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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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Running title: Gfi1 and polarization of AML-associated macrophages
Abstract (242 words, max 250 words)

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 here 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 the 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.
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. 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. Tumor cells induce the stroma and immune cells to express and partially secret various factors and cytokines that promote the growth of the tumor cells instead of activating the immune system to battle the malignant cells. This process of “polarization” is the result of a complex bi-directional interaction between the tumor and the stroma cells. Hence, the polarized macrophages in tumors are called tumor-associated macrophages (TAMs). The plasticity of macrophages is mostly tissue-specific and regulated by local and systemic signals. 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. Although meanwhile considered a simplification, 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), tumour 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). The M1 macrophages are involved in the host defence against different pathogens and play a role in the anti-tumor immunity. In contrast, M2 macrophages or alternatively activated macrophages have anti-inflammatory activity and are stimulated by interleukin 4 (IL-4) or IL-13. They secrete arginase, metalloproteinases, transforming growth factor-beta (TGFβ), IL-10 and other cytokines that cause immune suppression, angiogenesis and tissue repair. M2 macrophages have been further subdivided into M2a, M2b, M2c and M2d macrophages according to the polarizing cytokines. In contrast to M1 macrophages, which suppress the tumor growth, M2 macrophages play an important role in the development and progression of different tumors 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 a way that it supports the growth of LSCs instead of hematopoietic stem cells (HSCs).\textsuperscript{15} This might enhance the LSCs quiescence, leading to chemotherapy resistance.\textsuperscript{1,16-19} A recent study reported that the inhibition of SIRP\textalpha{} signalling in macrophages impairs engraftment of human LSCs in immunocompromised NSG mice.\textsuperscript{20} Clinically, the accumulation of TAMs in the lymph nodes of patients with classic Hodgkin’s lymphoma was associated with a poor prognosis.\textsuperscript{21} The most common form of adult leukemia is acute myeloid leukemia (AML)\textsuperscript{22}, which is characterized by an accumulation of myeloid blast cells in the BM. As AML patients have a poor prognosis\textsuperscript{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 it is crucial for normal lymphoid and myeloid hematopoiesis.\textsuperscript{23,24,25} Gfi1-deficient mice are characterized by a severe neutropenia and an overproduction of TNF-\alpha{} and other inflammatory mediators of macrophages when exposed to bacterial endotoxin or LPS.\textsuperscript{26} Using different mouse models of human AML we report here that AAMs support expansion of AML cells both \textit{in vivo} and \textit{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 after 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. AML-diagnosis 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 described²⁸. Wildtype (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, who approved on 21.07.2011 all studies on animals under the document number G1196/11.

AML cell lines

C1498GFP, a murine AML cell line²⁹, was a kind gift from Dr. Justin Kline from the University of Chicago. 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

Student's t-test was applied to calculate the differences between various groups. For the survival analysis, 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**

Expression of CD163 has been reported to be restricted to monocytes/macrophage lineage.\(^3\) Recently, CD163\(^+\) M2 TAMs have been reported to be involved in tumor progression in several hematological malignancies such as multiple myeloma\(^3\) or classical Hodgkin lymphoma (CHL).\(^3\) A common cell surface marker identified in TAMs is CD206.\(^3\) 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 (Supplementary Table 1). The frequency of CD163\(^+\)CD206\(^+\) M2-like macrophages in the BM of AML patients was significantly elevated compared to healthy volunteers (Supplementary Figure 1 A-C).

**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-ETO\(^9\)a, 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 pathogenicity in humans and are also used to model AML in mice.\(^3\),\(^3\) While AML1-ETO\(^9\)a-induced AML is associated with a rather good prognosis, MLL-AF9-driven AML has a rather bad prognosis.\(^3\),\(^3\) 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-ETO\(^9\)a cDNA fused to an IRES-GFP gene cassette and transplanted these cells into lethally irradiated mice alongside with 1.5x10\(^5\) competitive BM cells. Leukemic BM cells were then re-transplanted into secondary, sublethally irradiated recipient mice (Figure 1A). The expression of GFP alongside with 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\(^b\)Gr1\(^b\).\(^2\) The frequency of GFP\(^-\) AAMs in BM and spleen of leukemic mice
was significantly higher than in sublethally irradiated mice transplanted only with competitive normal BM cells (Figure 1 B and C). Also, when we defined AAMs as GFP–CD11b+Ly6G– cells\(^3\) (Figure 1 D), we found similar results (Figure 1 E). 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.\(^3\) Similarly, the percentage of AAMs in BM and spleen of leukemic NUP98-HOXD13 transgenic mice was higher than in WT non-leukemic mice (Supplementary Figure 2 A and B). We confirmed that, phenotypically, in both 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 respectively 70% (Supplementary Figure 2 C and D).

We then tested whether these AAMs would support the growth of murine AML cells \textit{in vitro}. We co-cultured BM-derived macrophages (BMDMs) with the murine AML cell line C1498GFP\(^2\) 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 1 F).

\textbf{Characterization of AAMs}

Macrophages are characterized by specific gene expression patterns, cytokine secretion and cell surface molecules.\(^7\) By using a similar gating strategy as reported earlier for studying TAMs in lung cancer\(^3\), we quantified the different mononuclear phagocyte subsets in BM and spleen of sublethally irradiated mice transplanted either with C1498GFP cell line or with MLL-AF9 or AML1-ETO9a BM leukemic BM cells from primary recipient mice (Figure 2 A). 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 2 B).\(^3\), \(^3\) In all leukemic mouse models, we found that not only the frequency (Figure 2 C) but also the absolute numbers (Supplementary Figure 3 A and B) of AAM1 cells, which are equivalent to the TAM1 phenotype (Ly6C–MHCII–), as well as the frequency of Ly6C\(^{int}\)MHCII\(^{low}\) immature leukemic macrophages\(^3\) (Supplementary Figure 4 A and B) 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 (Supplementary Figure 4 A and B).

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

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 3 A and B). Furthermore, they expressed significantly higher levels of Arg1 mRNA (Figure 3 C, left panel), which is characteristic for M2 macrophages with tumour-promoting functions.39 In contrast, the expression of IL-6 and Nos2 mRNA, characteristic for M1 macrophages10, were decreased compared to macrophages sorted from non-leukemic mice (Figure 3 C, 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 for the M2 activation profile, was significantly increased in AAMs from leukemic mice compared to macrophages from non-leukemic mice (Figure 3 D). There were no significant differences with regard to the secretion of IL-6 and IL-1B that are characteristic for M1 macrophages10 (data not shown).

Since Gfi1 is a transcription factor with an important role in macrophage development25,40, we next examined its expression in AAMs. Gfi1 expression was about two-fold upregulated in AAMs compared to non-leukemic macrophages (Figure 3 E), 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 murine C1498GFP AML cell line for 48 hours. The growth/proliferation of C1498GFP cells was significantly increased in the presence of AAMs (Figure 3 F). 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\(^8\)\(^1\)\(^1\) (Figure 4 A, Supplementary Figure 5 A). 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 4 B, Supplementary Figure 5 B and C). Furthermore, Gfi1-KO M1(LPS) macrophages expressed significant increased IL-6 and Nos2 mRNA levels and secreted more IL-6 and IL-1β (Figure 4 C and D). Also in Gfi1-KO M1(INF-γ) there was an almost three-fold increase in Nos2 mRNA levels, (Supplementary Figure 5 D), and two-fold increase in IL-1β secretion (Supplementary Figure 5 E). 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 4 E) and IL-10 secretion (Figure 4 F). *In vivo*, polarization of M1 and M2 macrophages can take place simultaneously depending on the signals and cytokines secreted from 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 surface marker expression were examined by flow cytometry (Figure 4 G). 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 4 I and H), whereas Gfi1-KO BMDMs showed less efficient CD206\(^+\)Ly6C\(^−\) M2 polarization and enhanced differentiation into Ly6C\(^+\)CD206\(^+\) and Ly6C\(^−\)CD206\(^−\) M1 macrophages (Figure 4 I and H). Together, these findings suggest that Gfi1 directs macrophage polarization towards a M2-like macrophage state.

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 5 A). Co-culture of Gfi1-WT BMDMs with C1498GFP cells significantly upregulated CD206 expression on macrophages (Figure 5 B) and resulted in an increased expression level of Arg1 mRNA (Figure 5 C left panel). Interestingly, Gfi1 was found to be highly upregulated in Gfi1-WT BMDMs co-cultured with C1498GFP cells (Figure 5 C, right panel). Although,
phenotypically, there was no difference in M1 or M2 macrophages polarization between Gfi1-WT and Gfi1-KO cultured in presence of C1498GFP cells, Gfi1-KO BMDMs showed a M1 response, as demonstrated by lower levels of Arg1 mRNA (Figure 5 D) and a significant increase in IL-6 secretion compared to Gfi1-WT BMDMs (Figure 5 E), confirming that 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 secondary irradiated Gfi1-WT and Gfi1-KO mice (Figure 6 A). Gfi1-KO mice that received MLL-AF9-expressing cells survived longer (Figure 6 B) and had significantly lower white blood cell count (WBC) in peripheral blood (PB) (Figure 6 C, left panel), reduced numbers of GFP+ leukemic cells in the BM (Figure 6 C, right panel) and decreased frequency of non-malignant macrophages (GFP+CD11bhiGr1int) in the BM and spleen (Figure 6 D) 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 6 E).

We validated these results in the NUP98HOXD13 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 BM and spleen of NUP98-HOXD13-expressing mice that developed AML (Figure 7 A). In agreement with the results presented above, the Gfi1-KOxNUP98-HOXD13-expressing leukemic mice survived longer (Figure 7 B) and were characterized by lower numbers of WBC in PB and decreased frequency of blast cells in the BM (Figure 7 C) compared to Gfi1-WTxNUP98-HOXD13-expressing leukemic mice. Furthermore, Gfi1-KOxNUP98-HOXD13 leukemic mice had a significantly decreased frequency of AAM1 in BM and spleen compared to Gfi1-WTxNUP98-HOXD13 leukemic mice (Figure 7 D and E). Also other macrophage populations such as immature macrophages, AAM2s and AML-associated dendritic cells (ADCs) were decreased in Gfi1-KOxNUP98-HOXD13-expressing leukemic mice (Supplementary Figure 6). The frequency of Ly6C+MHCII+ monocytes from which the different macrophage populations
are derived was increased in the BM and spleen of Gfi1-KOxNUP98-HOXD13 compared to Gfi1-WTxNUP98HOXD13 leukemic mice (Figure 7 F), suggesting that monocytes from Gfi1-KOxNUP98HOXD13 mice differentiate less efficiently into more mature macrophages than monocytes from Gfi1-WTxNUP98HOXD13 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 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 presence of AML and the leukemic environment leads to an infiltration of monocytes/macrophages and promotes their differentiation into AAMs. In very aggressive type of MLL-AF9 induced the absolute number of AAM is lower than in healthy mice (data not shown). Our hypothesis is that, the MLL-AF9 leukemic cells overgrow all other cells, including the AAM. However, in all cases, the relative percentage of AAM was always increased and the functional changes of AAM were similar from one type of AML to the next one.

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

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. We have demonstrated that, phenotypically, AAMs derived from BM and spleen of leukemic mice were M2-like macrophages (Ly6C-MHCII−) 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 BM and spleen of leukemic mice and the increased numbers of Ly6C+MHCII+ AAMs compared to non-leukemic mice, suggest that AAMs might be derived from Ly6C+MHCII+ monocytes. On the other hand, the accumulation of Ly6CintMHCII+ immature macrophages, which are the intermediate stage between Ly6C+MHCII+ monocytes and Ly6C+MHCII+ AAMs, in
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 engraftment of leukemic cells. It is known that irradiation can alter the stroma microenvironment to support the malignant transformation or to alter the macrophage subtypes. However, to ensure comparability, we always correlated our findings to sublethally irradiated mice transplanted with wildtype, 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, 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 favours the differentiation towards granulocytes and impedes monocyte development. However, it has been shown that there is a discrepancy between reduced Gfi1 mRNA level and elevated Gfi1 protein levels in monocytes. Thus, despite lower Gfi1 expression at 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. In our experiments, Gfi1 was two-fold upregulated at mRNA level in AAMs derived from 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 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 quantitative level with regard to certain pathways, but overall they can be regarded as macrophages.\textsuperscript{28, 48-50}

Our finding that *Gfi1*-KO AAMs express more IL-6, Nos2 and other inflammatory mediators at mRNA level \textit{in vitro} and \textit{in vivo} when exposed to LPS is in line with reports demonstrating a hyper-reactive response in *Gfi1*-deficient macrophages after exposure to LPS.\textsuperscript{28, 49} *Gfi1* exerts this function by its inhibitory effect on the Toll-like receptor 4 (TLR4) pathway through antagonizing the nuclear transcription factor kappa light chain enhancer of activated B cells (NF-κB).\textsuperscript{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) \textit{in vivo} and \textit{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. \textit{In vitro}, *Gfi1*-KO macrophages co-cultured with C1498GFP cells expressed higher levels of \textit{IL-6} and lower levels of \textit{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 \textit{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.\textsuperscript{25, 49}

We characterized on a functional level the interaction between macrophages and AML cells by using established procedures applied for analysis of the interaction between macrophages and solid cancers.\textsuperscript{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 \textit{in vivo} and \textit{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 expand the field on how the interaction between stroma and leukemic cells can be targeted as done for other pathways.\textsuperscript{51} Despite recent advances in the field
of immunotherapy of solid cancer, better understanding on how macrophages contribute to the growth of AML might open new AML therapy approaches.
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Author Contributions

R.H., U.D. and C.K. provided funding,
Y.A-M, L.B., B.O. and C.K. wrote manuscript,
U.D., B.O., J.S., T.S., R.H. and C.K. edited manuscript,
C.K. supervised study.

Competing financial interests statements:

No conflicts of interest to declare.
References


Al-Matary et al., Gfi1 and polarization of AML-associated macrophages

Figures legends

Figure 1. AML-associated monocytes/macrophages (AAM) proliferate and accumulate in 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*10⁵ MLL-AF9 or 5-7*10⁵ AML1-ETO9a GFP⁺ cells were transplanted into lethally irradiated (10Gy) primary recipient mice together with 5*10⁵ competitive BM cells. Leukemic BM cells (1*10⁵ GFP⁺ cells) were then re-transplanted into secondary sublethally irradiated (3Gy) mice. Macrophage surface markers from leukemic mice were subsequently analysed by flow cytometry.

B) Representative gating strategy for GFP⁻CD11b⁹Gr¹int 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⁻CD11b⁹Gr¹int 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⁻CD11b⁺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) 2-3x10⁵ BMDMs from mice transplanted with non-transduced or AML1-ETO9a or MLL-AF9-transduced cells were co-cultured with 5*10⁴ 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
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 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 expression of Ly6C and MHCII markers. Cells with a GFP^CD11b^Ly6G^-MHCII^-Ly6C^- phenotype were considered AAM1.

C) The frequency of AAM1 in 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 BM of the Gfi1-WTxNUP98-HOXD13 mouse model.

E) The frequency of AAM1 cells in 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
Figure 3. Characterization of AAMs by RT-PCR and ELISA

A) Schematic illustration of the experimental design. 1x10⁵-4x10⁵ 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 level 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) 5x10⁵ AAMs sorted from leukemic mice transplanted with C1498GFP cells (n=8) or 5x10⁵ CD11b⁺Ly6G⁻ non-leukemic macrophages sorted from mice transplanted with WT BM cells (n=8) were cultured in DMDM/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) 5x10⁴ C1498GFP⁺ cells were co-cultured with 1.5x10⁴ 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 independence 1, BMDM=Bone marrow derived macrophage, Arg1=arginase 1, Nos2=nitric oxide synthase 2, IL-6=interleukin 6, GAPDH=Glyceraldehyde 3-phosphate dehydrogenase
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 (20 ng/ml) for 48 hours. 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 level 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 (20 ng/ml) for 48 hours and M1 and M2 macrophages were characterized by flow cytometry.

I) Representative FACS plots showing different macrophages 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
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 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 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
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 1×10⁵ *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-Meyer 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⁺Gr-1⁻ 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.
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 analysed to determine the frequency of different macrophage types.

B) Kaplan-Meyer survival curve of *Gfi1*-KO (n=17) and *Gfi1*-WT (n=39) *NUP98-HOXD13* AML mice (p=0.01).

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
Figure 1.

A  
MLL-AF9 or AML1-ETO9a  
WT Lin−  
BM cells  
Primary recipient  
Secondary recipient  
Macrophage analysis by flow cytometry

B  
BM of mice transplanted with  
non-transduced cells  
BM of mice transplanted with  
AML1-ETO9a leukemic cells

C  
BM  
Spleen  
% Non-leukemic macrophages  
Non-leukemic, n=4  
MLL-AF9, n=5  
AML1-ETO9a, n=4

D  
BM of mice transplanted with  
non-transduced cells  
BM of mice transplanted with  
AML1-ETO9a leukemic cells

E  
BM  
Spleen  
% AAMs  
Non-leukemic, n=5  
MLL-AF9, n=4  
AML1-ETO9a, n=5

F  
M-CSF  
C1498GFP cells  
Leukemic and non-leukemic  
BM cells  
BMDM  
Co-culture for 6 days  
Fold change of C1498GFP cell no.  
No macrophages, n=30  
Non-leukemic, n=12  
MLL-AF9 leukemic, n=9  
AML1-ETO9a leukemic, n=9
Figure 2.

A. Transplantation of AML1-ETO9a or MLL-AF9 or C1498GFP leukemia cells into Gfi1 WT mice followed by analysis of different macrophage classes by flow cytometry.

B. Flow cytometry analysis of BM of mice transplanted with non-transduced cells and AML1-ETO9a leukemic cells.

C. Quantification of AAM1 macrophages in BM and Spleen from non-leukemic, C1498GFP, MLL-AF9, and AML1-ETO9a groups.

D. Flow cytometry analysis of BM from Gfi1-WTxNUP98-HOXD13 and Gfi1-WT mice.

E. Quantification of AAM1 macrophages in BM from WT, NUP98(tg), and WT, NUP98(tg) NUP9 mice.

F. Survival curve showing the effect of AAM1 on survival days.
Figure 3.

A) Transplantation of C1498GFP leukemic cells into Gfi1-WT mice followed by analysis of GFP-CD11b^+Ly6G^- monocytes/macrophages.

B) Flow cytometry analysis showing CD11b vs Ly6G expression with images of cells at 20µm scale.

C) Graphs showing fold change of Arg1 expression: Leukemic macrophages (n=6) vs Non-leukemic macrophages (n=6).

D) Graphs showing IL-10 levels (pg/ml) in Leukemic macrophages (n=8) vs Non-leukemic macrophages (n=8).

E) Graphs showing Gfi1 expression fold change in Leukemic macrophages (n=6) vs Non-leukemic macrophages (n=6).

F) Sort of C1498GFP sorted macrophages followed by analysis of fold change of C1498GFP cell number in +AAMs (n=9) vs -AAMs (n=9) condition.
Figure 4.

A

Gfi1-WT  Gfi1-KO

M-CSF  LPS 100 ng/ml  M1 macrophage

BMDMs  IL4 20 ng/ml  M2 macrophage

Gfi1-WT M1  Gfi1-KO M1

12.7%  47.5%

Ly6C  CD206

Ly6C  CD206

B

C

Gfi1-WT, n=6  Gfi1-KO, n=6

%Ly6C·CD206 M1

Fold changes of IL-6 expression

Fold changes of Nos2 expression

D

E

F

Gfi1-WT, n=8  Gfi1-KO, n=8

IL-1B in M1  IL-6 in M1

Arg1 in M2  IL-10 in M2

Fold changes of Arg1 expression

IL-1B pg/ml

IL-6 pg/ml

Fold changes

折叠变化

G

M-CSF  LPS 100 ng/ml  IL4 20 ng/ml

BMDMs  Detection of M1 and M2 surface markers by flow cytometry

Gfi1-WT  Gfi1-KO

0.2%  3%

2%  11%

Ly6C  CD206

Ly6C  CD206

I

H

Gfi1-WT  Gfi1-KO

%Ly6C·CD206 M2 cells

%Ly6C·CD206·M2 cells

%Ly6C·CD206·M1 cells

Gfi1-WT, n=6  Gfi1-KO, n=6

**  ***

0  1  2  3  4  5  6  7  8  9  10  11  12  13  14  15

75%  40%
Figure 5.

A

Gfi1-WT  
Gfi1-KO

M-CSF  
C1498GFP

BMDMs  
3 days

Flow cytometry and gene expression analysis

B

- C1498GFP

+ C1498GFP

Ly6C  
CD206

1%  
5%

CD206 (MFI)

C

- C1498GFP, n=6

+ C1498GFP, n=6

Fold change of Arg1 expression

Fold change of Gfi1 expression

D

Gfi1-WT, n=4

Gfi1-KO, n=4

Fold change of Arg1 expression

E

Gfi1-WT, n=4

Gfi1-KO, n=4

IL-6 pg/ml

*  
**  
***  
*

n=2
Figure 6.

A

Transplantation of Gfi1-WT MLL-AF9 leukemic cells to Gfi1-WT and Gfi1-KO mice. Survival of mice and flow cytometry analysis of C1498GFP co-culture with C1498GFP.

B

% AML free survival

\[ \% \text{ AML free survival} \]

Days

\[ p=0.01 \]

Gfi1-KO, n=3

Gfi1-WT, n=6

C

WBCs $10^3/\mu l$ in PB

\[ \text{WBCs} = 10^3/\mu l \]

\[ * \]

\[ ** \]

\[ n=4 \]

\[ n=3 \]

D

WT non-leukemic, n=4

Gfi1-WT leukemic, n=6

Gfi1-KO leukemic, n=3

BM

% Non-malignant macrophages

\[ * \]

\[ ** \]

Spleen

% Non-malignant macrophages

\[ ** \]

\[ ** \]

E

No monocytes, n=7

WT non-leukemic, n=9

Gfi1-WT leukemic, n=3

Gfi1-KO leukemic, n=12

Fold change of C1498GFP cells

\[ *** \]

\[ ** \]
Supplementary Material and Methods

Transplantation experiments

Leukemia in mice was induced by transplanting lineage-negative (Lin\(^{-}\)) BM cells transduced with retroviruses carrying the $\text{AML1-ETO9a}$ or the $\text{MLL-AF9}$ oncofusion genes as well as the GFP-encoding gene as previously described.\(^1\)\(^,\)\(^2\) For primary transplantations about 5-7x10\(^5\) $\text{AML1-ETO9a}$-transduced Lin\(^{-}\) BM cells or 1x10\(^5\) $\text{MLL-AF9}$-transduced Lin\(^{-}\) BM cells (GFP\(^+\)) from WT mice were injected together with 1.5x10\(^5\) competitive BM cells from $\text{Gfi1}$-WT mice into the tail vein of lethally irradiated (10 Gy) congenic recipient mice. After transplantation, the mice were monitored every second day and when moribund, the emerging BM leukemic cells were preserved in liquid nitrogen for subsequent experiments or directly used for secondary transplantations. For secondary transplantations, 1x10\(^5\) $\text{AML1-ETO9a}$ fresh GFP\(^+\) leukemic BM cells (or 1x10\(^5\) frozen $\text{MLL-AF9}$ leukemic BM cells were transplanted into sublethally irradiated (3 Gy) $\text{Gfi1}$-WT or $\text{Gfi1}$-KO (knock-out) secondary recipient mice. In an independent experiment, we created leukemia by transplanting 1-4x10\(^5\) $\text{C1498GFP}$ cells\(^3\) into sublethally irradiated (3 Gy) recipient mice.

**Macrophage co-culture with leukemic cell lines**

Bone marrow-derived macrophages (BMDMs) were prepared from BM cells of mice using macrophage colony stimulating factor (M-CSF), (Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described.\(^4\)\(^,\)\(^5\) In brief, 1.5-2x10\(^6\) BM cells were cultured in DMEM-Glutamax (Gibco) containing 10% fetal bovine serum (FBS) (PAN\(^\text{TM}\) BIOTECH), 1% penicillin/streptomycin (Gibco) and 10 ng/ml M-CSF. After 7 days the macrophages were harvested using trypsin-EDTA (Gibco) and the expression of CD11b and F4/80 surface markers was determined by flow cytometry. Normally, the macrophage purity was higher than 90%. 2-3x10\(^5\) macrophages per well were then cultured in 4 wells of 24 well plate (Falcon, New York, NY, USA). On day 1, the supernatant was discarded and 5x10\(^4\) $\text{C1498GFP}$ cells were added into three wells in the same medium and one well without $\text{C1498GFP}$ cells, as a negative control. After 6
days of co-culture, the suspension cells were collected and the non-adherent cells were washed with DPBS (Gibco) and collected too. The adherent cells were detached using trypsin-EDTA (Gibco) for 5 min at room temperature. The adherent and non-adherent cells in each well were counted manually and the frequency of live C1498GFP cells was determined at a FLOWScan flow cytometer (BD Biosciences, Heidelberg, Germany). Afterwards, the number of GFP$^+$ was calculated. In an alternative approach, we cultured sorted GFP$^-$ macrophages (CD11$^+$Ly6G$^-$) from transplanted leukemic mice as previously described. In brief, 1.5x10$^4$ of sorted cells were cultured per well in 48 well plates in DMEM-Glutamax containing 10% FBS and 1% penicillin/streptomycin. 5x10$^4$ C1498GFP cells were added to each well. After 48 hours, the non-adherent leukemic cells in each well were harvested by collecting supernatants and detaching the remaining cells using trypsin-EDTA. The cells were then manually counted and the frequency of live GFP$^+$ cells was determined by a FLOWScan flow cytometer.

**Macrophage polarization experiments**

BMDMs were prepared using M-CSF as described above. The macrophage purity (CD11b$^+$F4/80$^+$) was >95%. After 6 days, supernatants were discarded and wells were washed with DPBS (Gibco). BMDMs were then polarized into M1 macrophages using 2 ml of DMEM-Glutamax (Gibco) supplemented with 10% FBS, 1% penicillin/streptomycin and 100 ng/ml LPS (Sigma-Aldrich, Taufkirchen, Germany) or 100 ng/ml INF-γ (Peprotech INC, Hamburg, Germany) and into M2 macrophages using 20 ng/ml IL4 (MiltenyiBiotec). After 48 hours, the M1 and M2 polarization was examined by flow cytometry and RT-PCR. The supernatants were collected, filtered and frozen for subsequent ELISA measurement of cytokine productions.

**Flow cytometry and antibodies**

Murine leukemic BM cells were collected by flushing femurs, tibiae and humeri with FACS buffer (DPBS containing 2% FCS and 1% penicillin/streptomycin). Human mononuclear cells were extracted from the BM of leukemic patients and healthy volunteers using Ficoll density gradient centrifugation. The cells were stained and
analysed using LSR II or FLOWScan flow cytometers (BD Biosciences). A preparative FACS Vantage SE and Diva option flow cytometer (BD Biosciences) was used for cell sorting. Raw FACS data were analysed using the FlowJo software (Tree Star, Inc., OR, USA). The following anti-mouse antibodies from Biolegend (Fell, Germany) were used: CD11bPerCP clone: M1/70, (Cat. No. 101228), Ly6G PE clone: 1.A8, (Cat. No. 127607), MHC II APC clone: M5/114.15.2, (Cat. No. 107613), Ly6C PE/Cy7 clone: HK1.4, (Cat. No. 128017), F4/80 APC clone: MB8, (Cat. No. 1231115) and CD206 PE clone: C068C2, (Cat. No. 141705). For staining the human samples, the following anti-human antibodies from Biolegend were also used: PE/Cy5 CD206 clone: 15-2, (Cat. No. 321108) and APC CD163 clone: GHI161, (Cat. No. 333609). The anti-human antibody PE CD14 clone: M5E2, (Cat. No. 561707) was purchased from BD Bioscience (Heidelberg, Germany).

Quantitative RT-PCR

Total RNA was extracted from sorted macrophages (GFP^CD11^Ly6G^-) derived from transplanted mice or from sorted macrophages (CD11b^F4/80^) derived from in vitro co-culture experiments using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The Advantage® RT-for-PCR Kit from Clontech Laboratories (Takara, Kyoto, Japan) was used for cDNA synthesis according to the manufacturer’s instructions. Real-time quantitative PCR analysis was performed as previously described using the Real-Time PCR system One Step Plus (Applied Biosystems, Thermo Fisher Scientific, Schwerte, Germany). The following TaqMan assays (Applied Biosystems) were used: Gfi1 (Mm00515853_m1), Arg1 (Mm00475988_m1), IL-6 (Mm00446190_m1), Nos2 (Mm00440502_m1) and IL-10 (Mm01288386_m1). Gene expression was normalized to the endogenous control Gapdh1 (Mm03302249_g1) using the ΔΔCT method.

Measurement of cytokine production of macrophages

Supernatants from M1 and M2 macrophages were collected, filtered and stored in small aliquots at -80°C until further analysis. Also, 5x10^4 sorted macrophages (GFP^-CD11^-Ly6G^-) derived from transplanted leukemic and non-leukemic mice were cultured
in DMEM-Glutamax containing 10% FBS and 1% penicillin/streptomycin. After 24 hours the supernatants were collected, and the level of IL-1B, IL-6 and IL-10 was measured using the mouse ELISA Ready-SET (eBioscience, Frankfurt am Main, Germany), according to the manufacturer instructions.
References

Supplementary Figure 1. Accumulation of CD163^{+}CD206^{+} M2 like macrophages in BM of AML patients.

A) Schematic illustration of the experimental design. BM mononuclear cells from pre-diagnosed AML patients and healthy volunteers were isolated by Ficoll density gradient centrifugation and the frequency of M2 macrophages was evaluated using flow cytometry analysis.

B) The absolute numbers of BM CD163^{+}CD206^{+} M2 macrophages gated on CD14-expressing macrophage/monocyte cell population (out of 100000 cells acquired by FACS) assessed in 9 AML patients and 9 healthy volunteers, (**p=0.008).

C) Representative FACS plots from BM of one AML patient and one healthy volunteer demonstrating the frequency of CD163^{+}CD206^{+} M2 macrophages gated within total CD14^{+} monocytes.

Abbreviations: BM=bone marrow, AML=acute myeloid leukemia, FACS=fluorescence-activated cell sorting.
Supplementary Figure 2. Increased frequency of AAMs in BM and spleen of leukemic *NUP98-HOXD13* mice.

A) Schematic representation of the experimental design. Leukemic *Gfi1* WT\*NUP98-HOXD13 and healthy WT mice were sacrificed and the frequency of AAMs was determined by flow cytometry.
B) The frequency of CD11b⁺Ly6G⁻ monocytes/macrophages in BM (left panel) and spleen (right panel) of leukemic Gfi1-WT×NUP98-HOXD13 (n=4) and non-leukemic WT mice (n=4) (*p=0.05 for BM and *p=0.04 for the spleen).

C) Representative FACS plots showing that more than 90% of BM CD11b⁺Ly6G⁻ monocytes/macrophages are positive for the F4/80 surface marker.

D) Representative FACS plots showing that the majority of CD11b⁺Gr1⁺ monocytes/macrophages in BM are also positive for the F4/80 surface marker.

Abbreviations: BM=bone marrow, AAMs=acute myeloid leukemia associated macrophages, FACS=fluorescence-activated cell sorting, WT=wild type.
Supplementary Figure 3. The absolute number of AAM1 is significantly increased in BM and spleen of leukemic transplanted mice

A) The absolute numbers of Ly6C\(^{-}\)MHC II\(^{+}\) AAM1 assessed in BM of leukemic mice transplanted either with *AML-ETO9a* transduced cells (secondary recipients, n=5) or C1498GFP cell line (n=3) compared to control non-leukemic mice transplanted with non-transduced cells (n=5), (**p=0.008 for C1498GFP, *p=0.03 for *AML1-ETO9a*).  

B) The absolute numbers of Ly6C\(^{-}\)MHC II\(^{+}\) AAM1 assessed in spleen of leukemic mice transplanted with *AML-ETO9a* (n=5), *MLL-AF9* (n=3) or C1498GFP cell line (n=3) compared to control non-leukemic mice transplanted with non-transduced cells (n=5), (*p=0.02 for *MLL-AF9*, *p=0.02 for C1498GFP, ***p<0.0001 for *AML1-ETO9a*).  

Abbreviations: BM=bone marrow, AAM1=acute myeloid leukemia-associated macrophages type 1
Supplementary Figure 4. The distribution of different macrophage classes in BM and spleen of *AML1-ETO9a* leukemic mice.

A) Leukemia-associated monocytes (GFP^CD11b^-Ly6G^-) were classified into 6 classes according to the surface expression of Ly6C and MHCII markers. Representative FACS profiles showing the gating strategy for the 6 macrophages classes in leukemic mice transplanted with *AML1-ETO9a* transduced cells.

B) The frequency of different macrophage classes in BM (upper panels) and spleen (lower panels) of leukemic mice transplanted with *AML1-ETO9a* transduced cells (n=4) or non-transduced cells (n=6) (*p=0.05 for immature macrophages I in BM, **p=0.008 for Ly6C^+ monocytes in BM, **p=0.004 for immature macrophages I in spleen, *p=0.02 for immature macrophage II in spleen).
Abbreviations: AML=acute myeloid leukemia, AAM1=AML-associated macrophages type 1, AAM2=AML-associated macrophages type 2, AAD=AML-associated dendritic cells.
Supplementary Figure 5. *Gfi1* affects macrophage polarization by inhibiting M1 response in INF-y-treated macrophages.

A) Schematic representation of the *in vitro* polarization experiment. *Gfi1*-WT or *Gfi1*-KO BMDMs were stimulated with INF-y (100 ng/ml) for 48 hours. Medium was collected for ELISA and M1 macrophages were characterized by flow cytometric and gene expression analysis.

B) Representative FACS plots demonstrating the frequency of Ly6C⁺CD206⁻ M1 in BMDMs from *Gfi1*-WT and *Gfi1*-KO mice after INF-y stimulation.

C) The frequency of Ly6C⁺CD206⁻ M1 macrophages in BMDMs from *Gfi1*-WT (n=6) and *Gfi1*-KO (n=6) mice after INF-y stimulation, (**p=0.0001).

D) Fold change of Nos2 mRNA level in *Gfi1*-WT (n=4) and *Gfi1*-KO (n=4) M1 Ly6C⁺CD206⁻ macrophages after INF-y stimulation. RT-PCR results of duplicates from two independent experiments are shown (**p<0.0001).
E) The level of IL-1B in supernatants of M1 macrophages derived from Gfi1-WT (n=8) and Gfi1-KO (n=8) mice was measured using ELISA commercial kits. Results of duplicates from two independent experiments are shown (p<0.0004).

Abbreviations: BMDMs=bone marrow derived macrophages, INF-y=Interferon-y, Nos2=nitric oxide synthase 2, IL-1B=interleukin 1B; GAPDH=Glyceraldehyde 3-phosphate dehydrogenase, FACS=fluorescence-activated cell sorting, ELISA=enzyme-linked immunosorbent assay.
Supplementary Figure 6. The effect of Gfi1 on the distribution of different macrophage classes in the BM of NUP98-HOXD13 leukemic mice.

The frequency of different macrophages classes in BM of Gfi1-WTxNUP98-HOXD13 (n=6) and Gfi1-KOxNUP98-HOXD13 (n=5) leukemic mice evaluated using flow cytometry analysis (*p=0.02 for AAM2 and *p=0.16 for Ly6C+ monocytes).

Abbreviations: BM= bone marrow
**Supplementary Table 1: Characteristics of patient samples used for macrophage analysis**

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Abbreviations: F=female, M= male, AML=acute myeloid leukemia