Allograft inflammatory factor-1 is essential for acute monocytic leukemia infiltration

Extramedullary infiltration (EMI) occurs in 2-9% of newly diagnosed acute myeloid leukemia (AML) patients[1] and is a high-risk feature associated with relapse.[2,3] EMI involves tissues not typically involved in hematopoiesis, including skin, central nervous system, and gingivae, complicating diagnosis, prognosis, and treatment. Extensive investigation has found no clear association with intramedullary cytogenetics. However, EMI cases frequently show monoblastic/myelomonocytic differentiation (43-81% of cases)[4] and a higher prevalence of the FAB M5 subtype,[2,3] suggesting that distinct biological features of acute monoblastic/monocytic leukemia (AMoL) may underlie its tissue infiltration propensity. Here we reveal that infiltration-associated pathways are a distinct biological feature in AMoL, and identify AIF1 as a critical regulator of AMoL infiltration via the CCR2-MAPK axis.

To investigate whether infiltration-associated pathways represent a distinct biological feature of AMoL and to elucidate the underlying mechanisms driving the frequent occurrence of EMI, we conducted a comparative analysis of the expression profiles between AMoL and other AML subtypes across four public AML cohorts: TARGET, TCGA, GSE1159, and GSE6891. In line with the clinical phenotype, we identified specific enrichment of infiltration-associated characteristics related to cellular adhesion and migration in AMoL subtypes (*Online Supplementary Figure S1A*). We further identified eight infiltration-associated genes that are both relatively highly expressed in hematopoietic system and consistently up-regulated in AMoL compared to other AML subtypes across all four cohorts (*Online Supplementary Figure S1B*). Among these eight tightly interconnected genes, allograft inflammatory factor-1 (*AIF1*) emerges as the most highly connected hub, with computational predictions suggesting functional linkages to six others (*Online Supplementary Figure S1C*). *AIF1* has been reported to be associated with tumor growth and migration in breast cancer[5,6] and renal cell carcinoma.[7] However, its functional role in AML remains poorly defined.

To clarify the potential involvement of *AIF1* in AMoL, we performed a comprehensive analysis of *AIF1* using public datasets, complemented by experimental validation. We found that *AIF1* expression was specifically elevated in AMoL (FAB-M5) and acute myelomonocytic leukemia (AMML, FAB-M4), compared with other AML subtypes and healthy controls (Figure 1A, B, *Online Supplementary Figure S1D-F*). Furthermore, AML samples with monocytic differentiation, as defined by CD14 expression via flow cytometry, exhibited significantly higher *AIF1* levels relative to both primitive AML and healthy CD14$^+$ monocytes (Figure 1B, *Online Supplementary Figure S1D, E*). To assess whether *AIF1* expression was restricted to monocytic blasts, we analyzed single-cell RNA sequencing data from pediatric AMoL and AMML bone marrow samples,[8] revealing that *AIF1* expression was predominantly enriched in granulocyte/monocyte-like leukemic subpopulations (*Online Supplementary Figure S1G*). We first confirmed that AIF1 expression was significantly enriched at both the transcriptional and protein levels in M5 and a subset of M4 leukemia cell lines compared to other subtypes (Figure 1C). To investigate the functional role of *AIF1* in AMoL, we performed knockdown (KD) using lentiviral shRNA followed by reconstitution in the AMoL cell line THP-1. Both shRNA effectively reduced AIF1 expression at the transcriptional and protein levels (Figure 1D) and significantly inhibited AMoL cell proliferation *in vitro*. Notably, this proliferative defect was partially rescued by AIF1 re-expression (Figure 1E). This anti-proliferative effect of *AIF1* KD was consistently observed in additional AML cell lines with monocytic features, including U937, MV4-11, and OCI-AML3 (*Online Supplementary Figure S2A*), but had minimal impact on non-monocytic AML cells (Kasumi-1 and K562) (*Online Supplementary Figure S2B*). Mechanistically, *AIF1* KD reduced self-renewal capacity and induced apoptosis, with minimal impact on cell cycle (Figure 1F, *Online Supplementary Figure S2C*).

To further explore whether *AIF1* contributes to AMoL EMI, transwell assays and an adhesion assay were performed. *AIF1* KD remarkably reduced migration and infiltration capacities in both MLL-rearranged (THP-1) and non rearranged (U937) cells, and this phenotype could be rescued by *AIF1* re-expression (Figure 2A, B, *Online Supplementary Figure S2D*). Conversely, overexpression of *AIF1* in Kasumi-1 cells (an M2 line with low endogenous AIF1 expression) significantly enhanced their migratory capacity (*Online Supplementary Figure S2E*). AIF1 depletion also reduced adhesion to MS-5 stromal cells (*Online Supplementary Figure S2F*).

The transplantation of THP-1 cells into NSG mice has been reviewed and approved by the Ethics Committee of the Beijing Institute of Genomics, Chinese Academy of Sciences (China National Center for Bioinformation; approval N. 2023A018) and the Institutional Animal Care and Use Committee (IACUC) of Beijing Chaoyang Hospital, Capital Medical University (approval N. 25-2150). Given this, *in vivo*, following the transplantation of THP-1 cells into NSG mice, we detected varying degrees of leukemic infiltration in multiple organs, including the liver, spleen, kidneys, and even the brain. Notably, *AIF1* KD consistently attenuated this infiltration across all examined organs, with the most significant suppression observed in the liver (Figure 2C,

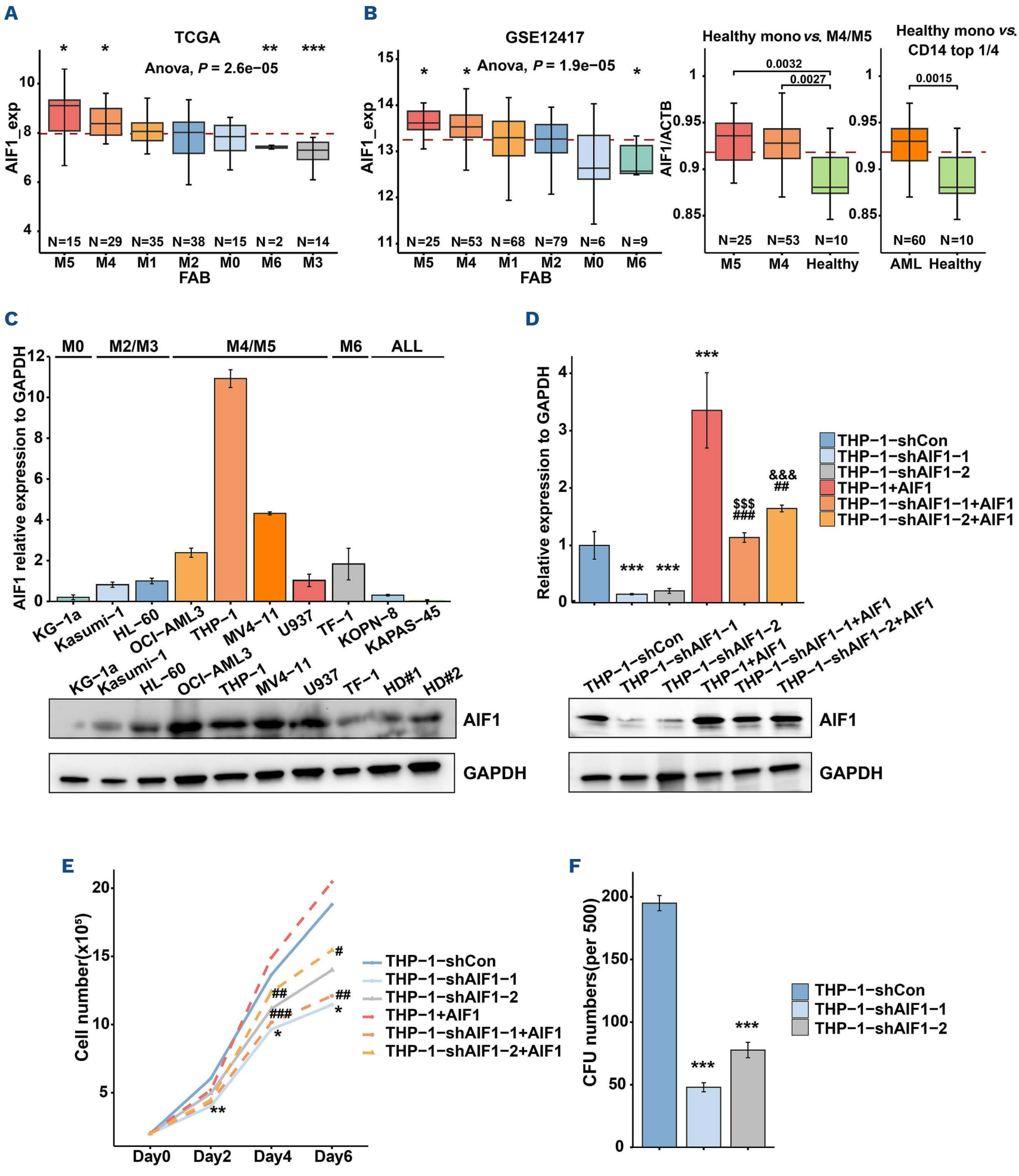


Figure 1. *AIF1* is highly expressed and plays an important role in acute monoblastic/monocytic leukemia. (A) Expression levels of *AIF1* across different FAB subtypes in the public TCGA acute myeloid leukemia (AML) cohort. The dashed line represents the mean expression level of *AIF1* across all samples. (B) The expression of *AIF1* in different AML FAB subtypes, and *AIF1*/ACTB ratios in CD14^{+} monocytes from healthy donors (HD) (N=10) *versus* M4, M5 (N=53, N=25) or CD14 top quartile AML patient blasts (N=60) in GSE12417. (C) AIF1 expression levels (transcriptional and protein) in various cell lines representing different subtypes and in HD. GAPDH expression is used as a control. (D) AIF1 expression levels (transcriptional and protein) in THP-1 cells transduced with scramble control (shCon), *AIF1* shRNA (shAIF1-1/2) and/or *AIF1* cDNA (+AIF1) by lentivirus. RNA was extracted 48 hours after cell sorting, and *AIF1* expression was quantified by real-time quantitative PCR. Relative *AIF1* expression of each sample was normalized to its

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GAPDH expression and then compared with shCon. Data are from a single experiment, representative of 3 independent experiments. (E) Growth curves of THP-1 cells were transduced with shCon, *shAIF1* and/or *AIF1*. Cell number (y axis) and days (x axis) are indicated. Data are from a single experiment, representative of 3 independent experiments. (F) Colony-forming unit (CFU) assay of THP-1 cells transduced with shCon or *shAIF1*, cells were planted in MethoCult™ H4230 with 20% FBS, and the cell colonies were counted after 14 days. Data are from a single experiment, representative of 3 independent experiments. ALL: acute lymphoblastic leukemia. **P*<0.05, ***P*<0.01, ****P*<0.001. Comparisons: **versus* shCon (*t* test) or mean expression level of *AIF1* (Anova test); #*versus* +AIF1; $*versus* shAIF1-1; &*versus* shAIF1-2 (*t* test).

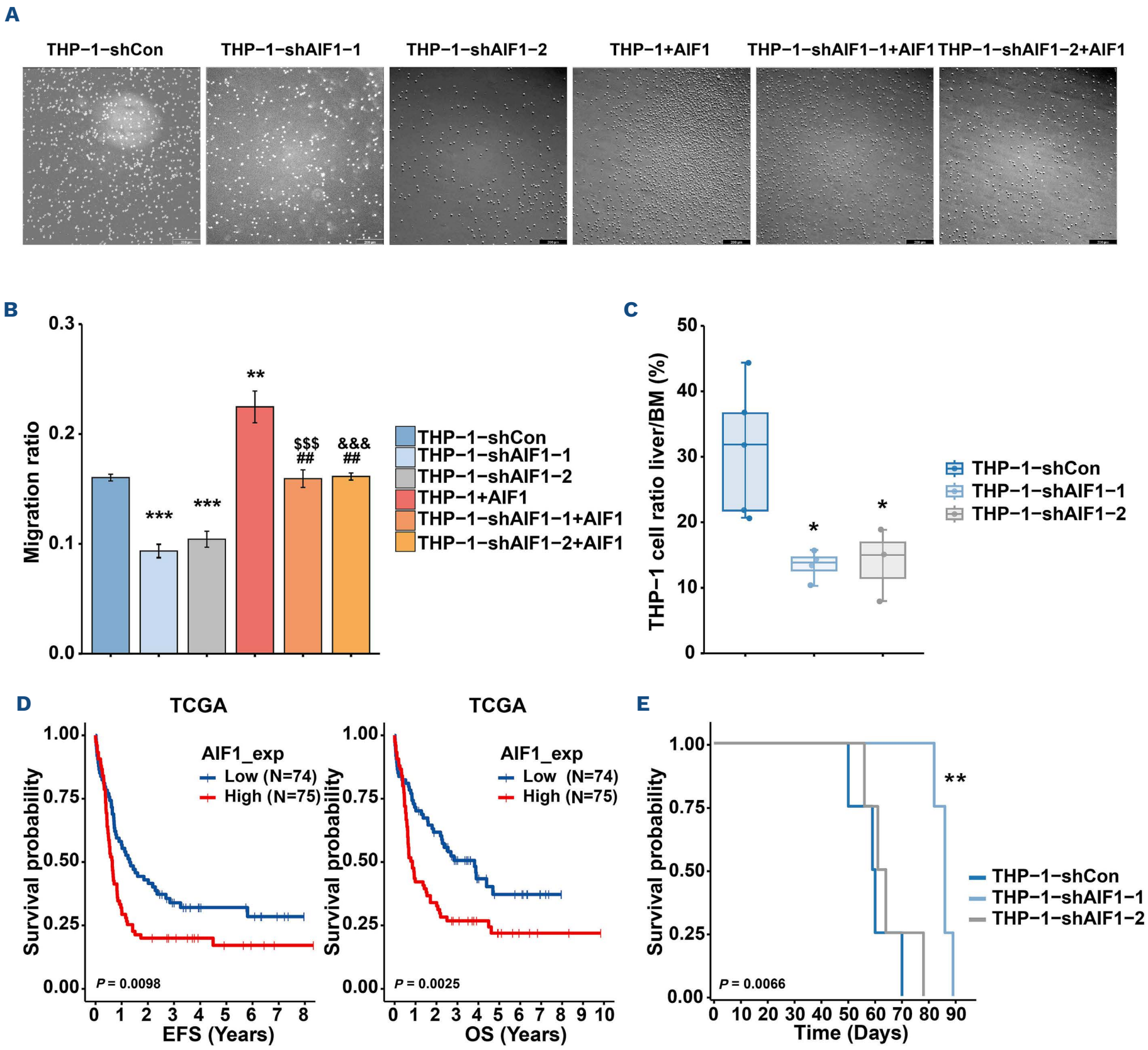


Figure 2. *AIF1* promotes the migration and infiltration of acute monoblastic/monocytic leukemia and impacts survival. (A) Transwell migration assay of THP-1 cells transduced with shCon, sh*AIF1* and/or *AIF1*. Cells were serum-starved for 20 hours, then seeded into the upper chamber. The lower chamber contained RPMI 1640 medium with 20% FBS. After 2 hours of incubation at 37°C with 5% CO_2, cells migrated to the lower chamber are counted to calculate migrate ratio. The images show cells that had migrated to the lower chamber, visualized under 10x magnification. Scale bar: 200 um. (B) Migration rate of THP-1 cells transduced with shCon, sh*AIF1* and/or *AIF1*, calculated as the ratio of cells that migrated to the lower chamber to the total number of cells seeded in the upper chamber. All experiments included plating controls to ensure equal seeding across groups. Quantifi-

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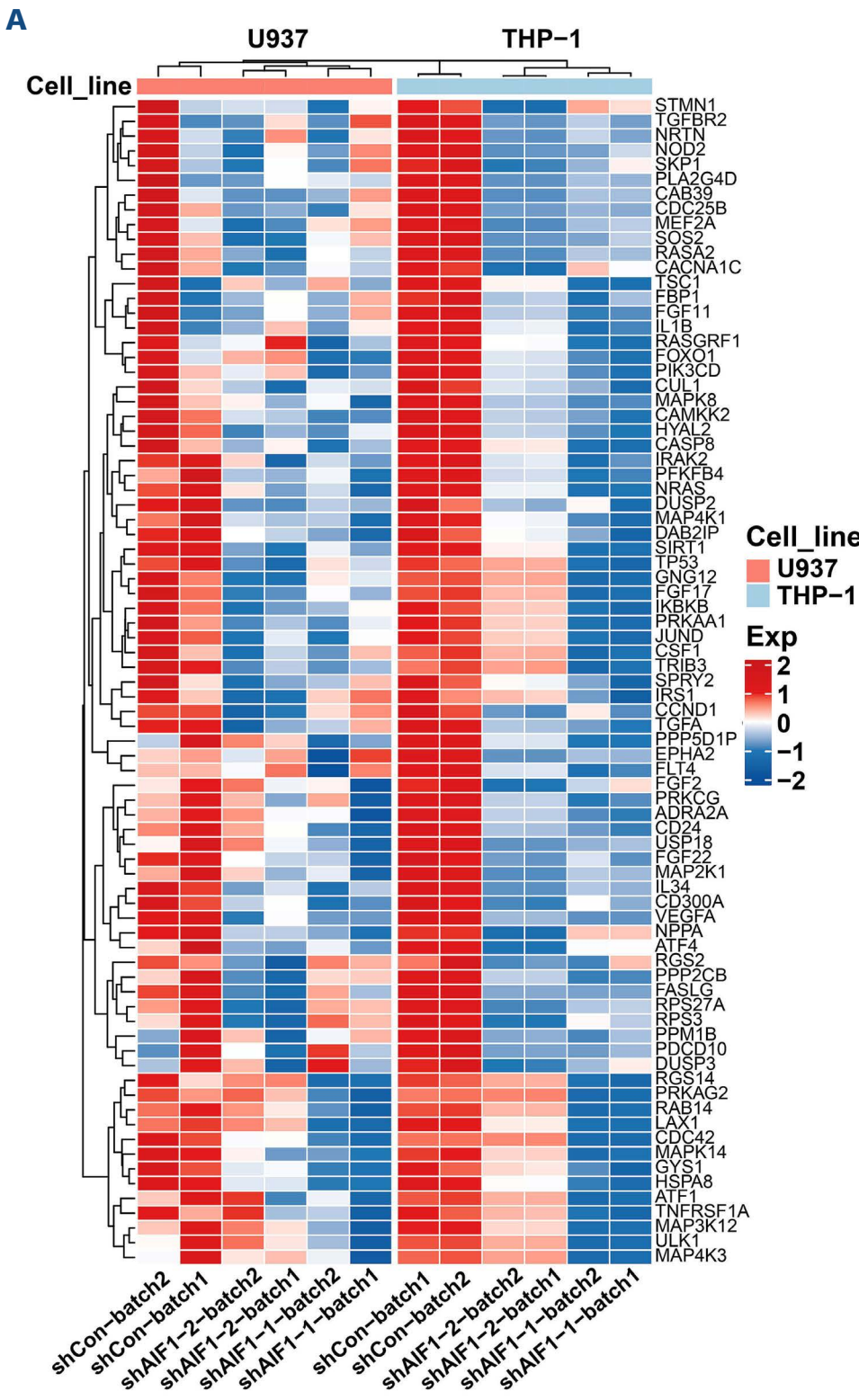
cation represents 3 biological replicates with 3 technical replicates each. (C) Liver infiltration of NSG mice receiving THP-1 cells transduced with shCon or sh*AIF1*, the infiltration ratio quantified as the ratio of THP-1 cell abundance in the liver relative to that in the bone marrow (BM) from the same mouse. (D) Kaplan-Meier curves showing the event-free survival (EFS) and overall survival (OS) of TCGA-AML patients, stratified by the median expression of *AIF1*. (E) Survival curves of NSGS mice receiving 250,000 sorted THP-1 cells transduced with shCon or sh*AIF1* (N=4). The overall *P* value is shown in the figure (log-rank test). For sh*AIF1* *versus* shCon: *P*=0.0067; for sh*AIF1*-2 *versus* shCon: *P*=0.3962. **P*<0.05, ***P*<0.01, ****P*<0.001. Comparisons: **versus* shCon; $^{\#}$*versus +AIF1*; $^{\$}$*versus* sh*AIF1*-1; $^{\&}$*versus* sh*AIF1*-2 (*t* test).

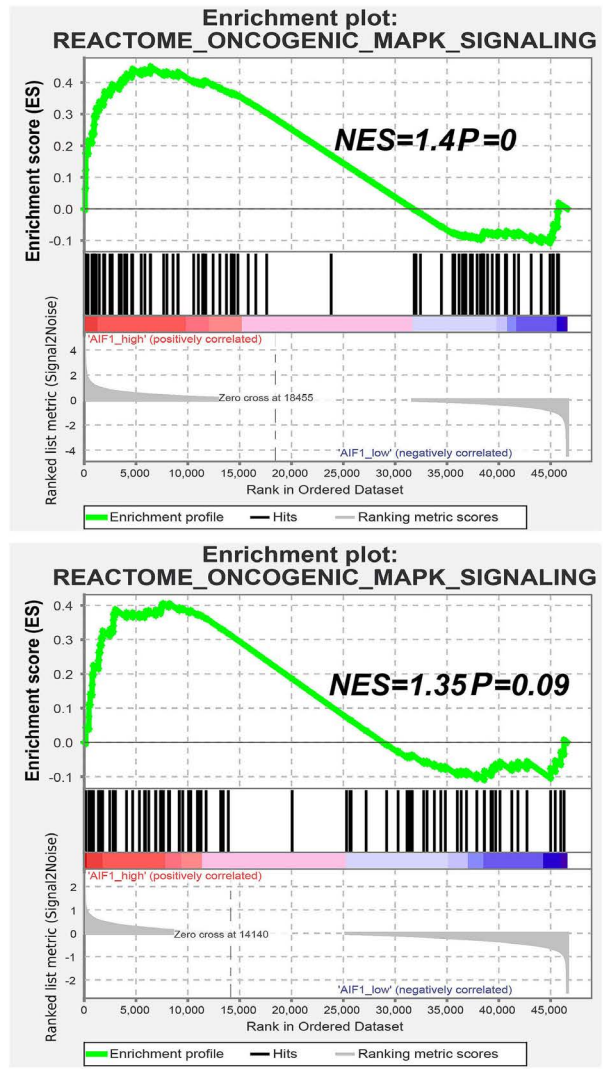
Online Supplementary Figure S2G). Moreover, high *AIF1* levels significantly correlated with inferior event-free survival (EFS) and overall survival (OS) in both pediatric (TARGET) and adult (TCGA) AML cohorts (Figure 2D, *Online Supplementary Figure S1H*), and were elevated in intermediate-/high-risk patients (*Online Supplementary Figure S1F*). Notably, *AIF1* levels further stratified EFS and OS outcomes within both M4/M5 or M1/M2 FAB subtypes (*Online Supplementary Figure S1 I, J*). Consistently, *AIF1* KD significantly delayed leukemia onset and prolonged survival in the THP-1-derived xenograft model (Figure 2E), reinforcing the role of *AIF1* as a potential adverse prognostic marker in AMoL.

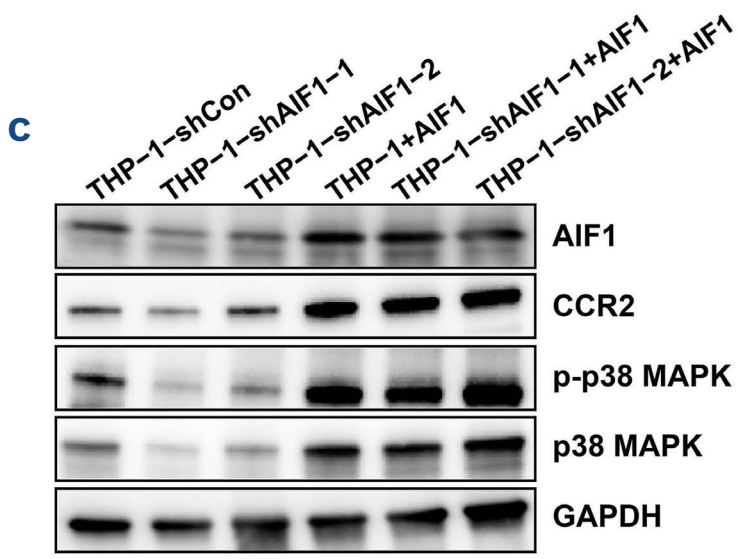
To investigate the underlying mechanism of *AIF1* in AMoL development and infiltration, transcriptomic sequencing (RNA-seq) was performed to identify the potential downstream genes and pathways regulated by AIF1. After *AIF1* KD, down-regulated genes were mainly enriched in cell adhesion, cell death, immune activation and kinase signaling pathways (*Online Supplementary Figure S3A*). To identify potential downstream effectors of AIF1, we focused on genes that were significantly up-regulated in FAB-M4 and M5 AML subtypes and exhibited strong correlation with *AIF1* expression in both our RNA-seq data and public AML datasets. Among these, *CCR2* (C-C chemokine receptor type 2) exhibited the most pronounced downregulation upon *AIF1* KD in both THP-1 and U937 monocytic AML cell lines, as validated by flow cytometry and quantitative PCR (*Online Supplementary Figure S3B-E*). Notably, CCR2 resides within the subset of six genes predicted to exhibit functional interactions with AIF1 among the eight candidate genes identified previously (*Online Supplementary Figure S1C*). Moreover, the expression of *CCR2* was specifically and consistently highly expressed in M4 and M5 patients and showed a significant positive correlation with *AIF1* expression across multiple public cohorts (*Online Supplementary Figure S3F, G*). CCR2 is a member of the G protein-coupled receptor (GPCR) family and functions as a receptor for chemokines such as CCL2 (monocyte chemoattractant protein-1, MCP-1). Previous studies have demonstrated that the CCL2–CCR2 signaling axis promotes tumor progression and migration through activation of the MAPK pathway.[9] Consistently, *AIF1* KD in AML cell lines led to significant downregulation of MAPK pathway-related genes in our RNA-seq data (Figure 3A). Simultaneously, GSEA results also indicate that in public cohorts, AML patients with high *AIF1* expression, regardless of disease subtype, exhibited activation of the MAPK pathway (Figure 3B). Western blot showed a decreased CCR2 expression and phosphorylation of p38 MAPK after *AIF1* KD (Figure 3C), suggesting that CCR2-MAPK axis supports AIF1-mediated leukemia cell migration and infiltration.

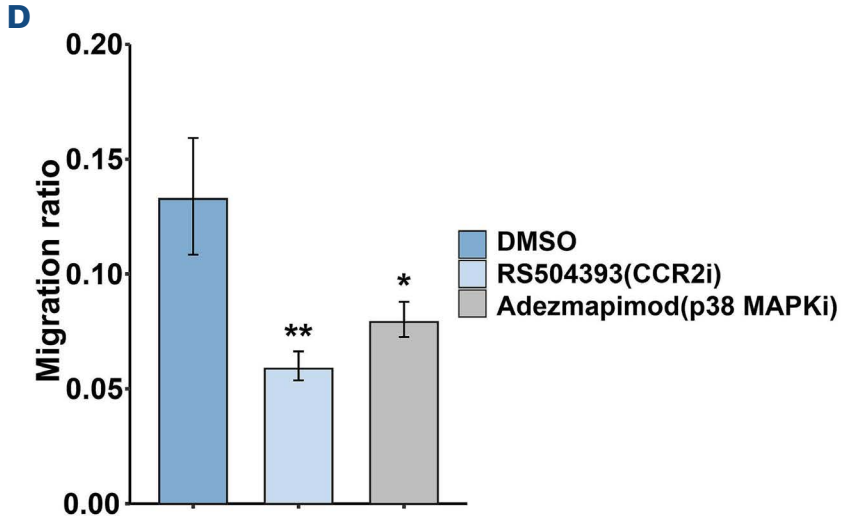
To further validate whether the CCR2-MAPK axis could serve as a potential target for inhibiting AMoL infiltration, we treated THP-1 cells with RS504393 (a CCR2 inhibitor) and Adezmapimod (a p38 MAPK inhibitor), and found both drugs could impair the migration capacity of AMoL cells *in vitro* (Figure 3D). Moreover, treatment with either RS504393 or Adezmapimod significantly reduced leukemic infiltration across multiple organs in a THP-1-derived xenograft model, with the most pronounced suppression observed in the liver and kidneys (Figure 3E). Collectively, these findings indicate that the AIF1–CCR2–MAPK axis plays a pivotal role in mediating AMoL infiltration and that therapeutic targeting of CCR2, in particular, may offer a promising strategy for the treatment of AMoL.

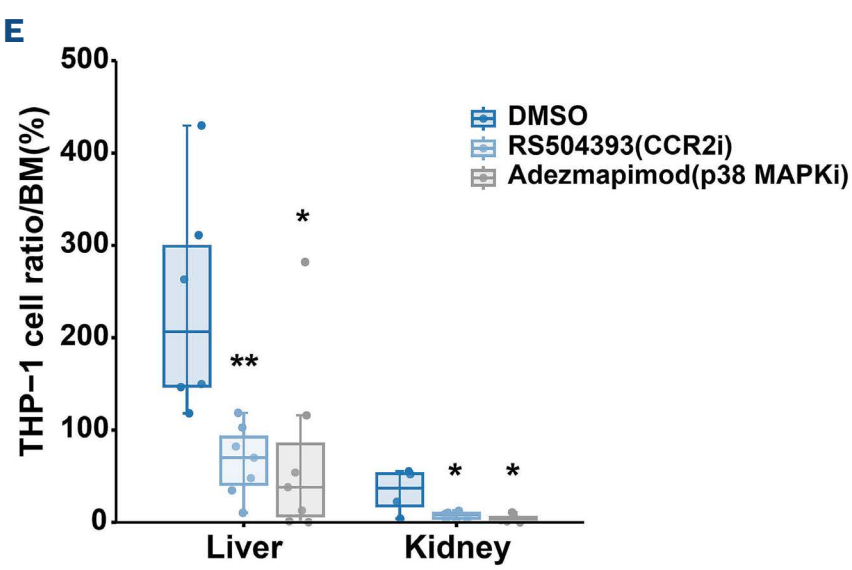
In this study, we demonstrate for the first time that *AIF1* overexpression promotes leukemic infiltration in AMoL, with CCR2 identified as a downstream effector. *AIF1* was originally discovered in rat cardiac allografts undergoing chronic rejection,[10] and is expressed in microglia, macrophages, T cells, synoviocytes, and adipocytes during inflammatory processes, playing a pivotal role in immune regulation and inflammation-associated pathologies.[11] Aberrant *AIF1* upregulation has been implicated in inflammatory disorders, transplant rejection, autoimmune diseases, and various malignancies. While the CCL2/CCR2 axis is a well-recognized pathway for monocyte migration,[12] its contribution in the context of monocytic leukemia cell trafficking has been understudied. Prior studies have primarily focused on the CXCR4–CXCL12 axis[13] and matrix metalloproteinase (MMP) family proteins,[14] highlighting a gap in our understanding of CCR2-mediated mechanisms in leukemic infiltration. Our findings identify AIF1 as a regulator of CCR2 and the MAPK signaling pathway. Notably, *AIF1* impaired monocytic leukemia cell migration and infiltration both *in vitro* and *in vivo* in a dose-dependent manner, correlating with the degree of KD efficiency. While the use of a p38 MAPK activator would further substantiate the rescue of the *AIF1* KD phenotype, the precise molecular mechanisms by which AIF1 governs this regulation warrant further investigation. Moreover, AIF1 has also been reported to bind directly to F-actin and Rho family GTPases such as Rac, thereby promoting membrane ruffling and phagocytosis,[15] whether this rep-











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Figure 3. The AIF1—CCR2—MAPK axis is essential for acute monoblastic/monocytic leukemia infiltration. (A) Expression of partial genes involved in the MAPK pathway after knockdown using sh*AIF1*-1 or sh*AIF1*-2 or empty control in THP-1 and U937 cell lines. (B) Enhanced MAPK pathway activity in the top one-third of acute myeloid leukemia (AML) patients with higher AIF1 expression compared to the bottom one-third of patients with lower AIF1 expression in FAB M2 and M5 subtypes of the BeatAML cohort (GSEA). (C) Endogenous CCR2, phosphorated p38 MAPK and p38 MAPK expression of THP-1 cells (knockdown or rescue *AIF1*) were detected by western blot, GAPDH was used as loading control. (D) Transwell migration assay of THP-1 cells treated with RS504393 (CCR2i) or Adezmapimod (p38 MAPKi). Cells were treated with no serum RPMI 1640 medium for 2 hours, then treated with DMSO, RS504393 (25 uM) and Adezmapimod (10 uM), respectively, placed into the transwell insert where the lower chamber contains RPMI 1640 medium with 20% serum, incubated at 37°C, 5% CO_2 for 2 hours, cells that had migrated to the lower chamber were counted to calculate migration ratios. (E) Liver and kidney infiltration ratio of NSG mice receiving THP-1 cells then treated with RS504393 or Adezmapimod. Both drugs were administered intraperitoneally at 5 mg/kg from the 10th day post transplantation, every other day for 5 doses. The infiltration ratio quantified as the ratio of THP-1 cell abundance in the liver/kidney relative to that in the bone marrow from the same mouse. **P*<0.05, ***P*<0.01, ****P*<0.001. Comparisons: **versus* DMSO (*t* test).

resents an alternative mechanism contributing to AMoL cell migration remains to be explored.

Our study also highlights the feasibility of targeting the CCR2 or MAPK pathways as potential therapeutic strategies for AMoL and EMI in AML. While previous studies suggest that EMI may facilitate disease relapse, its prognostic relevance in AML remains controversial.[1,4] In our analysis, *AIF1* was highly expressed in both AMML and AMoL (FAB M4 and M5), AMML frequently harbors the CBFβ–MYH11 fusion gene associated with favorable prognosis, which may partly explain the therapeutic dilemma in treating EMI cases. Our data further indicate that, in the context of AMoL, EMI may be associated with inferior clinical outcomes. Collectively, these findings provide novel insights into the pathogenesis of AMoL and propose new avenues for therapeutic intervention in EMI-associated AML.

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Contributions
ALC is responsible for study concept, funding acquisition, investigation, methodology, project administration, study supervision, validation, visualization, wrote the original draft, and reviewed and edited the manuscript. HYH is responsible for data curation, formal analysis, visualization, wrote the original draft, and reviewed and edited the manuscript. JYZ, SJY, BHZ and HYS are responsible for investigation and validation. HHZ is responsible for resources, and writing, reviewing and editing the manuscript. QFW is responsible for study concept, project administration, resources, study supervision, and writing, reviewing and editing the manuscript. All authors have read and approved the final manuscript for publication.

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Data-sharing statement

The bulk RNA-sequencing data generated in this study are deposited in the Genome Sequence Archive (GSA) under accession code HRA005186. The public datasets used in this study are available from The Cancer Genome Atlas (TCGA), the Therapeutically Applicable Research to Generate Effective Treatments (TARGET), the Beat AML cohort, and the Gene Expression Omnibus (GEO) under accession codes GSE1159, GSE6891, GSE12417, GSE14468, and GSE37642.

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