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Acute myeloid leukemia cells polarize macrophages towards a leukemia supporting state in a Growth factor independence 1 dependent manner

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ABSTRACT

The growth of malignant cells is not only driven by cell-intrinsic factors, but also by the surrounding stroma. Monocytes/Macrophages play an important role in the onset and progression of solid cancers. However, little is known about their role in the development of acute myeloid leukemia, a malignant disease characterized by an aberrant development of the myeloid compartment of the hematopoietic system. It is also unclear which factors are responsible for changing the status of macrophage polarization, thus supporting the growth of malignant cells instead of inhibiting it. We report herein that acute myeloid leukemia leads to the invasion of acute myeloid leukemia-associated macrophages into the bone marrow and spleen of leukemic patients and mice. In different leukemic mouse models, these macrophages support the *in vitro* expansion of acute myeloid leukemia cell lines better than macrophages from non-leukemic mice. The grade of macrophage infiltration correlates *in vivo* with the survival of the mice. We found that the transcriptional repressor Growth factor independence 1 is crucial in the process of macrophage polarization, since its absence impedes macrophage polarization towards a leukemia supporting state and favors an anti-tumor state both *in vitro* and *in vivo*. These results not only suggest that acute myeloid leukemia-associated macrophages play an important role in the progression of acute myeloid leukemia, but also implicate Growth factor independence 1 as a pivotal factor in macrophage polarization. These data may provide new insights and opportunities for novel therapies for acute myeloid leukemia.

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Introduction

The growth of various solid tumors, lymphomas and leukemias is not only the result of cell-specific changes at the genetic and epigenetic level, but is also affected by the surrounding microenvironment, the stroma and the cells therein.[1-4] The stroma is composed of many different cell types, among them fibroblasts, mesenchymal stem cells, vascular cells and a variety of immune cells including T and B lymphocytes, natural killer cells (NK-cells), neutrophils and macrophages.[4] Tumor cells induce the stroma and immune cells to express and partially secrete various factors and cytokines that promote the growth of the tumor cells, instead of activating the immune system to battle the malignant cells.[5,6] This process of "polarization" is the result of a complex bidirectional interaction between the tumor and the stroma cells.

Hence, the polarized macrophages in tumors are called tumor-associated macrophages (TAMs).[5] The plasticity of macrophages is mostly tissue-specific and regulated by local and systemic signals.[7] In response to different signals derived from the surrounding tissue, bacteria or activated lymphocytes, macrophages can differentiate into various polarization states with distinct functional phenotypes.[8] Although considered a simplification,[9] the M1/M2 is a straightforward classification for functionally distinct types of macrophages. M1 macrophages, known as classically activated macrophages, are stimulated by bacterial lipopolysaccharide (LPS), interferon-γ (IFN-γ), tumor necrosis factor (TNF)-α or granulocyte-macrophage colony-stimulating factor (GM-CSF), and are characterized by the production of numerous antimicrobial agents and inflammatory mediators, such as interleukin 6 (IL-6), reactive oxygen species (ROS) and nitric oxide (NO).[10] The M1 macrophages are involved in the host defense against different pathogens and play a role in anti-tumor immunity. In contrast, M2 macrophages or alternatively activated macrophages have anti-inflammatory activity and are stimulated by interleukin 4 (IL-4) or interleukin 13 (IL-13). They secrete arginase, metalloproteinases, transforming growth factor-β (TGFβ), interleukin 10 (IL-10) and other cytokines that cause immune suppression, angiogenesis and tissue repair.[11] M2 macrophages have been further subdivided into M2a, M2b, M2c and M2d macrophages, according to the polarizing cytokines.[12] In contrast to M1 macrophages, which suppress tumor growth, M2 macrophages play an important role in the development and progression of different tumors,[13,14] and are therefore also known as TAMs.

Despite a good understanding of the role of macrophages in solid tumors, little is known about the interaction between stroma cells and leukemic cells. Leukemic stem cells (LSCs) can modify the bone marrow (BM) niche in such a way that it supports the growth of LSCs instead of hematopoietic stem cells (HSCs).[15] This might enhance the LSCs quiescence, leading to chemotherapy resistance.[1,16-19] A recent study reported that the inhibition of SIRPα signalling in macrophages impairs engraftment of human LSCs in immunocompromised NSG mice.[20] Clinically, the accumulation of TAMs in the lymph nodes of patients with classic Hodgkin lymphoma was associated with a poor prognosis.[21] The most common form of adult leukemia is acute myeloid leukemia (AML),[22] which is characterized by an accumulation of myeloid blast cells in the BM. As AML patients have a poor prognosis,[22] novel therapy approaches are urgently needed. Furthermore, the function of AML-associated macrophages (AAMs) and their role in AML progression remains to be further investigated.

Transcription factors, key elements of gene regulation, show a distinct expression pattern and organ specificity. One such transcription factor is Growth factor independence 1 (Gfi1), a transcriptional repressor that plays an important role in HSCs maintenance and quiescence, and is crucial for normal lymphoid and myeloid hematopoiesis.[23-25] *Gfi1*-deficient mice are characterized by severe neutropenia and an overproduction of TNF-α and other inflammatory mediators of macrophages when exposed to bacterial endotoxin or LPS.[26] Using different mouse models of human AML we report herein that AAMs support the expansion of AML cells both *in vivo* and *in vitro*. Furthermore, we show that *Gfi1* has an important role in the process of macrophage polarization.

Methods

Human BM samples

Human BM samples were obtained following the informed consent of all subjects. All experiments with human samples were carried out in accordance with the approved protocol of the University of Duisburg-Essen ethics committee. The diagnosis of AML was confirmed based on cytological and flow cytometry examination.[22,27]

Mouse strains

NUP98-HOXD13 transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The *Gfi1-KO* mice have been previously described.[28] Wild-type (WT) mice (C57BL/6J) were provided by the animal facility of the University Hospital Essen. All animals were housed in single ventilated cages and specific pathogen-free conditions at the animal facility of University Hospital Essen. All animal experiments were carried out in accordance with the protocol of the government ethics committee for animal use, which on 21.07.2011 approved all studies on animals under document number G1196/11.

AML cell lines

C1498GFP, a murine AML cell line,[29] was a kind gift from Dr. Justin Kline from the University of Chicago, USA. The cells were maintained in DMEM (Gibco, Life Technologies, Darmstadt, Germany), supplemented with 10% fetal bovine serum (FBS) (PAN™ BIOTECH, Aidenbach, Germany) and 1% penicillin/streptomycin (Gibco).

Statistics

A student's *t*-test was applied to calculate the differences between various groups. For the survival analysis, a Kaplan-Meier test was performed. Differences were considered to be significant when the *P*-value was <0.05. The Graph Pad (version 6) software was used for applying all significance tests.

Results

AAMs proliferate and accumulate in the BM of AML patients

The expression of CD163 has been reported to be restricted to monocytes/macrophage lineages.[30] Recently, $CD163^+$ M2 TAMs have been reported to be involved in tumor progression in several hematological malignancies such as multiple myeloma[31] or classical Hodgkin lymphoma (CHL).[32] A common cell surface marker identified in TAMs is CD206.[33] To explore the ability of AML cells to educate macrophages and affect their polarization, we examined the rate of infiltration of $CD163^+CD206^+$ M2-like macrophages in the BM of AML patients and healthy volunteers (*Online Supplementary Table S1*). The frequency of $CD163^+CD206^+$ M2-like macrophages in the BM of AML patients was significantly elevated compared to healthy volunteers (*Online Supplementary Figures S1A-S1C*).

Leukemic cells polarize non-leukemic monocytes/macrophages that proliferate and accumulate in BM and spleen of recipient mice

To investigate the molecular mechanisms and the role of monocytes/macrophages in the development of AML, we used different established murine models of human AML. AML1-ETO9a, the product of the t(8;21)(q22;q22) translocation, and MLL-AF9, the product of the t(9;11)(p22;q23) translocation, are commonly involved in AML pathogenici-

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ty in humans, and are also used to model AML in mice.[34,35] While AML1-ETO9a-induced AML is associated with a rather good prognosis, MLL-AF9-driven AML has a rather bad prognosis.[34,35] To study the role of monocytes/macrophages in AML, we transduced lineage negative (Lin⁻) BM cells from WT mice with retroviruses encoding *MLL-AF9* or *AML1-ETO9a* cDNA fused to an IRES-GFP gene cassette, and transplanted these cells into lethally irradiated mice together with $1.5x10^5$ competitive BM cells. Leukemic BM cells were then re-transplanted into

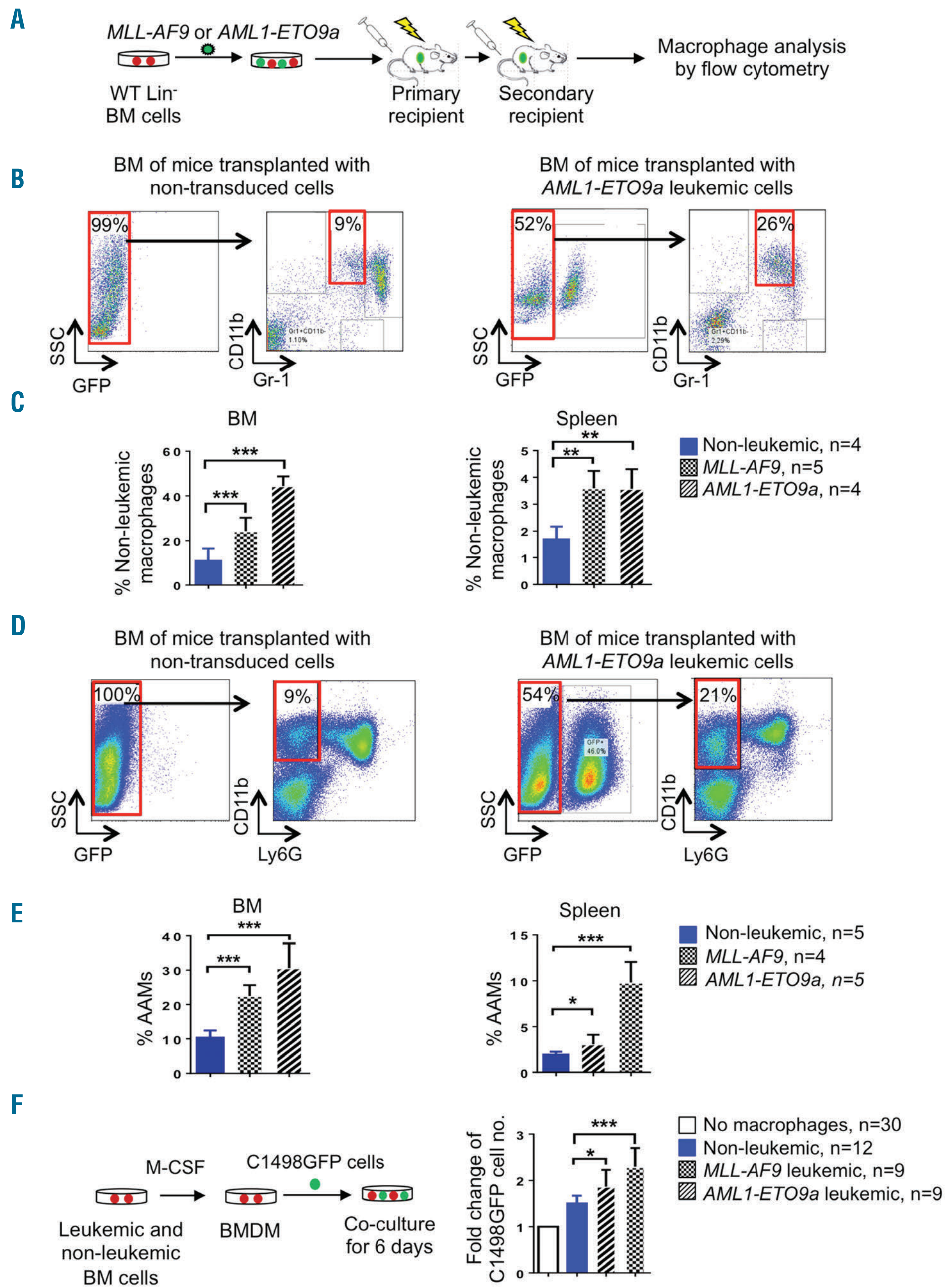

Figure 1. AML-associated monocytes/macrophages (AAM) proliferate and accumulate in the BM and spleen of AML mice. (A) Lin⁻ BM cells from WT mice were transduced either with *MLL-AF9* or *AML1-ETO9a* retroviruses and $1*10^5$ MLL-AF9 or $5\text{-}7*10^5$ AML1-ETO9a GFP⁺ cells were transplanted into lethally irradiated (10Gy) primary recipient mice together with $5*10^5$ competitive BM cells. Leukemic BM cells ($1*10^5$ GFP⁺ cells) were then re-transplanted into secondary sublethally irradiated (3Gy) mice. Macrophage surface markers from leukemic mice were subsequently analyzed by flow cytometry. (B) Representative gating strategy for $GFP^-CD11b^{hi}Gr1^{int}$ monocytes/macrophages in BM cells derived from mice transplanted with non-transduced (left panel) or *AML1-ETO9a*-transduced cells (right panel). (C) The frequency of non-leukemic $GFP^-CD11b^{hi}Gr1^{int}$ monocytes/macrophages in the BM (left panel) and spleen (right panel) of leukemic mice transplanted with *MLL-AF9* (n=5) or *AML1-ETO9a* transduced cells (n=5) compared to mice transplanted with non-transduced cells (n=4), (***P<0.0008 for BM, **P<0.001 for spleen). (D) Representative gating strategy for $GFP^-CD11b^+Ly6G^-$ monocytes/macrophages in BM cells derived from mice transplanted with non-transduced or AML1-ETO9a-transduced cells. (E) The frequency of non-leukemic $GFP^-D11b^+Ly6G^-$ macrophages in the BM (left) or spleen (right) of transplanted leukemic mice (n=5 for MLL-AF9 and n=5 for *AML1-ETO9a*), compared to mice transplanted with non-transduced cells (n=4), (***P<0.0001 for BM, *P=0.04 and ***P=0.0002 for spleen). (F) $2\text{-}3x10^5$ BMDMs from mice transplanted with non-transduced or *AML1-ETO9a* or MLL-AF9-transduced cells were co-cultured with $5*10^4$ C1498GFP cells for 6 days (left panel). Fold change of C1498GFP live cell numbers is given (right panel). Results from triplicates of 3 independent experiments for mice transplanted with *MLL-AF9* (n=9) and *AML1-ETO9a* (n=9) transduced cells and 4 independent experiments for mice transplanted with non-transduced cells (n=12) are shown, *P=0.03 for AML-ETO9a and ***P<0.001 for MLL-AF9 transgenic cells). BM: bone marrow; AAMs: acute myeloid leukemia associated macrophages; WT: wild-type; BMDM: bone marrow-derived macrophage; AML: acute myeloid leukemia.

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secondary, sublethally irradiated recipient mice (Figure 1A). The expression of GFP alongside the expression of either of the two different oncofusion proteins by the transduced pre-leukemic cells enabled the differentiation between leukemic and non-leukemic cells. To minimize any potential bias as a result of the irradiation, we used control mice that were sublethally irradiated but received only WT BM cells from healthy mice. In the BM and spleen of leukemic secondary recipient mice we first determined the fraction of GFP$^-$ AAMs defined as GFP$^-$CD11b^{hi}Gr1^{int}.[28] The frequency of GFP$^-$ AAMs in the BM and spleen of leukemic mice was significantly higher than in sublethally irradiated mice transplanted with competitive normal BM cells only (Figure 1B,C). Also, when we defined AAMs as GFP$^-$CD11b$^+$Ly6G$^-$ cells[36] (Figure 1D), we found similar results (Figure 1E). To confirm our findings and in order to rule out any effects of irradiation, we used the *NUP98-HOXD13* transgenic mouse model that mimics the t(2;11)(q31;p15) translocation, which is associated with human myeloid malignancies. These mice show features of human myelodysplastic syndrome (MDS), and some mice develop AML.[37] Similarly, the percentage of AAMs in the BM and spleen of leukemic *NUP98-HOXD13* transgenic mice was higher than in WT non-leukemic mice (*Online Supplementary Figures S2A and S2B*). We confirmed that, phenotypically, in both the GFP$^-$CD11b^{hi}Gr1^{int} and GFP$^-$CD11b$^+$Ly6G$^-$ monocyte population the expression of F4/80, the typical marker for BM macrophages, was more than 90% and 70%, respectively (*Online Supplementary Figure S2C and S2D*).

We then tested whether these AAMs would support the growth of murine AML cells *in vitro*. We co-cultured BM-derived macrophages (BMDMs) with the murine AML cell line C1498GFP for 6 days, counted the non-adherent C1498GFP cells and determined the number of GFP-expressing leukemic cells by flow cytometry. BMDMs from transplanted leukemic mice supported the proliferation of the C1498GFP cells better than BMDMs from non-leukemic mice (Figure 1F).

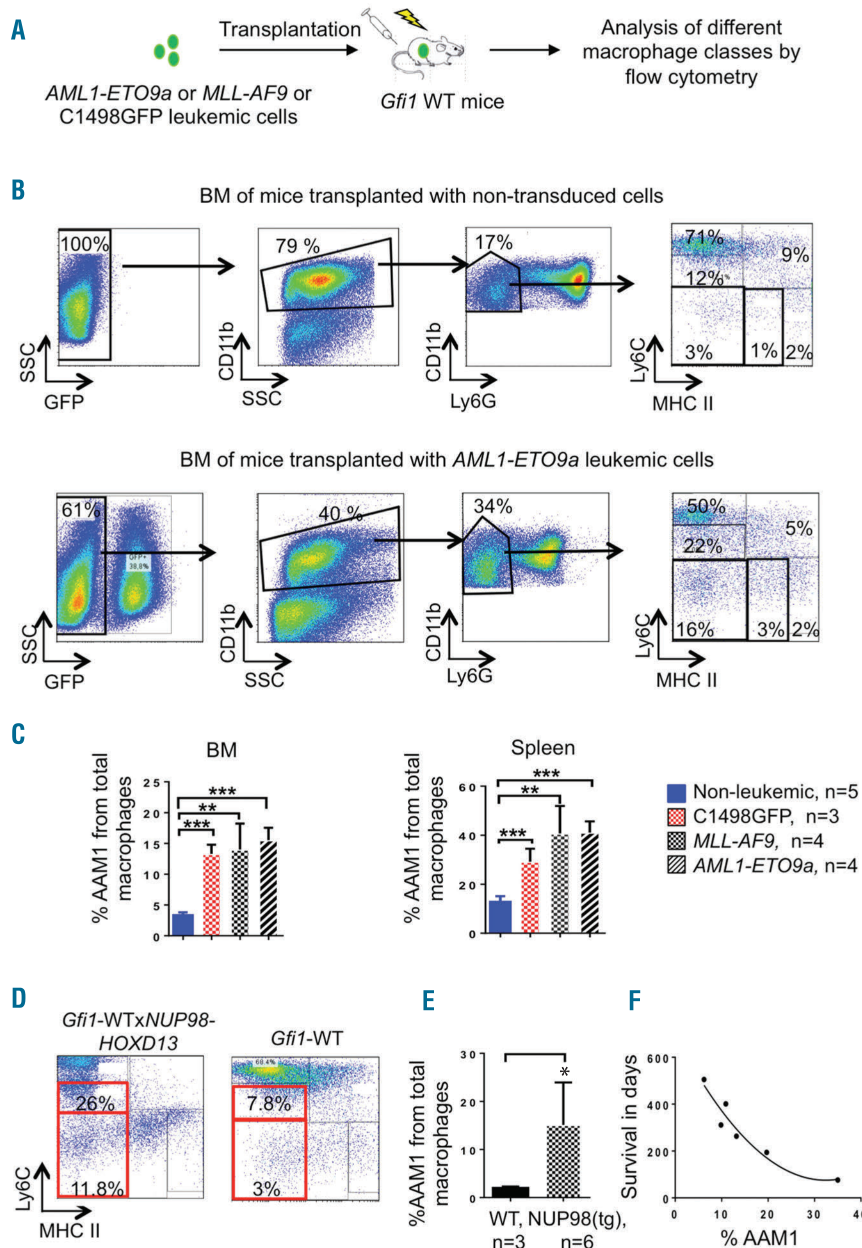

Figure 2.Characterization of AAMs by flow cytometry. (A) Schematic illustration of the experimental design. BM cells from *MLL-AF9* or *AML1-ETO9a* leukemic primary recipient mice or C1498GFP murine AML cells were transplanted into sublethally irradiated (3Gy) secondary recipient mice. When moribund, the mice were sacrificed and different macrophage classes were analyzed by flow cytometry. (B) Representative FACS plots from the BM of mice transplanted either with non-transduced or with *AML1-ETO9a* transduced cells showing the gating strategy used for classifying different types of macrophages according to the expression of Ly6C and MHCII markers. Cells with a GFP$^-$CD11b$^+$Ly6G$^-$MHCII-Ly6C$^-$ phenotype were considered AAM1. C) The frequency of AAM1 in the BM (left panel) and spleen (right panel) of leukemic mice transplanted with *AML1-ETO9a*, (n=4), *MLL-AF9* (n=4) or C1498GFP (n=3) compared to mice transplanted with non-transduced cells (n=5), (****P*<0.0001, ***P*=0.001). (D) Representative FACS plots showing macrophage classes in the BM of the *Gfi1*-WTx*NUP98-HOXD13* mouse model. (E) The frequency of AAM1 cells in the BM of leukemic *NUP98-HOXD13* mice (n=6) compared to WT mice (n=3), (**P*=0.04). F) Survival of the leukemic *NUP98-HOXD13* mice is inversely correlated with the percentage of AAM1 in the BM (R square=0.92). BM: bone marrow, AML: acute myeloid leukemia, AAMs: acute myeloid leukemia associated macrophages, WT: wild-type; Gfi1: growth factor independent 1.

Characterization of AAMs

Macrophages are characterized by specific gene expression patterns, cytokine secretion and cell surface molecules.[7] By using a similar gating strategy for studying TAMs in lung cancer, as reported earlier,[36] we quantified the different mononuclear phagocyte subsets in the BM and spleen of

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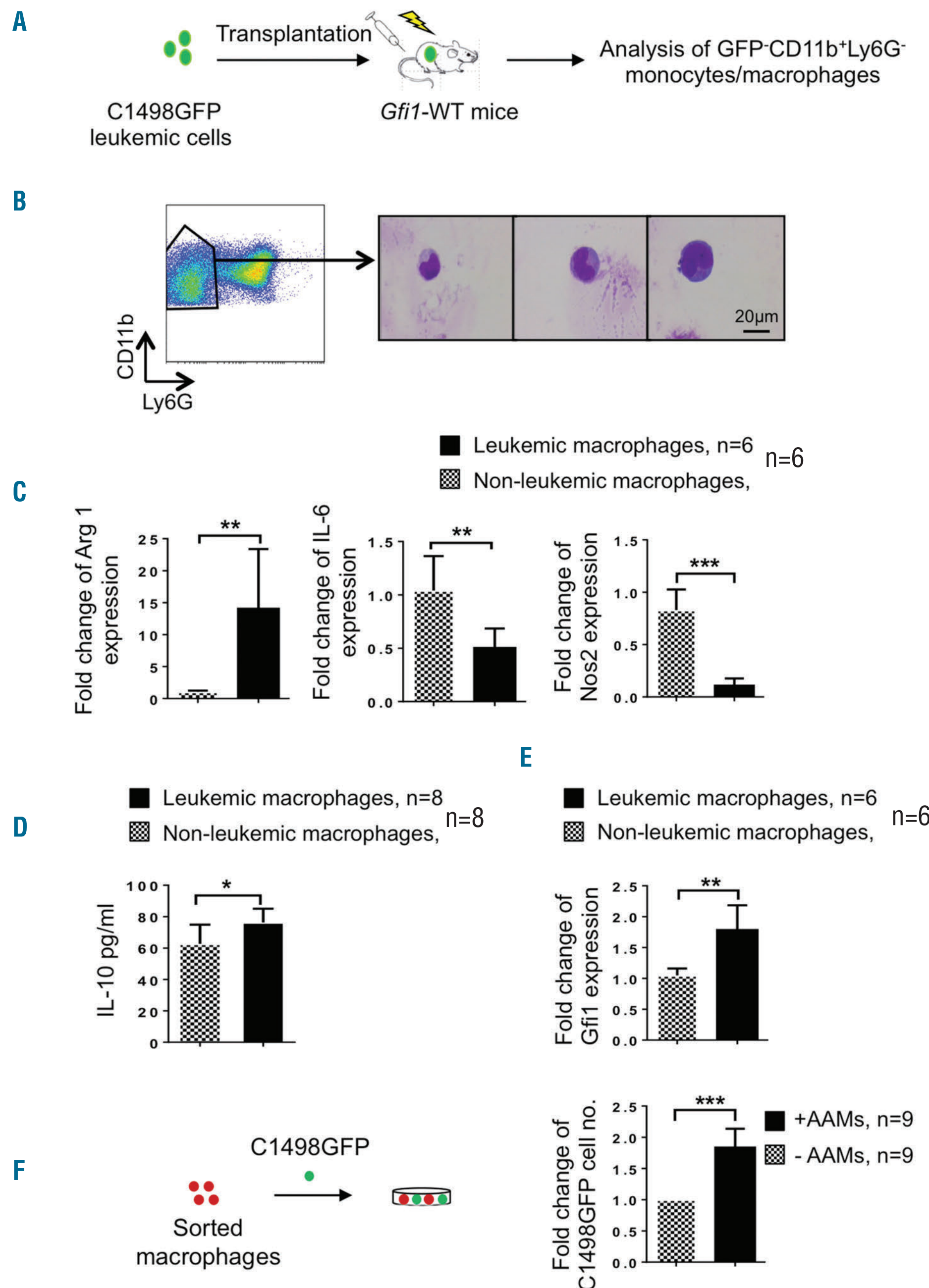

Figure 3. Characterization of AAMs by RT-PCR and ELISA. (A) Schematic illustration of the experimental design. 1x10^5-4x10^5 C1498GFP were transplanted into sublethally irradiated (3Gy) secondary recipient mice. When the mice developed AML, GFP$^-$CD11b$^+$Ly6G$^-$ BM macrophages were sorted for further experiments. (B) Cytospins were prepared from sorted AAMs (GFP$^-$CD11b$^+$Ly6G$^-$) and stained according to the May-Grunwald Giemsa protocol. Bar represents 20μm. (C) Fold change of Arg1, IL-6 and Nos2 mRNA levels in sorted AAMs from non-leukemic mice transplanted with WT BM cells (n=6) and leukemic mice transplanted with C1498GFP cells (n=6), normalized to GAPDH. Results of duplicates from three independent experiments are shown (***P*=0.006 for Arg1, ***P*=0.005 for IL-6 and ****P*<0.0001 for Nos2). (D) 5x10^5 AAMs sorted from leukemic mice transplanted with C1498GFP cells (n=8) or 5x10^5 CD11b$^+$Ly6G$^-$ non-leukemic macrophages sorted from mice transplanted with WT BM cells (n=8) were cultured in DMEM/glutamax supplemented with 10% FBS and 1% Pen/Strep. After 24 hours medium was collected, filtered and the levels of IL-10 were measured using an ELISA commercial kit. Results of duplicates from four independent experiments are shown (**P*=0.01). (E) Fold change of *Gfi1* mRNA level in sorted AAMs from leukemic mice transplanted with C1498GFP cells (n=6) and non-leukemic mice transplanted with WT BM cells (n=6), normalized to GAPDH. Results of duplicates from three independent experiments are shown (***P*=0.001). (F) 5x10^4 C1498GFP$^+$ cells were co-cultured with 1.5x10^4 sorted GFP$^-$CD11b$^+$Ly6G$^-$ cells (left panel). The numbers of C1498GFP$^+$ cells in the presence (n=9) or absence (n=9) of sorted AAMs are shown (right panel). Results of triplicates from three independent experiments are given (****P*=0.0009). BM: bone marrow, AML: acute myeloid leukemia, AAMs: acute myeloid leukemia associated macrophages, WT: wild-type; *Gfi1*: growth factor independent 1; BMDM: bone marrow derived macrophage; Arg1: arginase 1; Nos2: nitric oxide synthase 2; IL-6: interleukin 6; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; IL-10: interleukin 10.

sublethally irradiated mice transplanted either with C1498GFP cell line or with *MLL-AF9* or *AML1-ETO9a* leukemic BM cells from primary recipient mice (Figure 2A). Depending on the expression levels of Ly6C and MHCII surface markers, the GFP⁻CD11b⁺Ly6G⁻ monocytes/macrophages from non-leukemic and leukemic mice were divided into six populations (Figure 2B).[36,38] In all leukemic mouse models, we found that not only the frequency (Figure 2C) but also the absolute numbers (*Online Supplementary Figure S3A and S3B*) of AAM1 cells, which are

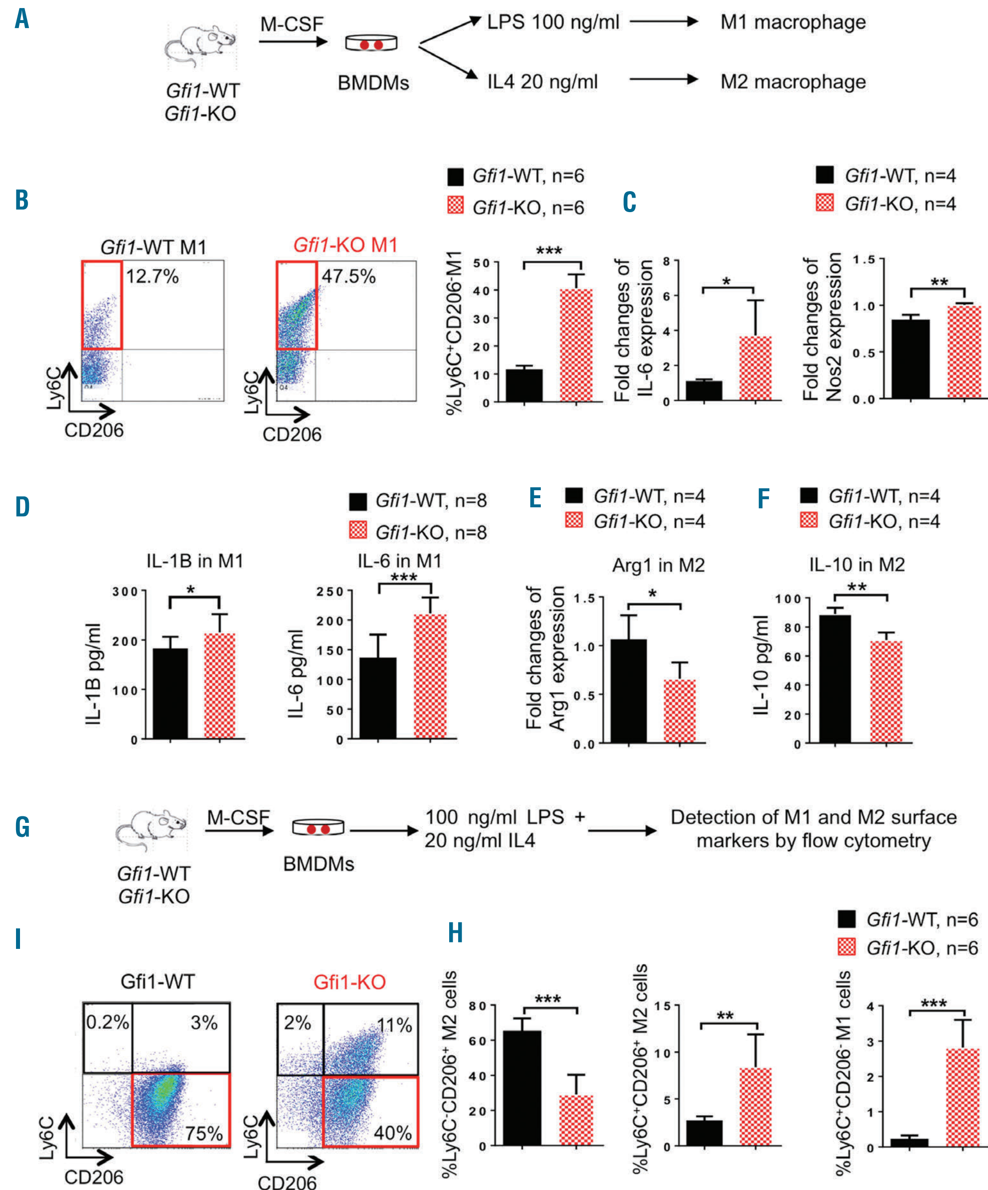

Figure 4. *Gfi1* enhances M2 polarization by IL-4 and suppresses the M1 polarization of macrophages by LPS *in vitro*. (A) Schematic representation of the *in vitro* polarization experiment. *Gfi1*-WT or *Gfi1*-KO BMDMs were stimulated with LPS (100 ng/ml) or IL-4 (20ng/ml) for 48 hours. The medium was collected for ELISA and M1 and M2 macrophages were characterized by flow cytometric and gene expression analysis. (B) Representative FACS plots of Ly6C⁺CD206⁻ M1(LPS) macrophages from *Gfi1*-WT and *Gfi1*-KO BMDMs (left panel). The frequency of polarized Ly6C⁺CD206⁻ M1(LPS) macrophages from *Gfi1*-WT (n=6) and *Gfi1*-KO (n=6) BMDMs (right panel), (***P<0.0001). Results of duplicates from three independent experiments are shown. (C) Fold change of IL-6 and Nos2 mRNA levels in *Gfi1*-WT (n=4) and *Gfi1*-KO (n=4) M1(LPS) macrophages, normalized to GAPDH. Results of duplicates from two independent experiments are shown (*P=0.03 for IL-6, **P=0.002 for Nos2). (D) The levels of IL-1B (left panel) and IL-6 (right panel) in the supernatants of *Gfi1*-WT (n=8) and *Gfi1*-KO (n=8) M1(LPS) macrophages. Results of duplicates from four independent experiments are shown (*P=0.05 for IL-1B, ***P<0.0001). (E) Fold change of Arg1 mRNA level in *Gfi1*-WT (n=4) and *Gfi1*-KO (n=4) M2(IL-4) macrophages, normalized to GAPDH. Results of duplicates from two independent experiments are shown (*P=0.04). (F) The levels of IL-10 in supernatants of *Gfi1*-WT (n=8) and *Gfi1*-KO (n=8) M2(IL-4) macrophages. Results of duplicates from four independent experiments are shown (**P=0.004). G) Schematic representation of the experimental design for simultaneous *in vitro* polarization of M1 and M2 macrophages. *Gfi1*-WT or *Gfi1*-KO BMDMs were stimulated with both LPS (100 ng/ml) and IL-4 (20ng/ml) for 48 hours and M1 and M2 macrophages were characterized by flow cytometry. (I) Representative FACS plots showing different macrophage classes derived from *Gfi1*-WT or *Gfi1*-KO mice polarized by both LPS and IL-4. H) BMDMs from *Gfi1*-WT (n=4) and *Gfi1*-KO (n=4) mice were polarized for 48 hours with LPS and IL-4. The frequency of Ly6C⁻CD206⁺ M2 macrophages (left panel), (***P<0.0001), Ly6C⁺ CD206⁺ macrophages (middle, **P=0.002) and Ly6C⁺CD206⁻ M1 macrophages (right, P<0.0001) are shown. Results of duplicates from two independent experiments are shown. LPS: lipopolysaccharide, BMDMs: bone marrow derived macrophages; Arg1: arginase1; Nos2: nitric oxide synthase 2; IL-6: interleukin 6; IL-4: interleukin 4; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; WT: wild-type; *Gfi1*: growth factor independent 1; IL-10: interleukin 10: IL-1B: interleukin 1β.

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equivalent to the TAM1 phenotype (Ly6C^{-}MHCII^{-}), as well as the frequency of Ly6C^{int}MHCII^{low} immature leukemic macrophages[38] (*Online Supplementary Figure S4A and S4B*) were significantly increased in the BM and spleen, whereas the frequency of Ly6C^{+}MHCII^{-} monocytes and the other macrophage subsets were decreased or not significantly changed (*Online Supplementary Figure S4A and S4B*).

We confirmed our findings in the *NUP98-HOXD13* mouse model, where the frequency of AAM1 in the BM and spleen of leukemic transgenic mice was higher than in the WT non-leukemic mice (Figure 2D,E). Notably, the survival of the leukemic *NUP98-HOXD13* mice was inversely correlated with the percentage of AAM1 in the BM (Figure 2F).

Evaluation of Wright-Giemsa stained cytospin preparations of sorted GFP^{-}CD11b^{+}Ly6G^{-} cells derived from C1498GFP transplanted leukemic mice, confirmed that these cells were indeed macrophages (Figure 3A,B). Furthermore, they expressed significantly higher levels of Arg1 mRNA (Figure 3C, left panel), which is characteristic for M2 macrophages with tumor-promoting functions.[39] In contrast, the expression of IL-6 and Nos2 mRNA, characteristic for M1 macrophages,[10] were decreased compared to macrophages sorted from non-leukemic mice (Figure 3C, middle and right panel). To further investigate the status of macrophage polarization, GFP^{-}CD11b^{+}Ly6G^{-} sorted cells were cultured in DMEM-Glutamax medium supplemented with 10% FBS, and after 24 hours the level of IL-10 secreted in the culture medium was measured. The production of IL-10, which is characteristic of the M2 activation profile, was significantly increased in AAMs from leukemic mice compared to macrophages from non-leukemic mice (Figure 3D). There were no significant differences with regard to the secretion of IL-6 and IL-1β that are characteristic of M1 macrophages[10] (*data not shown*).

Since Gfi1 is a transcription factor with an important role in macrophage development,[25,40] we next examined its expression in AAMs. Gfi1 expression was about two-fold upregulated in AAMs compared to non-leukemic macrophages (Figure 3E), indicating that higher levels of Gfi1 might be necessary for macrophage polarization. To investigate whether these AAMs can support the growth of leukemic cells *in vitro*, we co-cultured sorted GFP^{-}CD11b^{+}Ly6G^{-} AAMs from leukemic mice with the

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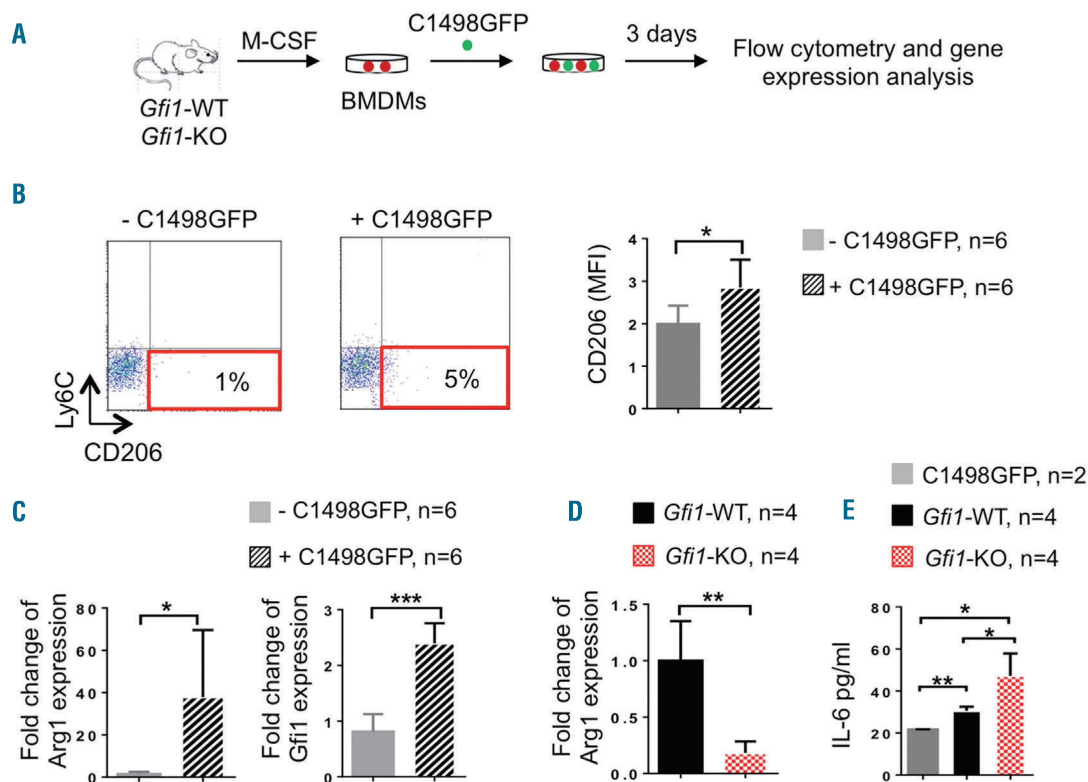

Figure 5. *Gfi1* is involved in the polarization of M2 macrophages by C1498GFP AML cell line *in vitro*. (A) Schematic representation of the procedure for co-culturing of BMDMs from *Gfi1*-WT or *Gfi1*-KO mice with C1498GFP murine AML cell line followed, after 3 days, by flow cytometric and gene expression analysis. (B) Representative FACS plots showing the frequency of Ly6C^{-}CD206^{+} M2 macrophages derived from *Gfi1*-WT mice co-cultured in the presence or absence of C1498GFP cells (left panel) and the corresponding quantification of MFI for CD206 surface marker expression (right panel), (*P=0.02). Results of duplicates from three independent experiments are shown. (C) Fold change of Arg1 and *Gfi1* mRNA expression in *Gfi1*-WT BMDMs cultured in the presence (n=6) or absence (n=6) of C1498GFP cells, normalized to GAPDH. RT-PCR results of duplicates from three independent experiments are shown (*P=0.02 for Arg1 and ***P<0.0001 for *Gfi1*). (D) Fold change in Arg1 mRNA expression in *Gfi1*-WT (n=4) and *Gfi1*-KO (n=4) BMDMs co-cultured with C1498GFP cells, normalized to GAPDH. RT-PCR results of duplicates from two independent experiments are shown (**P=0.004). (E) The level of IL-6 in supernatants of macrophages from *Gfi1*-WT (n=4) or *Gfi1*-KO (n=4) co-cultured with C1498GFP cells for 3 days. Results of duplicates from two independent experiments are shown (*P=0.02 and **P=0.003). AML: acute myeloid leukemia; BMDMs: bone marrow derived macrophages; MFI: mean fluorescence intensity; Arg1: arginase1; IL-6: interleukin 6; IL-4: interleukin 4; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; WT: wild-type; *Gfi1*: growth factor independent 1; RT-PCR: real-time PCR.

murine C1498GFP AML cell line for 48 hours. The growth/proliferation of C1498GFP cells was significantly increased in the presence of AAMs (Figure 3F). Together, these results indicate that the frequency and absolute numbers of AAM1 are increased in the BM of leukemic mice. Furthermore, these AAMs exhibit features of M2 macrophages.

The role of Gfi1 in macrophage polarization in vitro

To assess whether Gfi1 can affect macrophage polarization in response to M1 or M2 stimuli, *Gfi1*-KO and *Gfi1*-WT BMDMs were cultured in the presence of either LPS or INF-γ, which are both M1 stimulators, or IL-4, an M2 stimulator[8,11] (Figure 4A, *Online Supplementary Figure S5A*). In the absence of *Gfi1*, LPS or INF-γ activation resulted in a M1 response as demonstrated by a 2-4-fold increase in the frequency of Ly6C$^+$CD206$^-$ M1 macrophages (Figure 4B, *Online Supplementary Figure S5B and S5C*). Furthermore, *Gfi1-KO* M1(LPS) macrophages expressed significantly increased IL-6 and Nos2 mRNA levels and secreted more IL-6 and IL-1B (Figure 4C,D). Also, in *Gfi1-KO* M1(INF-γ), there was an almost 3-fold increase in Nos2 mRNA levels, (*Online Supplementary Figure S5D*) and 2-fold increase in IL-1B secretion (*Online Supplementary Figure S5E*). Although, phenotypically, there was no difference between the frequencies of M2-polarized macrophages derived from *Gfi1*-WT and *Gfi1*-KO mice (*data not shown*), IL-4 stimulation resulted in an M2 response in *Gfi1*-WT but not in the *Gfi1*-KO macrophages, as demonstrated by a significant increase in Arg1 mRNA expression in *Gfi1*-WT macrophages (Figure 4E) and IL-10 secretion (Figure 4F). *In vivo*, polarization of M1 and M2 macrophages can take place simultaneously depending on the signals and cytokines secreted from the tumor microenvironment. In an attempt to mimic the *in vivo* conditions, *Gfi1*-WT and *Gfi1*-KO BMDMs were challenged *in vitro* with both LPS and IL-4, and M1 and M2 sur-

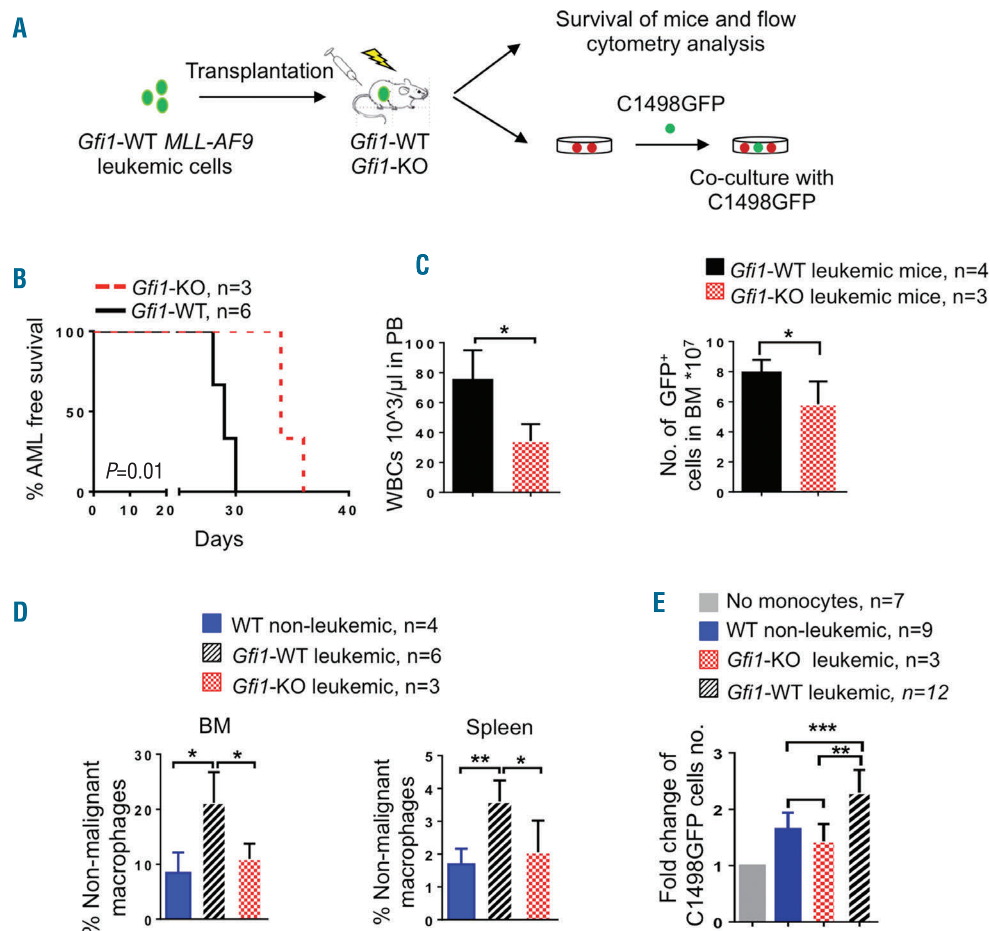

Figure 6. The role of *Gfi1* in polarization of AAMs in transplanted leukemic mice. (A) Schematic illustration of the experimental design. Sublethally irradiated (3Gy) *Gfi1*-WT or *Gfi1*-KO mice were transplanted with 1x10^5 *Gfi1*-WT *MLL-AF9* GFP$^+$ leukemic BM cells derived from primary recipient mice. The mice were monitored and sacrificed and analyzed when moribund. BMDMs from *Gfi1*-WT or *Gfi1*-KO mice were co-cultured with C1498GFP AML cells and after 6 days, C1498GFP counts were evaluated. (B) Kaplan-Meier survival curve of *Gfi1*-KO (n=3) and *Gfi1*-WT (n=6) transplanted with *Gfi1*-WT *MLL-AF9* leukemic cells (*P*=0.01). (C) Total white blood cell count (WBC) in peripheral blood (left) (**P*=0.02) and the number of GFP$^+$ leukemic blast cells in the BM (right) (**P*=0.04) of *Gfi1*-WT (n=4) and *Gfi1*-KO (n=3) leukemic mice. (D) The frequency of GFP$^-$CD11b^{hi}Gr-1^{int} non-malignant macrophages in the BM (left panel) and spleen (right panel) of *Gfi1*-WT (n=6) and *Gfi1*-KO (n=3) transplanted with MLL-AF9 transduced cells compared to mice transplanted with non-transduced cells (n=4) (**P*<0.01, ***P*=0.001). (E) Fold change of live C1498GFP cell number after 6 days of co-culturing with BMDMs from *Gfi1*-WT, *Gfi1*-KO MLL-AF9 transplanted leukemic mice or from mice transplanted with non-transduced cells. Results of triplicates from 3 and 4 independent experiments for *Gfi1*-WT leukemic (n=9) and non-leukemic mice (n=12) and from 1 experiment for *Gfi1*-KO leukemic mice (n=3) are shown (***P*=0.008 and ****P*=0.0004). BM: bone marrow; BMDM: bone marrow-derived macrophage; AML: acute myeloid leukemia; WT: wild-type; *Gfi1*: growth factor independent 1; GFP: green fluorescent protein: AAM: acute myeloid leukemia associated macrophage.

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face marker expressions were examined by flow cytometry (Figure 4G). In the presence of both stimuli, more than 60% of *Gfi1*-WT BMDMs were polarized into Ly6C⁻CD206⁺ M2-like macrophages without any differentiation into Ly6C⁺CD206⁻ M1 macrophages (Figure 4H,I), whereas *Gfi1*-KO BMDMs showed less efficient CD206⁺Ly6C⁻ M2 polarization and enhanced differentiation into Ly6C⁺CD206⁺ and Ly6C⁺CD206⁻ M1 macrophages (Figure 4H,I). Together, these findings suggest that *Gfi1* directs macrophage polarization towards a M2-like macrophage state.

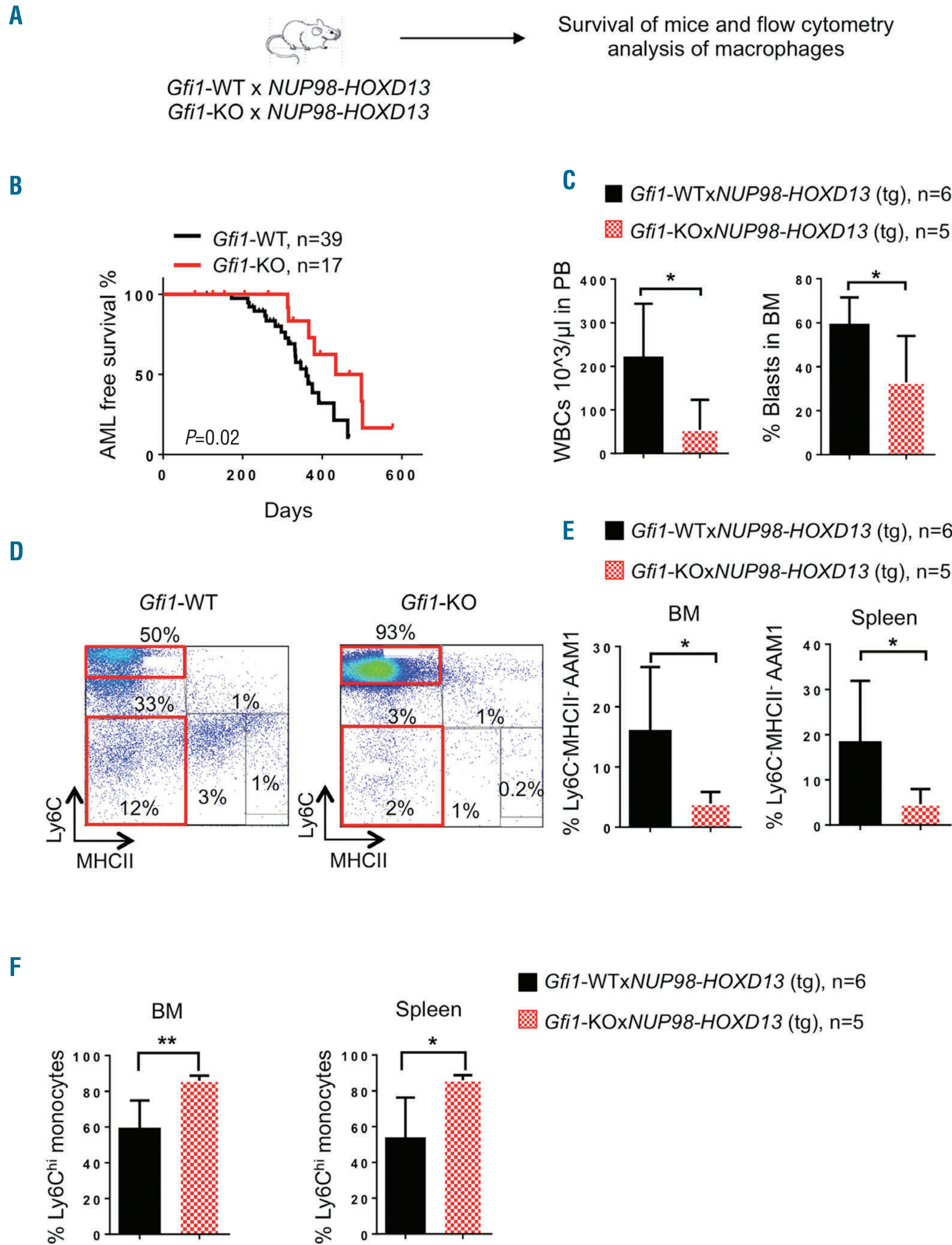

Figure 7. The role of *Gfi1* in polarization of AAMs *in vivo*. (A) Schematic representation of the experimental design. *Gfi1*-WT and *Gfi1*-KO mice were crossed to *NUP98-HOXD13* MDS/AML mouse model. Double transgenic mice were monitored for AML onset and survival. Leukemic mice were analyzed to determine the frequency of different macrophage types. (B) Kaplan-Meier survival curve of *Gfi1*-KO (n=17) and *Gfi1*-WT (n=39) *NUP98-HOXD13* AML mice (P=0.02). (C) Total white blood cells count (WBC) in peripheral blood (left) (*P=0.02) and the percentage of blasts in the BM (right) (*P=0.04) of *Gfi1*-WT (n=6) and *Gfi1*-KO (n=5) *NUP98-HOXD13* leukemic mice. (D) Representative FACS plots showing the frequency of AAM1 in a *Gfi1*-WTx*NUP98-HOXD13* and a *Gfi1*-KOx*NUP98-HOXD13* leukemic mouse. (E) The frequency of Ly6C⁻MHCII⁻ AAM1 in the BM (right) and spleen (left) of *Gfi1*-WTx*NUP98-HOXD13* (n=6) and *Gfi1*-KOx*NUP98-HOXD13* (n=5) leukemic mice (*P=0.02 for BM and *P=0.05 for spleen). (F) The frequency of Ly6C^{hi} monocytes in the BM (right) and spleen (left) of *Gfi1*-WTx*NUP98-HOXD13* (n=6) and *Gfi1*-KOx*NUP98-HOXD13* (n=5) leukemic mice (**P=0.005 for BM and *P=0.01 for spleen). AML: acute myeloid leukemia; AAM: AML-associated macrophages; BM: bone marrow; *Gfi1*: growth factor independent 1; WT: wild-type.

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To investigate the effect of AML cells on the macrophage phenotypes *in vitro*, we co-cultured *Gfi1*-WT and *Gfi1*-KO BMDMs with C1498GFP cells for 3 days (Figure 5A). Co-culture of *Gfi1*-WT BMDMs with C1498GFP cells significantly upregulated CD206 expression on macrophages (Figure 5B) and resulted in an increased expression level of Arg1 mRNA (Figure 5C, left panel). Interestingly, *Gfi1* was found to be highly upregulated in *Gfi1*-WT BMDMs co-cultured with C1498GFP cells (Figure 5C, right panel). Although, phenotypically, there was no difference in M1 or M2 macrophages polarization between *Gfi1*-WT and *Gfi1*-KO cultured in the presence of C1498GFP cells, *Gfi1*-KO BMDMs showed a M1 response, as demonstrated by lower levels of Arg1 mRNA (Figure 5D) and a significant increase in IL-6 secretion compared to *Gfi1*-WT BMDMs (Figure 5E), confirming that the loss of *Gfi1* shifts the macrophage phenotype towards an M1-like activation profile.

The role of Gfi1 in polarization of AAMs in vivo

To test the relevance of these findings and to investigate the effect of *Gfi1* ablation on the growth of leukemic cells *in vivo*, we transplanted *Gfi1*-WT MLL-AF9-expressing BM cells into sublethally irradiated secondary *Gfi1*-WT and *Gfi1*-KO mice (Figure 6A). *Gfi1*-KO mice that received MLL-AF9-expressing cells survived longer (Figure 6B) and had a significantly lower white blood cell (WBC) count in peripheral blood (PB) (Figure 6C, left panel), reduced numbers of GFP$^+$ leukemic cells in the BM (Figure 6C, right panel) and decreased frequency of non-malignant macrophages (GFP$^-$CD11b^{hi}Gr1^{int}) in the BM and spleen (Figure 6D), compared to *Gfi1*-WT mice transplanted with MLL-AF9-expressing cells. To further study the role of *Gfi1* in macrophage function, we co-cultured BMDMs from *Gfi1*-WT and *Gfi1*-KO leukemic mice with C1498GFP cells and found that *Gfi1*-KO BMDMs did not support the growth of C1498GFP cells *in vitro* to the same extent as *Gfi1*-WT BMDMs (Figure 6E).

We validated these results in the *NUP98-HOXD13* transgenic mouse model. We crossed these mice with *Gfi1*-WT or *Gfi1*-KO mice and analyzed their survival and the frequency of different macrophage classes in the BM and spleen of *NUP98-HOXD13*-expressing mice that developed AML (Figure 7A). In agreement with the results presented above, the *Gfi1*-KOx*NUP98-HOXD13*-expressing leukemic mice survived longer (Figure 7B), and were characterized by lower numbers of WBCs in PB and decreased frequency of blast cells in the BM (Figure 7C), compared to *Gfi1*-WT*xNUP98-HOXD13*-expressing leukemic mice. Furthermore, *Gfi1*-KOx*NUP98-HOXD13* leukemic mice had a significantly decreased frequency of AAM1 in the BM and spleen compared to *Gfi1*-WT*xNUP98-HOXD13* leukemic mice (Figure 7D,E). Other macrophage populations such as immature macrophages, AAM2s and AML-associated dendritic cells (ADCs) were also decreased in *Gfi1*-KOx*NUP98-HOXD13*-expressing leukemic mice (*Online Supplementary Figure S6*). The frequency of Ly6C$^+$MHCII$^-$ monocytes from which the different macrophage populations are derived was increased in the BM and spleen of *Gfi1*-KOx*NUP98-HOXD13* compared to *Gfi1*-WT*xNUP98-HOXD13* leukemic mice (Figure 7F), suggesting that monocytes from *Gfi1*-KOx*NUP98-HOXD13* mice differentiate less efficiently into more mature macrophages than monocytes from *Gfi1*-WT*xNUP98-HOXD13* mice.

Taken together, all of these results suggest that AAMs play an important role in the progression of AML, and *Gfi1* is crucial in the process of macrophage polarization, since its absence impedes macrophage polarization towards a leukemia-supporting state and favors an anti-tumor state.

Discussion

We investigated the interaction between AAMs and murine AML cells *in vivo* and *in vitro*. We observed an increased accumulation of monocytes/macrophages in the BM of AML patients and in the BM and spleen of several AML mouse models, indicating that the leukemic cells might induce BM monocyte/macrophage proliferation and/or infiltration. In addition, we found the same pattern of monocytes/macrophages infiltration in a *NUP98-HOXD13* transgenic MDS/AML mouse model. This suggests that the presence of AML and the leukemic environment leads to an infiltration of monocytes/macrophages and promotes their differentiation into AAMs. In the case of the very aggressive type of the MLL-AF9 induced AML, the absolute number of AAMs in the BM of the leukemic mice is lower than in the BM of healthy mice (*data not shown*). Our hypothesis is that the MLL-AF9 leukemic cells overgrow all other cells, including the AAMs. However, in all cases, the relative percentage of AAMs in the BM of leukemic mice was always increased compared to the situation found in the BM of healthy mice, and the functional changes of AAMs, with regard to supporting the growth of leukemic cells, were similar from one type of AML to the next.

The supporting role of TAMs in the growth of tumor cells has been studied in a number of different types of solid cancers.[41] Initially, the concept of M1 and M2 macrophages have been helpful in exploring the new field of TAMs,[13,41-43] but it has been recently redefined. For example, what we describe herein as M2 macrophages[44] has recently been proposed to be IL-4 macrophages, and the M1 macrophages as LPS or IFN-γ macrophages.[41] Also, distinct expression profiles and secretion patterns have been used to better characterize different macrophage classes.[9,45]

Although TAMs are mostly M2-like macrophages, some studies showed that TAMs have a gene expression profile similar to both, M1- or M2-like macrophages.[36] We have demonstrated that, phenotypically, AAMs derived from the BM and spleen of leukemic mice were M2-like macrophages (Ly6C$^-$MHCII$^-$)[33,36] that express higher levels of Arg1 and lower levels of IL-6 and Nos2 mRNA, and secrete more IL-10 than non-leukemic macrophages. The decrease in the frequency of Ly6C$^+$MHCII$^-$ monocytes in the BM and spleen of leukemic mice, and the increased numbers of Ly6C$^-$MHCI$^-$ AAMs compared to non-leukemic mice, suggest that AAMs might be derived from Ly6C$^+$MHCII$^-$ monocytes. On the other hand, the accumulation of Ly6C^{int}MHCII$^-$ immature macrophages, which are the intermediate stage between Ly6C$^+$MHCII$^-$ monocytes and Ly6C$^-$MHCII$^-$ AAMs[36,38] in the BM and spleen of leukemic mice, indicates that the differentiation process of Ly6C$^+$MHCII$^-$ monocytes towards an AAM phenotype is active during leukemia development.

In our first set of experiments, mice were subjected to sublethal irradiation to enable the engraftment of leukemic cells. It is known that irradiation can alter the stroma microenvironment to support the malignant transformation[46] or to alter the macrophage subtypes.[13,47] However, to

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ensure comparability, we always correlated our findings to sublethally irradiated mice transplanted with wild-type, non-malignant BM cells.

In terms of the functional characterization of AAMs *in vitro*, we cannot exclude that the differentiation of AAMs *via* M-CSF might alter their function, but as we obtained similar results in a murine model of AML in which AAMs were sorted and co-cultured with AML cells without prior M-CSF co-culture, we believe that the cytokine-induced differentiation is not *per se* artificial.

As *Gfi1* is required for the differentiation and maturation of HSCs into myeloid and lymphoid cells,[25,40] we hypothesized that *Gfi1* might play an important role in the polarization of macrophages in leukemic mice. It is known that within the myeloid lineage/compartment, Gfi1 favors the differentiation towards granulocytes and impedes monocyte development.[24-26,28] However, it has been shown that there is a discrepancy between reduced Gfi1 mRNA levels and elevated Gfi1 protein levels in monocytes.[48] Thus, despite lower Gfi1 expression at the mRNA level, Gfi1 is present at the protein level, and is required for the proper differentiation of monocytes towards macrophages and other monocyte-derived cell types.[48] In our experiments, *Gfi1* was 2-fold upregulated at mRNA levels in AAMs derived from the BM of transplanted leukemic mice and in macrophages co-cultured with AML cells, indicating that *Gfi1* indeed plays a role in macrophage differentiation. Leukemic *Gfi1*-KO mice survived longer, and had a lower percentage of leukemic cells in PB and BM and decreased numbers of AAMs than *Gfi1*-WT leukemic mice. These results indicate that various *Gfi1*-deficient stroma elements, including AAMs, were not well polarized to support the growth of AML cells *in vivo*. This might be explained by the fact that the loss of *Gfi1* shifts the cells toward a M1-like activation profile, which counteracts the growth of malignant cells rather than supporting it. It could be argued that *Gfi1*-deficient macrophages are too different from their WT counterparts. A number of publications have examined *Gfi1*-WT and *Gfi1*-KO macrophages and found that *Gfi1*-KO macrophages might differ on a quantitative level with regard to certain pathways, but overall they can be regarded as macrophages.[28,48-50]

Our finding that *Gfi1*-KO AAMs express more IL-6, Nos2 and other inflammatory mediators at mRNA level *in vitro* and *in vivo* when exposed to LPS, is in line with reports demonstrating a hyper-reactive response in *Gfi1*-deficient macrophages after exposure to LPS.[28,49] Gfi1 exerts this function by its inhibitory effect on the Toll-like receptor 4 (TLR4) pathway through antagonizing the nuclear transcription factor κ-light-chain-enhancer of activated B cells (NF-κB).[49] In contrast to the inhibitory effect of *Gfi1* on M1 macrophage polarization, our results indicate that *Gfi1* enhances the polarization of AAMs (M2-like macrophages) *in vivo* and *in vitro*. The upregulation of *Gfi1* in response to M2 stimuli underlines this. We observed that transgenic *Gfi1*-KOx*NUP98-HOXD13* leukemic mice had a lower frequency of AAMs and a higher percentage of Ly6C$^+$ monocytes than *Gfi1*-WTx*NUP98-HOXD13* leukemic mice. We hypothesize that in the absence of *Gfi1*, the differentiation of immature macrophages into AAMs is disturbed. *In vitro*, *Gfi1*-KO macrophages co-cultured with C1498GFP cells expressed higher levels of IL-6 and lower levels of Arg1 mRNA than *Gfi1*-WT macrophages. *Gfi1* might regulate M1 and M2 polarization through its suppressive function on genes that are associated with M1 polarization. The increased *Gfi1* expression in AAMs *in vivo* might impede M1 macrophage polarization and function, resulting in a shift of polarization towards a M2 phenotype. Additionally, *Gfi1* is required by AAMs or M2 macrophages to secrete enzymes and cytokines, such as Arg1 and IL-10, which play important roles in the suppression of the immune system. There are, however, many open questions on how *Gfi1* polarizes AAMs and which pathways might be involved.[25,49]

On a functional level, we characterized the interaction between macrophages and AML cells by using established procedures applied for the analysis of the interaction between macrophages and solid cancers.[6,36] AML cells induce the expansion and/or migration of tissue-resident macrophages. They function as AAMs since they support the growth of AML cells both *in vivo* and *in vitro*. Furthermore, the polarization of AAMs depends on the presence of *Gfi1*, which is a potential new regulator of AAMs and macrophage polarization. We show one possibility of how the polarization of AAMs might be regulated, and targeting *Gfi1* could be a novel approach to AML therapy by inhibiting the function of AAMs, expanding the possibility of stroma targeting approaches.[51] Despite recent advances in the field of immunotherapy of solid cancers, a better understanding on how macrophages contribute to the growth of AML might open new AML therapy approaches.

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