SUPPLEMENTAL MATERIALS

CD36 drives metastasis and relapse in acute myeloid leukemia

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MATERIAL AND METHODS

Cell lines, primary cultures, and culture conditions

Primary AML cells, peripheral blood or bone marrow patients' samples were frozen in FCS with 10% DMSO and stored in liquid nitrogen. The percentage of blasts was determined by flow cytometry and morphologic characteristics before purification. Cells were thawed at 37 °C, washed in thawing media composed of IMDM, 20% FBS. Cells were maintained in IMDM, 20% FBS, and 1% Pen/Strep (GIBCO) for all experiments.

Human AML cell lines were maintained in MEMa-media (Gibco) supplemented with 10% FBS (Invitrogen) in the presence of 100 U/ml of penicillin and 100 µg/ml of streptomycin, and were incubated at 37 °C with 5% CO2. Cells were split every 2–3 days and maintained in an exponential growth phase. All AML cell lines were purchased at DSMZ or ATCC. These cell lines have been routinely tested for Mycoplasma contamination in the laboratory. The U937 cells were obtained from the DSMZ in February 2012 and from the ATCC in January 2014.

Bioluminescent xenograft mouse models

Briefly, 6 to 10-week-old NOD.Cg-Prkdc scid/J (NSG) mice were obtained from Charles River France or bred in-house and maintained on a 12-h light and 12-h dark cycle, under specific pathogen-free conditions with sterilized food and water provided ad libitum. Animals were injected via tail vein at day 0 with 0.2x106 viable luciferase-expressing AML cell lines in 0.1mL sterile PBS. A bioluminescence measurement was performed at day 1, then once a week, following injection of endotoxin-free luciferin (Promega #E6552, 30 mg/kg) using an Optima PhotonIMAGER imaging system and analyzed using M3vision software (Biospace Lab, Nesles-la-Vallée, France). Daily monitoring of mice for symptoms of disease (weight loss >20%, ruffled coat, hunched back, weakness, reduced motility) determined the time of killing for injected animals with signs of distress. After the final time point, for ex vivo organ imaging, 10 min after receiving luciferin, mice were killed and organs were collected and washed with PBS. Images were then immediately acquired in indicated organs. Results were expressed as percentage of total signal (100*(organ signal/total bioluminescence).

Assessment of leukemic engraftment

At the end of the experiment, NSG mice were humanely killed in accordance with European ethics protocol. Bone marrow (mixed from tibias and femurs), spleen and liver were dissected and flushed in HBSS with 1% FBS. Mononuclear cells from bone marrow and spleen were labeled with anti-hCD33, anti-mCD45.1, anti-hCD45, anti-hCD3, and/or anti-hCD44 (all from BD) antibodies to determine the fraction of viable human blasts (hCD3−hCD45+mCD45.1−hCD33+/hCD44+AnnV− cells) by flow cytometry. Adipose tissues and lungs were dissociated with collagenase NB4 (Standart Grade from Coger) 0.4 U/mL for 30 minutes. Acquisitions were performed on a CytoFLEX flow cytometer with CytoExpert software v2.0 (Beckman Coulter), and analyses done with Flowjo v10.4.2. The number of AML cells/µL of peripheral blood and number of AML cells in total leukemia burden (in bone marrow, spleen, subcutaneous and perigonadic adipose tissues, liver and lungs) were determined using CountBright beads (Invitrogen) as described in the provided protocol.

Single cell analysis

Next generation sequencing of single cell gene expression (NextSeq 550) was performed on each of the two experimental groups (vehicle or AraC) of the PDX patient TUH07 (GEO accession GSE178910) published in (1), to produce raw FASTQ reads. Gene expression data had been rendered from FASTQ reads using CellRanger software by 10x Genomics. The quality control, principle component analysis (PCA), and t-SNE/UMAP dimensionality reduction procedures to integrate and visualize the scRNAseq expression data of the patients was performed using R package Seurat 3.0 by Satija Lab. The corresponding input parameters and functional R scripts to execute the aforementioned procedures are publicly available on the Zenodo repository (accession 5137701). Cells were classified into their corresponding clusters on a UMAP projection based upon their phenotypic characteristic at diagnosis and response to therapies. Clusters significantly increasing in cell count after Arac treatment in proportion to the entire cell population were considered “enriched”, while clusters that significantly decreased in cell count were considered “sensitive”. A standard Z-test for difference in proportions had

been utilized to facilitate this classification and determine the statistical significance of each change in cluster size according to its calculated z-value (**Table S2**).

In order to assign enrichment values to the metadata of the single-cell samples, the Seurat function "AddModuleScore" had been implemented for the gene expression signatures: CD36, FRIDMAN_SENESCENCE_UP, and WU_MIGRATION (**Table S2**) with the "search" parameter set as "TRUE". The enrichment values pertaining to each cell were z-standardized using the base-R "scale" function.

To visualize the enrichment regions of the aforementioned gene expression signatures, the method "FeaturePlot" was used on the PDX category subset by patient number and treatment type. To maintain visual continuity across each FeaturePlot, the maximum and minimum z-scores for the color gradient were standardized to the 0.999 and 0.001 percentile expression values respectively in order to mitigate the effect of outliers. For the correlation analyses performed among the gene expression signatures at the cellular level, the function "FeatureScatter" had been implemented to both derive linear Pearson correlation coefficients (r) and to visualize the effect of each individual cluster on the overall relationship between the variables examined. Differential CD36 signature expression was derived for each cluster using the "col_t_welch" function from the matrixTests R library, which produced the relevant test statistics for a two-sample t-test of unpaired means for different variances (**Table S2**). The same process was then performed for senescence and migratory signatures (**Table S2**).

The cluster 8 was isolated using base-R command "subset" (with parameter 'subset = "seurat_clusters" == 8') on the Seurat object belonging to patient TUH07-AraC. To better assess the overlapping effects of CD36 with senescence and migration within this cluster, it had been bifurcated into two groups "CD36 High" and "CD36 Low" by assigning a new "CD36" field to the cluster's Seurat metadata. Using the base-R "ifelse" function, all cells with z-scores greater than zero in CD36 signature expression were assigned "CD36 High", and cells having less than z = 0 were assigned "CD36 Low" and recorded on the "CD36" metadata field. To facilitate the rendering of the blended plot visualizing the overlap of senescence and migratory activity exclusive to the CD36 enrichment within cluster 8, the "FeaturePlot" Seurat function was utilized with its functions: "blend = TRUE", "features = c("FRIDMAN_SENESCENCE_UP", "WU_CELL_MIGRATION")", and "split.by = "CD36"".

GSEA analysis

GSEA analysis was performed using GSEA version 4.0 (Broad Institute). Gene signatures used in this study were from Broad Institute database, literature, or in-house built. Following parameters were used: Number of permutations = 1000, permutation type = gene_set. Other parameters were left at default values.

Plasmid cloning, shRNA, lentiviral production, and leukemic cell transduction

Control shRNA (MFCD07785395), CD36 shRNA (TRCN0000056998) and TSP1 shRNA plasmids (TRCN0000226405 and TRCN0000219072) on a pLKO.1 backbone were purchased from Sigma. shRNA, lentiviral production and cell transduction was performed as previously described (2).

SA-β-gal activity measurement by C12-FDG staining

C12-FDG staining was performed as previously (3). Briefly, cells were treated with 100 nM Bafilomycin A1 (Invivogen tlrl-bafa1) for 2 hours then incubated with 20 µM C12-FDG (Abcam ab273642) in the dark for 1 hour at 37 °C. Cells were then washed with PBS and stained for flow cytometry as per usual.

Number of viable cells

U937 and OCIAML3 cells (300,000/mL) were seeded for 72-96h in MEMa-media (Gibco) supplemented with 10% FBS (Invitrogen) in the presence of 100 U/ml of penicillin and 100 µg/ml of streptomycin, and diluted in half with fresh media at 48h. Two technical replicated were seeded in a 24-well plate for each condition. Alive cells were counted every 24h on a Malassez chamber using Trypan Blue staining.

Primers

Primers sequences are : CCL2 (human) Forward - CATGAAAGTCTCTGCCGCCC and Reverse - GGGGCATTGATTGCATCTGGC), **P21 (human)** Forward - TGCCGAAGTCAGTTCCTTGT and Reverse - GTTCTGACATGGCGCCTCC; **P16 (human)** Forward - GGGGTCGGGTAGAGGAGG and Reverse - GCCCATCATCATGACCTGGA; **P15 (human)** Forward -

GGGACTAGTGGAGAAGGTGC and Reverse - CCATCATCATGACCTGGATCG; **IL1-β (human)** - Forward - AGCCATGGCAGAAGTACCTG and Reverse - CCTGGAAGGAGCACTTCATCT; **TNF-α (human)** - Forward - GCCCATGTTGTAGCAAACCC and Reverse - TATCTCTCAGCTCCACGCCA; **TSP1 (human)** - Forward - CTCAGGACCCATCTATGATAAAACC and Reverse - AAGAAGGAAGCCAAGGAGAAGTG; **CD45 (human)** Forward - CACTGCAGGGATGGATCTCA and Reverse - TGCGTAGAGCTTTTACCACTTGAA; **RPLP0 (human)** Forward - TAGTTGGACTTCCAGGTCGC and Reverse - CGTCCTCGTGGAAGTGACAT and **36b4 (mouse)** Forward - AGTCGGAGGAATCAGATGAGGAT and Reverse - GGCTGACTTGGTTGCTTTGG.

Molecular analysis

The presence of *FLT3-ITD* was tested as described (4). Electrophoregram peaks were quantified using GeneMarker 2.2 (SoftGenetics, State College, PA). Extended DNA resequencing was performed using Illumina NextSeq500 and Haloplex HS (Agilent, Santa Clara, CA) targeted on the complete coding regions of 52 genes: *ASXL1*, *ASXL2*, *ATM*, *BCOR*, *BCORL1*, *CBL*, *CCND2*, *CEBPA*, *CSF3R*, *CUX1*, *DDX41*, *DHX15*, *DNMT3A*, *EP300*, *ETV6*, *EZH2*, *FLT3*, *GATA1*, *GATA2*, *IDH1*, *IDH2*, *JAK2*, *KDM5A*, *KDM6A*, *KIT*, *KMT2D*, *KRAS*, *MGA*, *MPL*, *MYC*, *NF1*, *NPM1*, *NRAS*, *PHF6*, *PIGA*, *PPM1D*, *PRPF8*, *PTPN11*, *RAD21*, *RUNX1*, *SETBP1*, *SF3B1*, *SMC1A*, *SMC3*, *SRSF2*, *STAG2*, *TET2*, *TP53*, *U2AF1*, *WT1*, *ZBTB7A*, and *ZRSR2*. Data were processed through two algorithms from GATK (https://software.broadinstitute.org/gatk), HaplotypeCaller (scaling accurate genetic variant discovery) to tens of thousands of samples (5). The mean depth was 2,190 reads. Identified variants were curated manually and named according to the rules of the Human Genome Variation Society (hgvs.org). Molecular data are stored in the European Nucleotide Archive (https://www.ebi.ac.uk/ena/).

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SUPPLEMENTAL FIGURES

Fig. S1. Characterization of CD36 role in lipid metabolism in blasts. a-d. Cell surface CD36 expression (**a, b**) of viable human AML blasts in the BM of vehicle- or AraC-treated AML PDX. **c**. Representative flow cytometry histograms of basal CD36 expression in U937 and OCIAML3 cells. **d.** CD36 protein expression in AML cells treated with AraC for 24h, relative to that of PBS-treated cells. AraC concentrations used were 2µM for U937 and 10µM for OCIAML3 cells (n=5). **e-f.** Viable cell number of shCtrl and shCD36 U937 (**e**) and OCIAML3 (**f**) cells proliferating over 72h (n=4). **g-h.** Viable cell number of U937 (**g**) and OCIAML3 (**h**) cells proliferating over 96h in the presence of FA6-152 antiCD36 antibody (0-10 µg/ml) (n=3). **i**. Representative flow cytometry histograms of CD36 expression in shCtrl and shCD36 U937 cells treated with vehicle or 2µM AraC for 24h. **j-k.** CD36 expression assessed by flow cytometry in shCtrl or shCD36 cells treated with vehicle or AraC (2µM for U937 (**j**), 10µM for OCIAML3 (**k**)) for 24h (n=4-6). Values are normalized to control. **l-m.** Neutral lipid staining measured by flow cytometry using the Bodipy probe, in viable shCtrl or shCD36 U937 (**l**) and OCIAML3 (**m**) cells treated with vehicle or AraC (2µM for U937, 10µM for OCIAML3) for 24h (n=3-4). Values are normalized to control. **n-o.** Neutral lipid content measured by flow cytometry using the Bodipy probe, in viable U937 treated with vehicle or AraC (2µM) and/or CD36 blocking antibody (JC63.1, (**n**) or FA6-152 (**o**) for 24h (n=3-4). Values are normalized to control. **p.** Representative flow cytometry histograms of CD36 expression in siCtrl and siCD36 U937 cells treated with vehicle or 2µM AraC for 24h. **q.** CD36 expression in siCtrl and siCD36 U937 cells treated with vehicle or 2µM AraC for 24h (n=4). Values are normalized to control. **r, s.** 14C palmitate uptake **(r)** and oxidation **(s)** in siCtrl and siCD36 U937 cells treated with vehicle or AraC (2µM) for 24h (n=4). Values are normalized to the number of viable cells per condition and reported as a fold change to control. **t-u.** 14C palmitate uptake **(t)** and oxidation **(u)** in U937 cells treated with vehicle or AraC (2µM) and/or CD36 blocking antibody (FA6-152) for 24h (n=4). Values are normalized to the number of viable cells per condition and reported as a fold change to control. **v.** Representative flow cytometry histograms of CD36 expression in siCtrl and siCD36 OCIAML3 cells treated with vehicle or 2µM AraC for 24h. **w.** CD36 expression in siCtrl and siCD36 OCIAML3 cells treated with vehicle or 10µM AraC for 24h (n=4). Values are normalized to control. **x-y.** 14C palmitate uptake **(x)** and oxidation **(y)** in siCtrl and siCD36 OCIAML3 cells treated

with vehicle or AraC (10μM) for 24h (n=4). Values are normalized to the number of viable cells per condition and reported as a fold change to control. **z-aa.** 14C palmitate uptake **(z)** and oxidation **(aa)** in OCIAML3 cells treated with vehicle or AraC (10μM) and/or CD36 blocking antibody (FA6-152) for 24h (n=4). Values are normalized to the number of viable cells per condition and reported as a fold change to control. Values are expressed as mean ± SEM. a, Mann-Whitney or unpaired t test depending on sample distribution, with or without Welch's correction depending on sample variance. b, Wilcoxon matched-pairs signed rank test. d, one sample t test. e-h, ordinary two-way ANOVA. j-aa, ordinary one-way ANOVA with Tukey's multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Fig. S2. CD36 triggers OCIAML3 migration and analysis of blasts dissemination in vivo. a. GSEA gene signature of "GO_POSITIVE_REGULATION_OF_MONONUCLEAR_ CELL_MIGRATION" was performed in CD36 overexpressing patients from BEATAML, Verhaak and TCGA cohorts compared to the baseline CD36 expressers. **b-c.** Number of migrating cells in shCtrl or shCD36 OCIAML3 cells (**b**) and in siCtrl or siCD36 OCIAML3 cells (**c**) in the lower chamber of the transwell assay. Data are normalized to shCtrl/siCtrl for each experiment (n=8 and n=3). **d.** Number of migrating OCIAML3 cells treated or not with CD36 blocking antibodies (FA6-152 or JC63.1 as indicated) in the lower chamber of the transwell assay. Data are normalized to control (n=3). **e.** TSP1 mRNA expression in shCtrl or shTSP1 U937 cells. Data are normalized to shCtrl (n=3). **f.** TSP1 mRNA expression in shCtrl or shTSP1 OCIAML3 cells. Data are normalized to shCtrl (n=3). **g.** Number of migrating shCtrl or shTSP1 OCIAML3 cells in the lower chamber of the transwell assay. Data are normalized to control (n=3). **h.** Number of migrating shCtrl or shCD36 OCIAML3 cells in the lower chamber of the transwell assay, in the presence or absence of TSP1 blocking antibody (A6.1). Data are normalized to shCtrl (n=5). **i.** Number of migrating OCIAML3 cells treated or not with recombinant TSP1, in the presence or not of CD36-blocking antibody (FA6-152). Data are normalized to control (n=3). **j.** Acquisition of bioluminescent signals from organs harvested from mice injected with the indicated AML cell line stably expressing a luciferase reporter, at final time point. **k.** Quantification of bioluminescent signals in each organ (percentage compared to total bioluminescent signal). **l.** Quantification of human cells in the bone marrow (BM), spleen (SP), perigonadal adipose tissue (PGAT), and liver after intra-venous injection of U937 cells in NSG mice by RT-qPCR (n=2-3). hCD45 mRNA normalized to m36B4, data expressed as $2\Delta Ct$ (values multiplied by 1000). **m.** Flow cytometry analysis of human viable CD45+ AML blasts in the bone marrow (BM), spleen (SP), subcutaneous adipose tissue (SCAT), perigonadal adipose tissue (PGAT), liver, lung and blood 24 days after intra-venous injection of OCIAML3 (n=6). Values are expressed as mean ± SEM. **n.** OCIAML3 cells incubated with IGG or FA6-152 anti-CD36 antibody (1 µg/ml) for 30 minutes were injected intra-venously in NSG mice. Mice were treated with either control IgG or FA6-152 anti-CD36 antibody injected 3 times/week. At day 10 after injection, tumor burden was analyzed by flow cytometry in different organs including bone marrow (BM), perigonadal adipose tissue (PGAT), liver and lung. a, unpaired t test with or without Welch's correction depending on sample

variance. b-h, one sample or Wilcoxon t test depending on sample distribution. i, ordinary one-way ANOVA with Tukey's multiple comparisons test. n. Mann-Whitney tests. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Fig. S3. Effect of AraC on monocytes markers expression and senescence in OCIAML3 cells, and PDX TUH07 single-cell RNAseq correlation plots. a. CD11b, CD14, CD15 and CD44 cell surface expression in viable OCIAML3 cells in control conditions or in the presence of 10µM AraC for 96h (n=4). **b**. FSC/SCC analysis on gated CD36low and CD36high cells from OCIAML3 treated or not with 10µM AraC for 4 days (n=3). **c.** C12FDG staining (MFI) in gated CD36low and CD36high OCIAML3 cells treated or not with 10µM AraC for 4 days. Data are normalized to control CD36low cells (n=3). **d.** Expression of senescence-associated genes in AraC-treated OCIAML3 cells expressed as a Log2 fold change in comparison to untreated cells. mRNA of the gene of interest was normalized to the human housekeeping gene, data expressed as 2ΔΔCt. Data are normalized to control (n=3). **e.** C12FDG staining on OCIAML3 cells treated or not with 10µM AraC and CD36 blocking antibody (FA6-152) for 4 days. Data are normalized to control (n=4). **f-g.** U937 cells were treated with 2µM AraC for 4 days and viable cells were FACS sorted and submitted to migration assay. C12FDG staining was then performed in migrating and non-migrating cells. **f.** Representative histogram of C12FDG staining in non-migrating and migrating AraC-treated cells. **g.** Quantification of C12FDG staining performed in **f.** Data are normalized to non-migrating AraC-treated cells (n=4). **h.** Correlation between expression levels of gene sets related to migration (WU_CELL_MIGRATION) and the CD36 gene signature. **i.** Correlation between expression levels of gene sets related to senescence (FRIDMAN_SENESCENCE_UP) and the CD36 gene signature. **j.** Correlation between expression levels of gene sets related to senescence (FRIDMAN_SENESCENCE_UP) and migration (WU_CELL_MIGRATION). Values are represented as mean ± SEM. a and b, Mann-Whitney or unpaired t test depending on sample distribution, with or without Welch's correction depending on sample variance. c and e, ordinary one-way ANOVA with Tukey's multiple comparisons test. d and g, one sample t test. *P<0.05, **P<0.01, **P<0.001, ****P<0.0001.