D-2-hydroxyglutarate supports a tolerogenic phenotype with lowered major histocompatibility class II expression in non-malignant dendritic cells and acute myeloid leukemia cells

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Supplement

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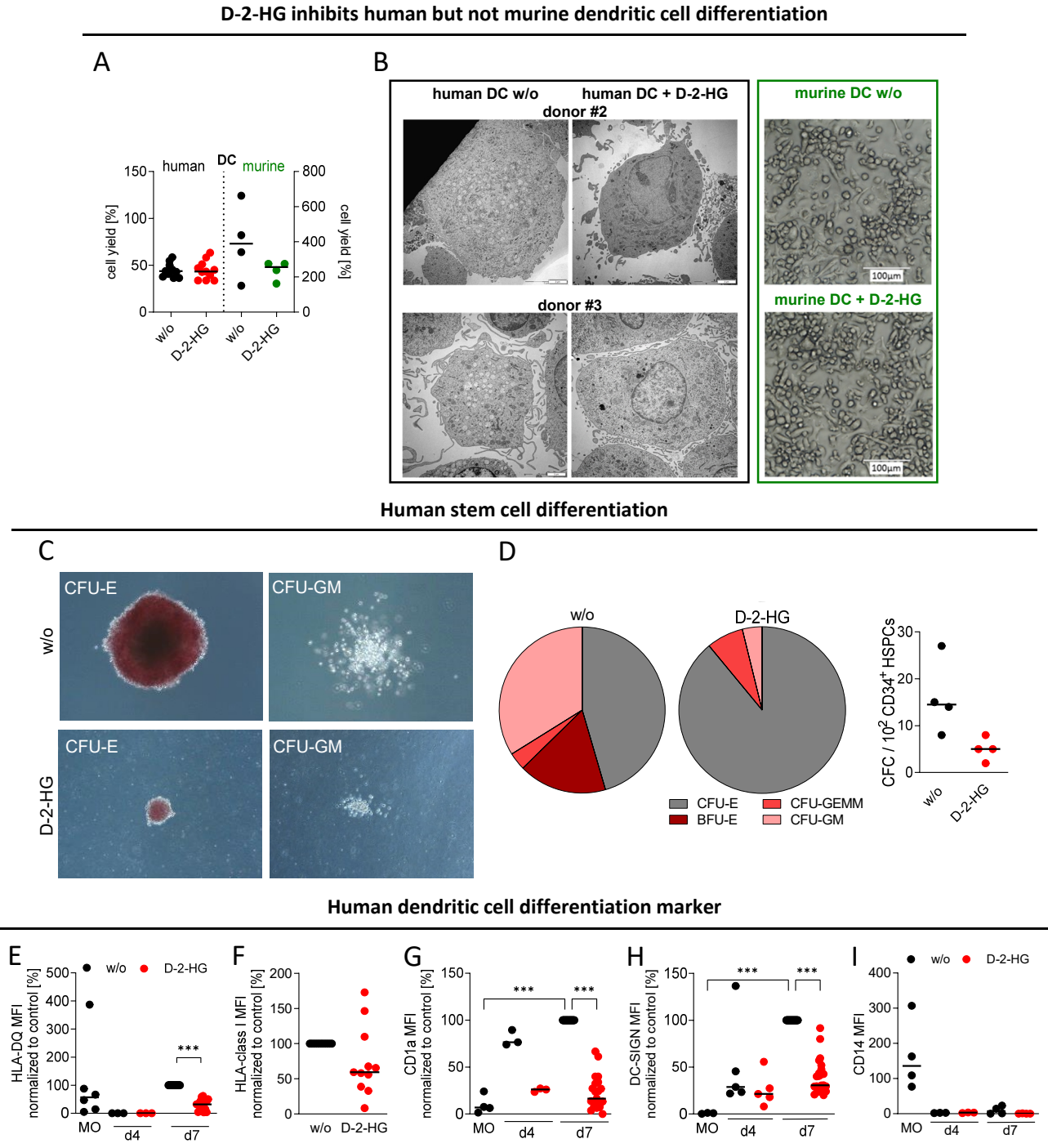
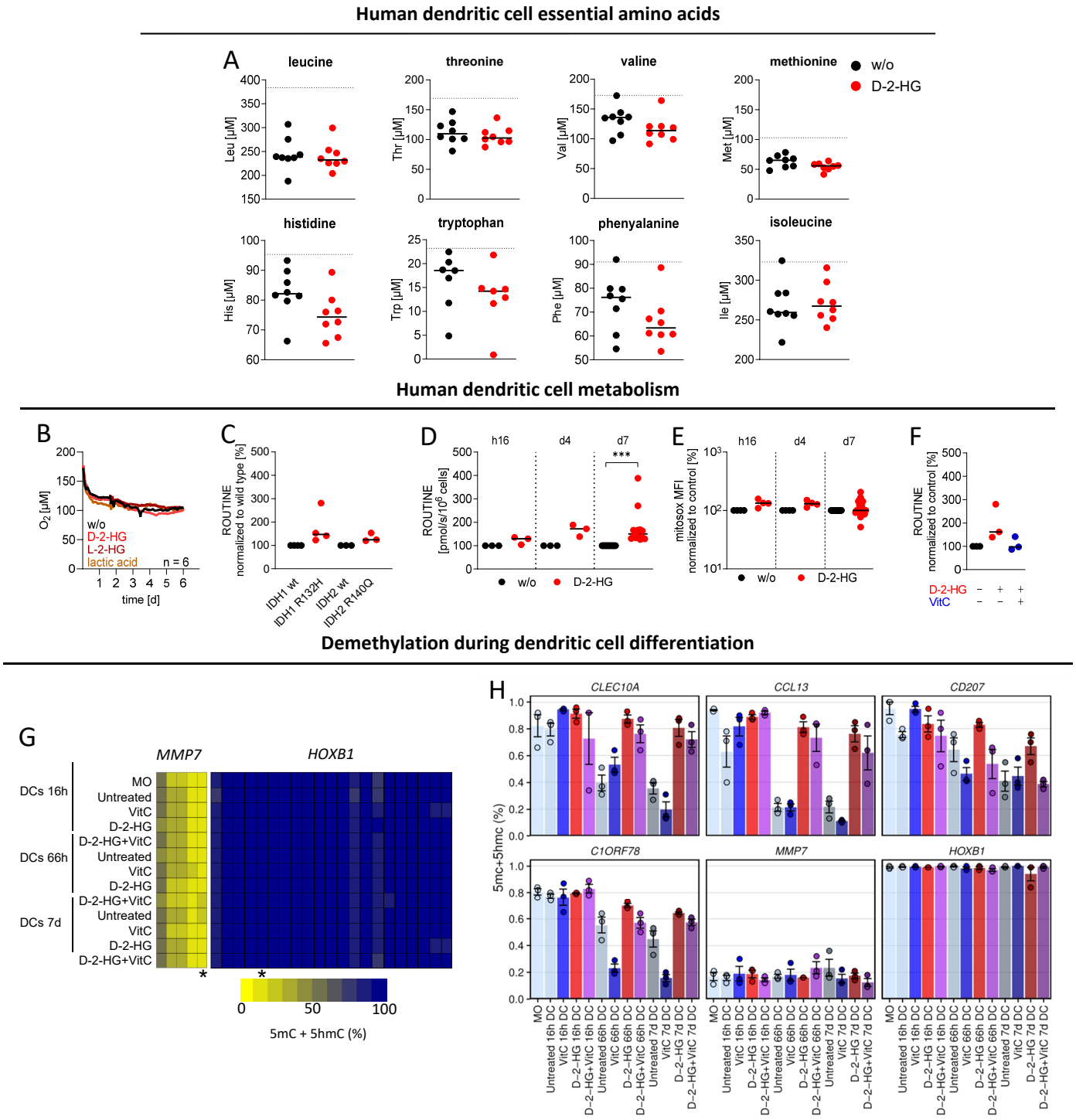
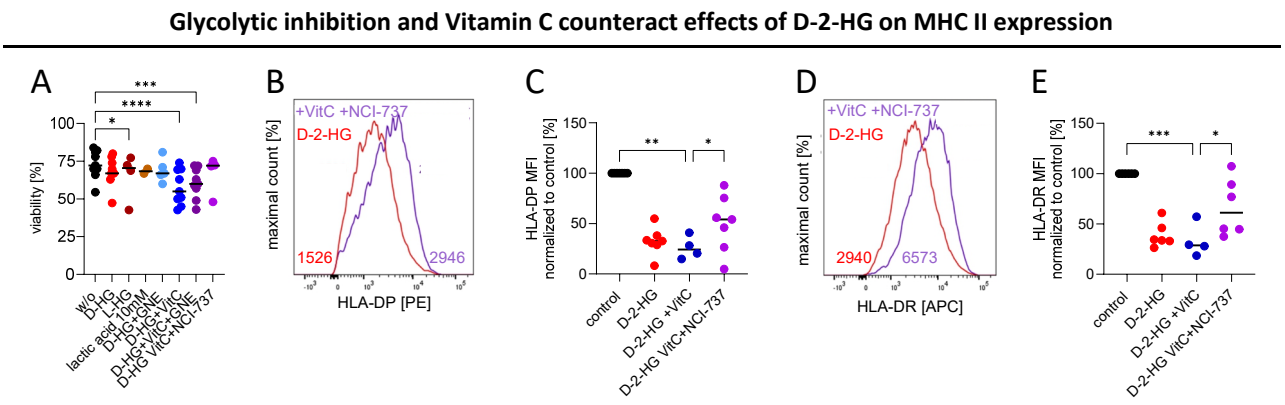


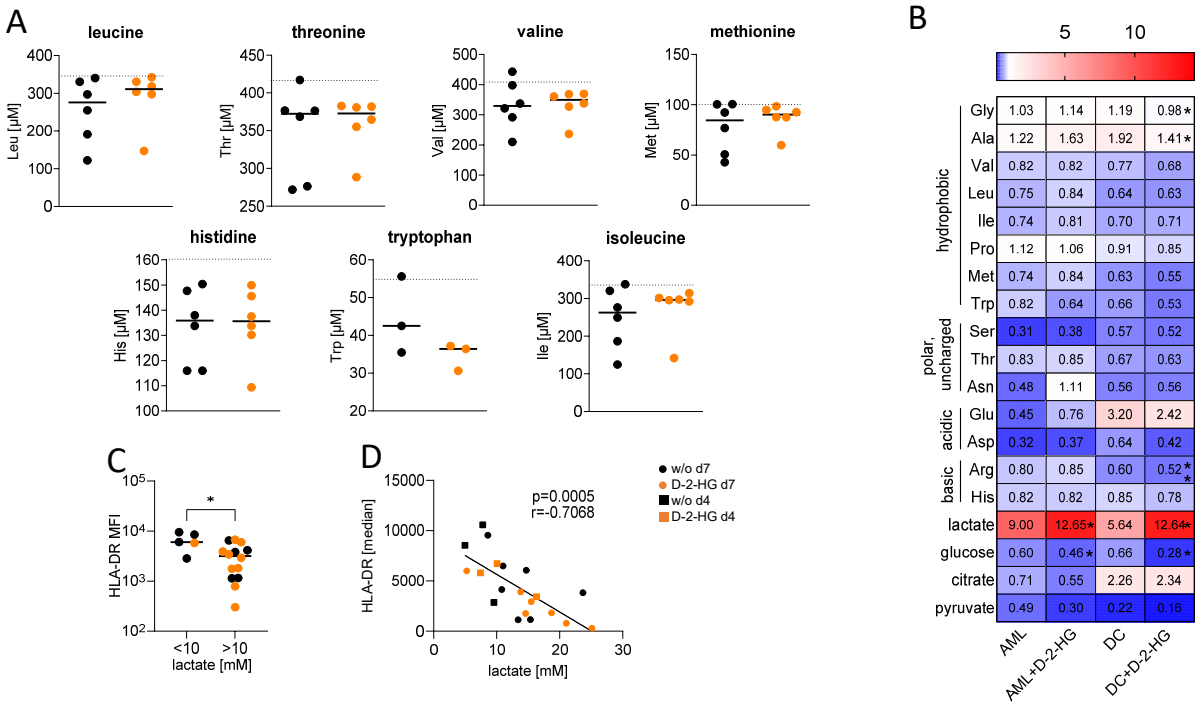
Supplementary Figure S1: D-2-HG inhibits human hematopoiesis and myeloid differentiation. (**A**) cell yield of human (n = 11 donors) and murine DCs (n = 4 mice) analyzed after 7 (human) or 10 (murine) days of culture. (**B**) Transmission Electron Microscopy of human DCs cultured for 7 days in the presence or absence of 10 mM D-2-HG. Representative pictures of two out of four donors are shown at a magnification of 10,000x. Morphology of murine bone marrow culture in the absence or presence of 20 mM D-2-HG assayed on day 10. Cell morphology from one representative mouse out of 4 different is shown.(**C**) Colony forming assay was performed with G-CSF mobilized linage negative $CD34^+$ hematopoietic stem and progenitor cell (HSPCs) of healthy stem cell donors (n = 4) cultured for 14 days in the presence or absence of 20 mM D-2-HG. Pictures show representative CFU-E and CFU-GM colonies (100x magnification). (**D**) The pie chart summarizes percentages of different colony units. Erythrocyte (BFU-E, CFU-E), CFU granulocyte-macrophage (CFU-GM) and CFU granulocyte-erythrocyte-monocyte-macrophage (CFU-GEMM). Total number of colonies formed in each treatment are summarized in the scatter dot plot on the right. (**E**) HLA-DQ (MO n = 6, day 4, n = 3; day 7 n = 18), (**F**) HLA class I molecules (day 7, n = 11) and (**G**) expression of CD1a and (**H**) DC-SIGN on untreated monocytes before start of culture in comparison to DCs on day 4 (CD1a n = 4, DC-SIGN n=3 donors) and 7 (CD1a n = 21, DC-SIGN n = 23) of culture (with and w/o 20 mM D-2-HG) by flow cytometry. (**I**) CD14 (MO n = 4; day 4, n = 3; day 7, n = 4) expression on untreated monocytes before start of culture in comparison to DCs on day 4 and 7 of culture (presence or absence 20 mM D-2-HG) analyzed by flow cytometry. Symbols represent individual donors analyzed in independent experiments and horizontal bars mark median values. For two-group comparison Wilcoxon test, multiple-group comparison Kruskal-Wallis and post-hoc Dunn's test were performed. $P < 0.05$ was considered significant (*$P < 0.05$, ***$P < 0.001$).



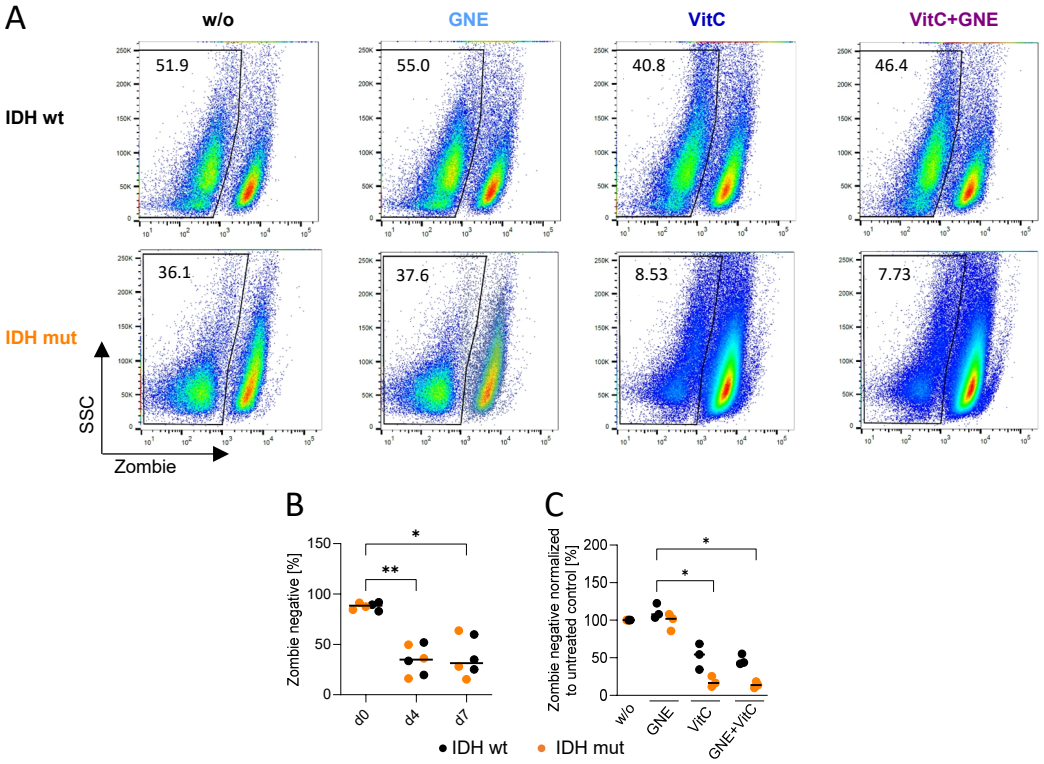
Supplementary Figure S2: D-2-HG did not affect essential amino acid levels but increased DC respiration after 7 days of culture. (**A**) Levels of nonessential amino acids measured by LC-MS/MS in culture supernatants of DCs in the presence or absence 20 mM D-2-HG (n = 3). Dashed lines indicate media (RPMI) concentrations. (**B**) Oxygen concentration was monitored in the absence or presence of 20 mM D-2-HG or L-2-HG or 10mM lactic acid for 7 days by PreSens technology, mean values of 6 independent experiments are shown. (**C**) ROUTINE respiration of DCs expressing wild-type (IDH1 n = 4, IDH2 n = 3) or mutant IDH1 (R132H, n = 4) or IDH2 (R140Q, n = 3) upon RNA transfection was analyzed by respirometry on day 7. (**D**) Scatter blot shows ROUTINE respiration after 16 h (n = 3), 4 (n = 3) or 7 days (n = 14) of D-2-HG treated monocytes and DCs analyzed by respirometry. (**E**) Scatter blot shows mitochondrial ROS after 16 h (n = 4), 4 (n = 4) or 7 (n = 21) days of D-2-HG treated monocytes and DCs. (**F**) ROUTINE respiration of DCs in the presence or absence of 20 mM D-2-HG and or 2 mM vitamin C (Vit. C) was measured on day 7. (**G**) Bisulfite-converted DNA of DCs was analyzed by MassARRAY Epityper analysis. Shown are two control regions with no change in methylation (MMP7, HOXB1). The methylation ratio (including 5mC and 5hmC, which cannot be distinguished after bisulfite treatment) at single CpGs dinucleotides (individual boxes) is indicated by shades of yellow to blue (yellow: no methylation, dark blue: 100 % methylation). Grey boxes indicate CpGs not detected by MALDI-TOF MS. (**H**) Barplot presentation of representative CpGs (marked with asterisks in Figure 2F and S2G) showing active demethylation. Symbols represent individual donors and horizontal bars mark median values. For two-group comparison Mann Whitney test or Wilcoxon test were performed. $P < 0.05$ was considered significant (***$P < 0.001$).



Supplementary Figure S3: Glycolytic inhibition and Vitamin C support MHC II expression. (**A**) Viability of DCs treated with HG, lactic acid with or without different inhibitors. (**B, C**) HLA-DP and (**D, E**) HLA-DR expression of DCs treated with inhibitors (1 mM Vit C, 0.1 µM NCI-737) and analyzed by flow cytometry on day 7. Numbers in representative blots indicate median fluorescence intensity values. Symbols in data summarized represent individual donors, analyzed in independent experiments, and horizontal bars mark median values. For multiple-group comparison one way ANOVA (mixed-effect analysis) and post-hoc Dunnet test were performed. $P < 0.05$ was considered significant (*$P < 0.05$, **$P < 0.01$).



Supplementary Figure S4: Essential amino acid levels of IDH wt AML cells are not influenced by D-2-HG but lactate level correlate with HLA-DR expression. (**A**) Level of essential amino acids in supernatants of 20 mM D-2-HG treated IDH wt AML cells (n = 6) after 7 days of culture determined by LS-MS/MS. Symbols represent individual donors analyzed in independent experiments and horizontal bars mark median values. Tryptophan concentrations were above detection limit in 3 of 6 analyzed donors. (**B**) Heat map of metabolite concentrations in supernatants of AML cells (n = 8) and DCs (n = 6) treated with or without 20 mM D-2-HG and measured on day 7 of culture. Fold changes over media concentrations are shown in the heatmap and are color-coded as depicted in the legend. Significance of treatment was tested against untreated controls. (**C**) HLA-DR surface expression of IDH wild type AML blasts cultured for either 4 or 7 days separated by lactate concentrations below and above 10 mM. Mann Whitney test was performed. $P < 0.05$ was considered significant. (**D**) Correlation of lactate concentration and HLA-DR expression is shown. AML samples without D-2-HG are displayed in black, samples treated with D-2-HG in orange, samples after 4 days are shown as squares and after 7 days in dots (< 10 mM n = 6, > 10 mM n = 14). Spearman's rank correlation coefficient was calculated.



Supplementary Figure S5: Vitamin C impairs survival of cultured primary AML. Primary cultured AML cells expressing wild type (black) and mutant IDH (orange) were analyzed before (d0) and after 4 days and 7 days of culture in the absence of presence of 2 mM vitamin C and 1µM GNE140. Cells were stained with the viability dye Zombie; Zombie negative cells are regarded as viable. (**A**) Representative FACS plots of cultured AML in the presence or absence of vitamin C and GNE140 on day 7. (**B**) Summarized data of viable untreated AML cells expressing wild type (black) and mutant IDH (orange) on day 4 and day 7. (**C**) Percentage of viable AML cells after treatment with vitamin C and GNE140 on day 7, normalized to the percentage of untreated cells. For multiple-group comparison Friedman or Kruskal Wallis and post-hoc Dunn's test were performed. $P < 0.05$ was considered significant (*$P < 0.05$, **$P < 0.01$).

Supplementary Table S1: Genomic position of analyzed CpG residues

Gene	Chromosomal Location (GRCh38/hg38)	Amplicon (for bisulfite-treated DNA)
CCL13	chr17:34356259-34356559	Epi00109_CCL13.1 [a]
STAT5	chr17:42283625-42283783	Epi00104_STAT5A.2 [a]
C9ORF78	chr9:129839252-129839471	Epi00148_C9ORF78.3 [a]
MMP7	chr11:102530563-102530766	Epi00162_MMP7.1 [a]
HOXB1	chr17:48530488-48530489	Epi00193_HOXB1_01 [a]
CD207	chr2:70837911-70838410	Epi00116_CD207.2 [a]
CLEC10A	chr17:7079646-7080109	Epi00184_CLEC10A.1 [a]

[a]Amplicons described in Ref. (Klug et al. 2010, 2013)

Supplementary Materials and Methods

Mixed lymphocyte reaction (MLR) and T cell assays

DCs ($1x10^4$ DCs) differentiated in the presence or absence of 20 mM D-2-HG were activated (100 ng/mL LPS) and co-cultured with allogeneic CD4+ T cells ($1x10^5$ T cells) that were isolated from PBMCs by immunomagnetic beads technology (Miltenyi Biotec, Bergisch Gladbach, Germany) in RPMI supplemented with 10 % human serum, 100 U/mL penicillin, 100 µg/mL streptomycin (GIBCO), 100 µM MEM NEA (Lonza, Cologne, Germany), 2 mM glutamineand 25 IU IL-2 (Proleukin, San Diego, CA, USA). On day 5, cell supernatant was collected for cytokine analysis and on day 7 cells were counted.

Lysis of HLA-typed (DPB1*04:01 positive) primary AML blasts by DPB1*04:01-specific TCR modified CD8 T cells was analyzed in standard [^{51}Cr]-release assay in duplicates as reported (Dörrschuck et al., 2004). TCR modified T cells were generated by electroporation of pre-stimulated CD8 T cells with *in-vitro* transcribed RNA encoding an HLA-DPB1*04:01 specific TCR as previously described (Klobuch et al., 2020). TCR expression was confirmed by TCR-Vβ13.2 staining 16 h after electroporation by flow cytometry (data not shown).

Metabolite analysis

Intracellular 2-HG determination

Intracellular 2-HG levels were determined by HPLC-MS/MS in different cell types. To do so, monocytes ($2.5x10^6$/well) were seeded in 6-well plates in a final volume of 4 mL in the presence or absence of 10 mM D-2-HG for 20 h in DC culture medium and harvested. Macrophages ($2×10^6$/well) were seeded in 6-well plates in a final volume of 4 mL in the presence or absence of 10 mM D-2-HG for 20 h in macrophage medium. PBMCs were isolated as described above and immediately harvested. CD3 T cells were isolated from PBMCs by immunomagnetic beads technology (Miltenyi Biotec) and subsequently cultured in RPMI supplemented with 10 % human serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µM MEM NEA (Lonza), 2 mM glutamine and 25 IU IL-2 (Proleukin, San Diego, CA, USA) for 3 days and subsequently harvested. Primary fibroblasts were generated and cultured as previously described (Nonn et al., 2008) and cultured with or without D-2-HG in DMEM supplemented with supplemented with 10 % fetal calf sera, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine for 20 h and harvested. AML blast were thawed and immediately harvested. Care was taken to wash the cells twice with cold PBS to remove extracellular traces of 2-HG and pellets were stored at -80 °C. Cell extraction and achiral HPLC-MS/MS analysis was performed as described (Berger et al., 2021). Intracellular 2-HG amounts were normalized to total protein amount.

Lactate, pyruvate, citrate, glucose and amino acid determination

Dendritic cells and AML cells were cultured as described above in the presence or absence of 20 mM D-2-HG. After 7 days cells were harvested and washed two times with cold PBS and pellets were stored at -80 °C for further determination.

Lactate, pyruvate, citrate and glucose in supernatants were analyzed by GC-MS as recently described (Schöller et al., 2021). Sample injection was performed at 280 °C in split mode using a split ratio of 8:1 and an injection volume of 1 µL.

Amino acid analysis in supernatants was performed by HPLC-MS/MS after propyl chloroformate derivatization as previously described (van der Goot et al., 2012).
Sample preparation was modified. Briefly, 10 µL supernatant were mixed with 10 µL of an aqueous stable isotope-labeled standard mix (^{13}C, ^{15}N labeled canonical amino acid mix (MSK-CAA-1, Eurisotop GmbH, Saarbrücken, Germany), Orn-d7, hippuric acid-d5, 10 µM each) and 180 µL water was added. Derivatization was carried out by adding 80 µL of reagent 1 (77 % n-propanol, 23 % 3-picoline, vol/vol) and 50 µL of reagent 2 (17.4 % propyl chloroformate in isooctane, vol/vol) with mixing steps in between. Derivatives were extracted with 250 µL ethyl acetate. 200 µL of the ethyl acetate phase was collected, evaporated to dryness and reconstituted in 100 µL of a solvent mixture (38 % water, 62 % methanol, vol/vol) for HPLC-MS/MS analysis. Supernatant concentrations were not normalized as DCs do not proliferate and D-2-HG treatment had no impact on AML proliferation.

Enzymatic lactate determination
For determination of lactate concentrations in Fig. 3A, 5C, 5J, 5L culture supernatants were collected and frozen at -20 °C until further analysis. Lactate concentrations were measured enzymatically using an ADVIA 1650 instrument (Bayer, Tarrytown, NY, USA) and specific reagents (Roche, Mannheim, Germany). Lactate values were corrected for basic lactate concentration of the culture medium.

Transmission Electron Microscopy and image analysis
$1x10^7$ cells were transferred in a 1.5 mL tube and centrifuged at 300 g for 5 minutes at room temperature. For ultrastructural studies cell pellet were fixed in Karnovsky-fixative (0.1 M cacodylate-buffer with 2.5 % glutaraldehyde and 2 % paraformaldehyde) for at least one day. The pellet was then enclosed with 4 % low-melting agarose and postfixed in 1 % osmium tetroxide for 2 h at pH 7.3, followed by dehydration in graded ethanols, and embedding in the EMbed-812 epoxy resin (all reagents from Science Services, Munich/Germany; automated LYNX-tissue processor Leica, Bensheim, Germany). After 48 h heat polymerization at 60° C, semithin (0.8 µm) sections were cut, stained with toluidine blue and basic fuchsin, and after light microscopic selection of representative cells in the section the epon block was trimmed for ultrathin sectioning. Ultrathin (80 nm) sections were cut with a diamond knife on a Reichert Ultracut-S ultramicrotome (Leica, Bensheim, Germany) and double stained with aqueous 2 % uranyl acetate and lead citrate solutions for 10 min each. The sections were examined in a LEO912AB electron microscope (Zeiss, Oberkochen, Germany) operating at 100 kV, equipped with a side-mounted CCD-camera (TRS, Lagerlechfeld, Germany) capable to record images with 2k x 2k pixels. Documentation was done with the iTEM-software (Olympus Soft Imaging Solutions GmbH, Muenster, Germany). For the evaluation of the ultrastructural images the software RADIUS (EMSIS, Muenster, Germany) was used. Vacuoles of 10 cells per group were annotated and counted to compare their median number per cell.

Protein isolation, SDS protein isolation, SDS-PAGE and Western Blot
Peripheral blood of AML patients was thawed; DCs were generated as described above. Proteins were extracted with RIPA buffer (Sigma/Merck) containing a protease inhibitor cocktail (Roche, Basel, Switzerland) and separated on a denaturing 10-12 % acrylamide gel. After western blotting, membranes were stained with rabbit anti-HLA-DP (EPR11226)

(Abcam, Cambridge, UK), rabbit anti-β-actin (polyclonal) (Sigma/Merck), rabbit anti-c-Myc/N-Myc (D3N8F) (Cell Signaling Technology, Danvers, MA, USA), monoclonal mouse anti-LDHB (Santa Cruz, Dallas, TX, USA), polyclonal rabbit anti-LDHA (Cell Signaling) ,and anti-rabbit immunoglobulins/HRP (polyclonal) (Agilent Dako, Santa Clara, CA, USA) in dry milk (5 %) and detection was performed by chemiluminescence (ECL). Actin expression was used as loading control (Sigma/Merck). Densitometric analyses were performed by means of the ChemiDoc MP Imaging System and the Image LabTM software (Bio-Rad Laboratories, Hercules, CA, USA). Quantification of signal was done with ImageJ (Version 1.53K, National Institutes of Health, New York, NY, USA).

Online-measurement of oxygen concentration and pH value in cell culture

The SDR SensorDish® Reader (PreSens Precision Sensing GmbH, Regensburg, Germany) is a 24-channel oxygen and pH meter. The optical oxygen (OxoDish®) or hydrogen (HydroDish) sensor is integrated at the bottom of each well of a 24-well multi-dish. The sensors are luminescent dyes embedded in an analyte-sensitive polymer. The luminescence lifetime of the dyes depends on the amount of analyte. The sensors are read out non-invasively through the bottom of the multi-dish by the SensorDish® reader. The resulting signal is converted automatically to the respective parameter (dissolved oxygen or pH value) using calibration parameters stored in the software. 7×10^5 cells/mL medium were incubated with or without 20 mM D-2-HG and 2 mM vitamin C. The SensorDish® Reader was used in the incubator for the entire 7-day cultivation period and measurements were performed in 5-minute intervals.

Flow cytometry, antibodies, MitoSox, and cytokine measurements

Flow cytometry was performed on a FACS Calibur, Celesta (BD Biosciences, Heidelberg, Germany) and analyzed using FlowJo X 10.8.1 software (Tree Star). Fluorochrome-labeled monoclonal antibodies (mAb) were anti-human HLA-ABC-PE (G46-2.6), anti-human HLA-DQ-FITC (Tu169), anti-human HLA-DR-APC (L243), anti-human CD14-FITC (M5E2), anti-human CD1a-PE-Cy7 (HI149), anti-mouse I-A/I-E-PE (M5/114.15.2) (all BD Biosciences), anti-human HLA-DP-PE (BRAFB6; Santa Cruz) for DCs, anti-human HLA-DP-PE (B7/21 Leinco Technologies Inc., St. Louis, USA) for AML blast, anti-human DC-SIGN-PE (FAB161P, R&D Systems, Minneapolis, MN, USA), anti-human HIS-PE (GG11-8F3.5.1, Miltenyi), anti-human TCRVβ 13.2-PE (H132, Beckman Coulter, CA, USA). Mitochondrial ROS was stained with 5 µM MitoSox (Thermo Fischer Scientific) according to the manufacturer's protocol at 37 °C for 10 min. Directly after staining cells were analyzed by flow cytometry. For analyses of cytokine concentrations, cell culture supernatants were collected and analyzed by commercially available ELISA kits (Duoset ELISA, R&D Systems).

Colony forming cell (CFC) assay

PBMCs were isolated by density centrifugation on LSM 1077 Lymphocyte Separation Medium (PAA Laboratories GmbH, Pasching, Austria) and HSPC were enriched using immunomagnetic beads (Dynabeads®, Invitrogen) followed by cell sorting for $Lin^- CD34^+$ cells on a FACSAria II (BD Biosciences) cell sorter. Burst-forming and colony-forming units erythrocyte (BFU-E, CFU-E), CFU granulocyte-macrophage (CFU-GM) and CFU granulocyte-erythrocyte-monocyte-macrophage (CFU-GEMM) were assayed as described before (Grassinger et al., 2014). In brief, defined numbers of cells (100 $CD34^+$ cells per dish)

were plated in Human Methylcellulose Complete Medium (R&D Systems) in the presence (20 mM) or absence of D-2-HG and colonies were scored according to count and morphology on day 14 using an inverted microscope (magnification 100x; Axiovert 200 M MAT and AxioVision Rel. 4.8 software; Carl Zeiss GmbH, Jena, Germany).

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