in a monocyte-intrinsic fashion.

M-CSFR signaling blockade impairs the proliferation of peripheral and tumor-infiltrating Ly6C⁶0 monocytes

M-CSFR signaling may drive monocyte proliferation and as such contribute to tumor-infiltrating mononuclear cell numbers. BrdUrd pulse labeling in combination with an intravenous injection of CD45-PECy7 1 minute before sacrifice revealed that the proliferative capacity of Ly6C⁶0 monocytes in isotype-treated mice dropped while migrating from peripheral blood (11.7% ± 2.5%) to the tumor vasculature (4.8% ± 0.9%) and finally to the tumor mass (0.27% ± 0.05%; Fig. 1D). In all sites, the proliferation of Ly6C⁶0 monocytes depended on M-CSFR signaling, as it significantly dropped after M-CSFR blockade (Fig. 1D). Of note, co-staining with AnnexinV/7AAD revealed that M-CSFR antibody
treatment had no effect on the viability of Ly6C^{hi} monocytes nor TAM subsets (Supplementary Fig. S2). Thus, M-CSFR signaling blockade impairs the recruitment and proliferation of Ly6C^{hi} monocytes, resulting in a reduced mononuclear tumor infiltrate. Notably, the amount of Ly6C^{hi} monocytes was unaltered in the peripheral blood and spleen and was even increased in the bone marrow after M-CSFR blockade (Supplementary Fig. S3), possibly resulting from the combined effect of a lower proliferation rate and reduced recruitment to the tumor.

M-CSFR signaling blockade impedes the differentiation of Ly6C^{hi} monocytes into MHC-II^{lo} TAM

We next assessed whether M-CSFR signaling affected the differentiation pathway of the Ly6C^{hi} monocytes that were still able to infiltrate the tumor. Upon anti-M-CSFR treatment, proportionally more monocytes and immature TAM (gates 1 + 2 + 3) and less mature TAM (gates 4 + 5), with especially a strong effect on MHC-II^{lo} TAM in gate 4) were found within the CD11b^{+} Ly6G^- SiglecF^- tumor infiltrate (Fig. 2A and Supplementary Fig. S4A and S4B), suggesting a monocyte-to-macrophage differentiation block. This conclusion is corroborated by the diminished gene expression level of the macrophage differentiation regulators *Pu.1* and *Mafb* in tumor-associated Ly6C^{hi} monocytes from anti-M-CSFR-treated mice (Fig. 2B). In this context, it is important to note that especially M-CSF, but much less the alternative M-CSFR ligand IL-34, is secreted in the tumor microenvironment (Supplementary Fig. S5A) and that blocking the M-CSFR tends to reduce the presence of this cytokine in tumors (Supplementary Fig. S5B).

Interestingly, mainly the generation of MHC-II^{lo} TAM was hampered upon M-CSFR blockade, as the MHC-II^{lo}/MHC-II^{hi} TAM balance shifted from an excess of MHC-II^{lo} TAM towards equal amounts of both TAM subsets (Fig. 2A and Supplementary Fig. S4). Notably, also the presence of tumor-associated dendritic cells (gate 6) was not affected. Accordingly, the differentiation pathway of adoptively transferred GFP^{+} Ly6C^{hi} monocytes shifted from yielding mainly MHC-II^{lo} TAM in isotype-treated mice to predominantly MHC-II^{hi} TAM upon anti-M-CSFR treatment (Fig. 2C). Importantly, the altered MHC-II^{lo}/MHC-II^{hi} TAM balance caused by M-CSFR blockade was also seen in TS/A (Supplementary Fig. S6).

M-CSFR and GM-CSFR signaling play opposing roles in determining the phenotype of MHC-II^{lo} and MHC-II^{hi} TAM

Finally, we wondered whether M-CSFR signaling also influences the phenotype of the mature TAM subsets. Both MHC-II^{lo} and MHC-II^{hi} TAM expressed the M-CSFR, but the expression level being associated with the distinct TAM subsets, it became clear

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**Figure 2.**

M-CSFR regulates the maturation and preferential differentiation of Ly6C^{hi} monocytes to MHC-II^{lo} TAM. A, single-cell suspensions of 13-day-old 3LL-R tumors treated with isotype or α-M-CSFR antibody were stained for myeloid populations. Ly6C versus MHC-II plots of the gated CD11b^{+} Ly6G^- SiglecF^- population were shown and the indicated populations are 1, Ly6C^{hi} monocytes; 2, MHC-II^{lo} immature macrophage (Mφ); 3, MHC-II^{lo} immature Mφ; 4, MHC-II^{lo} TAM; 5, MHC-II^{lo} TAM, and 6, tumor-associated dendritic cells (TADC). Graphs show the percentage of all these subsets within the CD11b^{+} Ly6G^- SiglecF^- gate. B, expression of the macrophage maturation markers *Pu.1* and *Mafb* in tumor-associated Ly6C^{hi} monocytes from anti-M-CSFR-treated mice (Fig. 2B). In this context, it is important to note that especially M-CSF, but much less the alternative M-CSFR ligand IL-34, is secreted in the tumor microenvironment (Supplementary Fig. S5A) and that blocking the M-CSFR tends to reduce the presence of this cytokine in tumors (Supplementary Fig. S5B).

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Finally, we wondered whether M-CSFR signaling also influences the phenotype of the mature TAM subsets. Both MHC-II^{lo} and MHC-II^{hi} TAM expressed the M-CSFR, but the expression level being associated with the distinct TAM subsets, it became clear
that most (but not all) MHC-IIlo TAM-associated genes (3, 4), such as Mrc1, Arg1, Cd163, and Mmp9 were downregulated in the MHC-IIlo TAM upon M-CSFR inhibition, while several MHC-IIhi TAM-associated genes, such as Mgl2, Cdh1, Cldn1, Retnl, Pges2, and Ccl17 were upregulated in these cells (Fig. 3A). Hence, M-CSFR blockade enhanced the MHC-IIhi TAM gene signature in MHC-IIlo TAM. Accordingly, the protein expression level of markers highly expressed on MHC-IIlo TAM (MMR, SR-A and IL4Rα) significantly dropped after M-CSFR blockade (Fig. 3B). The drop in IL4Rα expression may imply that part of the anti-M-CSFR-mediated effects on gene expression are due to diminished IL4Rα signaling. Although some genes were indeed regulated by IL4Rα in the tumor microenvironment, the effects of M-CSFR blockade are largely maintained in IL4Rα-deficient mice and are thus IL4/IL13-independent (Supplementary Fig. S7). Notably, the inherent phagocytic capacity of the MHC-IIlo TAM, which is one of its hallmark functions (3), was strongly diminished by M-CSFR blockade as evidenced by lower uptake of latex beads ex vivo (Fig. 3C). In addition, the capacity of both MHC-IIlo and MHC-IIhi TAM to stimulate naive CD4+ and CD8+ T cells was increased following M-CSFR blockade, further evidencing a functional shift (Supplementary Fig. S8).

M-CSF and GM-CSF were reported to have opposing effects on the macrophage activation state (5, 6). Both TAM subsets expressed high levels of the GM-CSFR, with the highest expression seen on the MHC-IIhi population (Fig. 2D). To assess whether GM-CSF regulates the TAM phenotype, 3LL-R tumors were inoculated in GM-CSFR-deficient mice. Notably, the abundance of the mononuclear tumor infiltrate was comparable between GM-CSFR KO and WT tumors, the Ly6Chi monocytes expressed similar levels of maturation markers (Fig. 3D) and the relative abundance of TAM subsets was unaltered (Fig. 3D). These data illustrate that GM-CSF does neither regulate monocyte extravasation nor their intratumoral differentiation. However, several genes that are...
highly expressed by MHC-IIhi TAM (3, 4), such as Mgl2, Cdhl1, Cldn1, Retnla, Ptg2, Ccl17, and Il1b were strongly downregulated in GM-CSFR KO MHC-IIhi TAM (Fig. 4A). Conversely, genes that are typically associated with the MHC-IIlo TAM were either unchanged or upregulated in the GM-CSFR KO MHC-IIlo population (except Arg1). A similar general trend was observed in GM-CSFR KO MHC-IIlo TAM (Fig. 4A). At the protein expression level, the MHC-IIhi TAM marker CD11c dropped in GM-CSFR KO MHC-IIhi TAM, while surface markers associated with a MHC-IIlo TAM signature (IL4Rα, MMR, and SRA) increased or remained unaltered (Fig. 4B). In addition, the phagocytic capacity of both TAM subsets was enhanced in the absence of GM-CSFR, while having no effect on the Ly6C hi monocytes (Fig. 4C).

Interestingly, treatment of GM-CSFR-deficient mice with blocking anti-M-CSFR mAb caused a significant reduction of the CD11b+Ly6G− SiglecF+ tumor infiltrate (Supplementary Fig. S9A), hampered the generation of MHC-IIlo TAM (Supplementary Fig. S9B), and enhanced the MHC-IIhi TAM gene and protein signature in MHC-IIlo TAM (Supplementary Fig. S9C and S9D), similar to blocking M-CSFR signaling in WT mice (Figs. 1A, 2A, and 3A and B). Hence, the induction of a more M1-like TAM phenotype upon M-CSFR blockade is GM-CSF–independent.

Together, these data validate the distinct role of M-CSFR and GM-CSFR signaling in macrophage polarization in tumors in vivo (Fig. 4D).

Discussion

Strong reductions in the number of TAM have been reported in various tumor models upon blocking M-CSFR signaling using...
several M-CSFR blockers (12). However, the underlying mechanisms remain largely unexplored. In this study, we show that M-CSFR, but not GM-CSFR, signaling, is responsible for the recruitment, extravasation, proliferation, and maturation of tumor-infiltrating Ly6c<sup>hi</sup> monocytes. The exact mechanism by which M-CSFR signaling regulates monocyte extravasation remains unknown, but appears not to be CCL2 driven like in some other tissues (7). However, we cannot exclude the possibility that the process of extravasation is driven by other M-CSFR–regulated chemokines in these tumors. Another M-CSFR–regulated molecule, involved in monocyte and macrophage migration and activation is the GTPase Rac2 (16), which could be an interesting candidate for further investigation.

We also show that M-CSFR, but not GM-CSFR, inhibition abrogated the differentiation of Ly6c<sup>hi</sup> monocytes to M2-like MHC-II<sup>hi</sup> TAM. M-CSFR blockade was previously shown to induce a shift in phenotype from MHC-II<sup>lo</sup> to MHC-II<sup>hi</sup> TAM in a syngeneic mouse model of BRAFV600E–driven melanoma (9), to drive the repolarization of protumoral M2 to M1 TAM in a mouse glioblastoma model (8) and to cause the preferential depletion of M2-like MMR<sup>lo</sup> TAM (10). Notably, M2-like MMR<sup>lo</sup> TAM may be MHC-II<sup>lo</sup> in some models and are also depleted by M-CSFR blockade (11, 17). Overall, these data suggest that M-CSFR blockade preferentially depletes mature M2-like TAM. Administration of M-CSFR blocking mAbs to patients with diffuse-type giant cell tumors even led to clinical responses, validating M-CSF and its receptor as a therapeutic target (18).

We now show for the first time that M-CSFR and GM-CSFR signaling are in fact opposing driving forces in the tumor microenvironment, regulating the MHC-II<sup>lo</sup> and MHC-II<sup>hi</sup> TAM phenotype, respectively. While M-CSFR predominantly regulated the differentiation and M2-like properties of MHC-II<sup>lo</sup> TAM in a GM-CSFR–independent fashion, GM-CSFR fine-tuned the M1-like MHC-II<sup>hi</sup> phenotype. A differential expression of the respective receptors may mediate part of this effect. However, as distinct TAM subsets are differentially located within the tumor, with the MHC-II<sup>lo</sup> TAM associated with more hypoxic regions (3, 4), a locoregional production of M-CSF and GM-CSF in different compartments of the tumor microenvironment may also be involved. Interestingly, many of the genes that were shown to be regulated by GM-CSF in MHC-II<sup>hi</sup> TAM in the current study were reported before as typical GM-CSF–induced genes in bone marrow–derived macrophages in vitro (Cldn1, Cldn1, C17F2, Il1b; ref. 19).

Hence, even in the complex tumor microenvironment harboring a multitude of potential macrophage-regulating cues, M-CSFR and GM-CSF signaling are important for skewing macrophage polarization.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Van Overmeire, B. Stijlemans, F. Heymann, J. Keirsse, Y. Morias, C. Abels, Q. Lahmar, L. Vereecke, F. Tacke, D. Laoui

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