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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Abstract

D-2-hydroxyglutarate (D-2-HG) accumulates in patients with acute myeloid leukemia (AML) with mutated isocitrate dehydrogenase (*IDH*) and in other malignancies. D-2-HG suppresses antitumor T-cell immunity but little is known about potential effects on non-malignant myeloid cells. Here we show that D-2-HG impairs human but not murine dendritic cell differentiation, resulting in a tolerogenic phenotype with low major histocompatibility class II expression. In line with this, IDH-mutated AML blasts exhibited lower expression of HLA-DP and were less susceptible to lysis by HLA-DP-specific T cells. Interestingly, besides its expected impact on DNA demethylation, D-2-HG reprogrammed metabolism towards increased lactate production in dendritic cells and AML. Vitamin C accelerated DNA demethylation, but only the combination of vitamin C and glycolytic inhibition lowered lactate levels and supported major histocompatibility complex class II expression. Our results indicate an unexpected link between the immunosuppressive metabolites 2-HG and lactic acid and suggest a potentially novel therapeutic strategy with combinations of anti-glycolytic drugs and epigenetic modulators (hypomethylating agents) or other therapeutics for the treatment of AML.

Introduction

Heterozygous somatic mutations in isocitrate dehydrogenase (*IDH*) were originally identified in patients with glioma and acute myeloid leukemia (AML), but they are also detected in patients with other tumor entities.[1] Mutated IDH1 and IDH2 gain neomorphic function to convert α-ketoglutarate (α-KG) to the oncometabolite D-2-hydroxyglutarate (D-2-HG), which accumulates in tumor tissues as well as in sera of patients.[2] In tumor tissues of glioma patients 2-HG levels range from 5-35 mM,[3] whereas levels in sera of AML patients range from 2-300 μM.[4] Despite the absence of *IDH* mutations, elevated 2-HG levels have also been described in other tumor entities. In breast cancer, MYC overexpression triggers glutamine uptake and glutaminolysis resulting in 2-HG accumulation,[5] whereas in renal cell carcinoma,[6] the L-enantiomer of 2-HG accumulates due to reduced expression of its degrading enzyme L-2-HG dehydrogenase. Moreover, other enzymes such as malate dehydrogenase and lactate dehydrogenase (LDH) can catalyze the conversion

of α-KG to 2-HG under hypoxic and/or acidic conditions, a process termed "enzyme promiscuity".[7]

In some cancer entities, D-2-HG accumulation is associated with worse prognosis, whereas an IDH mutation is a favorable prognostic marker in glioma.[1,2,5] The current view is that the structural similarity between D-2-HG and α-KG causes competition and inhibition of α-KG-dependent enzymes, such as Jumonji-C domain histone demethylases. D-2-HG also inhibits the ten-eleven translocation (TET) family of 5-methylcytosine hydroxylases, a family of enzymes involved in the first step of active DNA demethylation.[8] IDH1 or IDH2 mutated AML display global DNA hypermethylation and expression of mutated IDH2 in mouse myeloid progenitor cells *in vitro* increases DNA methylation while inhibiting their differentiation.[9] Despite its effects on epigenetic programming, expression of mutated IDH1 alone does not cause murine leukemic transformation but promotes leukemogenesis only in cooperation with an additional driver, such as HoxA9.[10] In contrast, it has been shown by others that D-2-HG, but not its L-enantiomer, is sufficient to promote leukemogenesis in a human and murine cell-based model.[11] Collectively, these data suggest that *IDH* mutations and D-2-HG accumulation dysregulate the epigenetic machinery, which in turn disturbs differentiation. Besides myeloid cells, endogenously produced 2-HG also affects DNA methylation and differentiation of murine lymphoid cells.[12] In primary human T cells, however, Bunse *et al*. demonstrated that gene expression but not the methylation pattern changed after D-2-HG treatment,[13] indicating differences in D-2-HG susceptibility between murine and human immune cells. The same group recently demonstrated IDH-dependent immunosuppression related to D-2-HG-induced metabolic changes in tryptophan degradation by tumor-associated macrophages.[14]

Here, we investigated the differentiation of another important population of myeloid cells, namely dendritic cells (DC). In contrast to published data on murine DC differentiation,[15] we describe the inhibition of human DC differentiation by D-2-HG via induction of MYC, accelerated glycolysis and reprogramming of the epigenetic machinery. Our data suggest that D-2-HG might influence cell differentiation by both induction of epigenetic changes and modulation of cellular metabolism.

Methods

Primary cells and ethics statement

Peripheral blood mononuclear cells were isolated from leukapheresis products or leukocyte reduction system cones of healthy donors by density gradient centrifugation over Ficoll/Hypaque. Primary AML blasts were isolated by density gradient centrifugation over Ficoll/Hypaque from peripheral blood of leukemia patients at initial diagnosis of AML, before they had started leukemia therapy. Human $CD34^+$ hematopoietic stem and progenitor cells were isolated from stem cell leukapheresis products of healthy donors after the donors had been stimulated with granulocyte colony-stimulating factor. The studies were conducted in accordance with the Declaration of Helsinki. All donors gave their informed consent to participation in the study and the protocols were approved by the Ethics Committee of the University Hospital Regensburg (permission numbers 05-097, 17-587-101, 13-101-0240, 13-101-0238, and 10-101-0099).

Monocyte isolation and macrophage generation

Mononuclear cells from healthy donors were separated by leukapheresis, followed by density gradient centrifugation over Ficoll/Hypaque. Monocytes were isolated from mononuclear cells by countercurrent centrifugal elutriation in a J6M-E Beckmann centrifuge with a large chamber and a JE-5 rotor at 2,500 rpm and a flow rate of 110 mL/min in Hanks' balanced salt solution with 2% human plasma. Elutriated monocytes were >80% pure as determined by morphology and CD14 expression. For the generation of monocyte-derived human macrophages, purified monocytes were cultured on teflon foils (Biofolie 25, Heraeus Hanau, Germany) for 7 days at a cell density of $1x10^6$ cells/mL in RPM1 1640 supplemented with 2% pooled human AB group serum.

Dendritic cell generation

Bone marrow-derived murine DC were generated as described previously[16] in the presence or absence of 20 mM D-2-HG.

For the generation of human DC, monocytes were seeded in RPMI supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (100 U), streptomycin (100 μg/mL), interleukin 4 (150 U/mL), and granulocyte-macrophage colony-stimulating factor (230 U/mL) at a concentration of $1x10^6$/well in a 24-well plate in 1 mL of medium or $7x10^6$ in a T25 culture flask in 10 mL medium. D-2-HG was added once, immediately after seeding the cells, for the whole period of culture. Cells were cultured for 7 days to generate immature DC. Where indicated, the culture medium was supplemented with D-2-HG (10 mM, 20 mM), the LDHA inhibitor GNE140 (1 μM, starting on day 2) or pyrazole-based LDHA/B inhibitor NCI-737[17] (0.1 μM, starting on day 2) and vitamin C (2 mM), respectively. Maturation of DC was induced by the addition of 100 ng/mL lipopolysaccharide on day 7. DC expressing mutated IDH were generated by *in-vitro* transcribed RNA electroporation as described elsewhere.[18] In brief, the coding DNA region of wild-type IDH2 including a HIS tail was synthesized by GeneArt and inserted into the pGEM4Z-64A vector for *in vitro* mRNA transcription. With a QuikChange Site-Directed Mutagenesis Kit, site-directed mutagenesis was performed to change the nucleotide responsible for the exchange of arginine to glutamine in the amino acid sequence of IDH2 (IDH2 R140Q). Both constructs were used to electroporate DC for T-cell stimulation.

Acute myeloid leukemia cell culture

Primary AML blasts were thawed and cultured in AIM-V medium (GIBCO) supplemented with 10% pooled human serum, 50 ng/mL stem-cell factor (PeproTech, Rocky Hill, NJ, USA), 50 ng/mL granulocyte colony-stimulating factor (Hospira, Lake Forest, IL, USA) with or without 20 mM D-2-HG (Sigma/Merck, Darmstadt, Germany), 2 mM vitamin C (Sigma/Merck) and 1 μM GNE140 (1 μM, Selleckchem, Housten, TX, USA) from day 0, at a density of $2x10^6$ cells/mL for 7 days.

Cell numbers and viability

Cell numbers, cell size and viability were determined using either a Neubauer chamber or a CASY cell analyzer. Cells were resuspended and 50 μL of the cell suspension were mixed with the same volume of trypan blue. This solution was transferred into a Neubauer chamber and the contents of two large squares were counted.

The CASY cell analyzer system (Casy® Model TT, OLS Omni Life Science, Bremen, Germany) obtains signals when a cell passes in a low-voltage field through the system's high-precision measuring pore. The system was used according to the manufacturer's instructions.

Other methods

The mixed lymphocyte reaction and T-cell assays are described in the *Online Supplementary Methods*, together with detailed information on the metabolite analyses. The *Online Supplementary Methods* also provides information on transmission electron microscopy, as well as protocols for protein isolation, sodium dodecylsulfate polyacrylamide gel electropheresis and western blotting.

Oxygen consumption, oxygen concentration and pH

Mitochondrial respiratory activity was determined by high-resolution respirometry as described elsewhere.[19] Online-measurement of oxygen concentration and determination of pH values in cell cultures were performed as detailed in the *Online Supplementary Methods.*

Flow cytometry, antibodies, MitoSox, and cytokine measurements

Details are described in the *Online Supplementary Methods*.

Colony-forming cell assay

The colony-forming cell assay was performed as described previously[20] and detailed in the *Online Supplementary Methods*.

The Cancer Genome Atlas data analysis

AML RNA sequencing data (normalized expression values per gene, displayed as reads per kilo base per million mapped reads) and available clinical information were downloaded from The Cancer Genome Atlas data portal (*https://portal.gdc.cancer.gov*). Normalized transcription levels of major histocompatiblity complex (MHC) class II α and β chain genes (*HLA-DP, -DQ* and *-DR*) and class II MHC transactivator (*CIITA*) were compared in AML blasts expressing wild-type or mutated IDH.

DNA methylation analysis

Methylation analysis was done as previously described.[21] Analyzed amplicons and gene regions are listed in *Online Supplementary Table S1*.

Statistical analysis

The statistical analyses were performed with Graphpad Prism, version 9 (La Jolla, CA, USA). Sample sizes are given in the respective figure legends. Comparisons between groups were made using the appropriate statistical methods depending on Gaussian distribution and number of groups and variables (Friedman test, Wilcoxon test, Kruskal-Wallis test, Mann-Whitney and one-way analysis of variance). Differences were considered statistically significant for *P* values of <0.05 (**P*<0.05, ***P*<0.01, ****P*<0.001).

Results

D-2-hydroxyglutarate alters the morphology of human but not murine dendritic cells

High D-2-HG levels are detected in tumor tissues and it is known that immune cells can take up exogenous D-2-HG, which may limit their anti-tumor potential.[13] We measured endogenous 2-HG levels in peripheral blood mononuclear cells, T cells, monocytes, macrophages and fibroblasts by liquid chromatography-tandem mass spectrometry. T cells, fibroblasts and myeloid cells (i.e., monocytes and macrophages) exhibited higher levels of endogenous 2-HG compared to peripheral blood mononuclear cells, and these levels could be further increased by supplementation with D-2-HG, demonstrating that the cells had a capacity for active uptake of this metabolite (Figure 1A).

We, therefore, investigated whether high exogenous D-2-HG levels might alter monocyte to DC differentiation over a 7-day culture period with interleukin-4 and granulocyte-macrophage colony-stimulating factor. It has been reported that D-2-HG does not affect murine DC differentiation.[15] Here, we compared differentiation of human blood monocytes into DC with differentiation of murine (C57BL/6) bone marrow cells to bone marrow-derived DC. D-2-HG did not affect the viability of either murine or human DC (Figure 1B), but cell yields measured by CASY cell counting technology were slightly lower for murine bone marrow-derived DC treated with D-2-HG (*Online Supplementary Figure S1A*). Interestingly, electron microscopy revealed profound changes in cell morphology with significantly decreased numbers of vacuoles and impaired dendrite formation in D-2-HG-treated human DC compared to untreated controls (Figure 1C, *Online Supplementary Figure S1B*). Bright

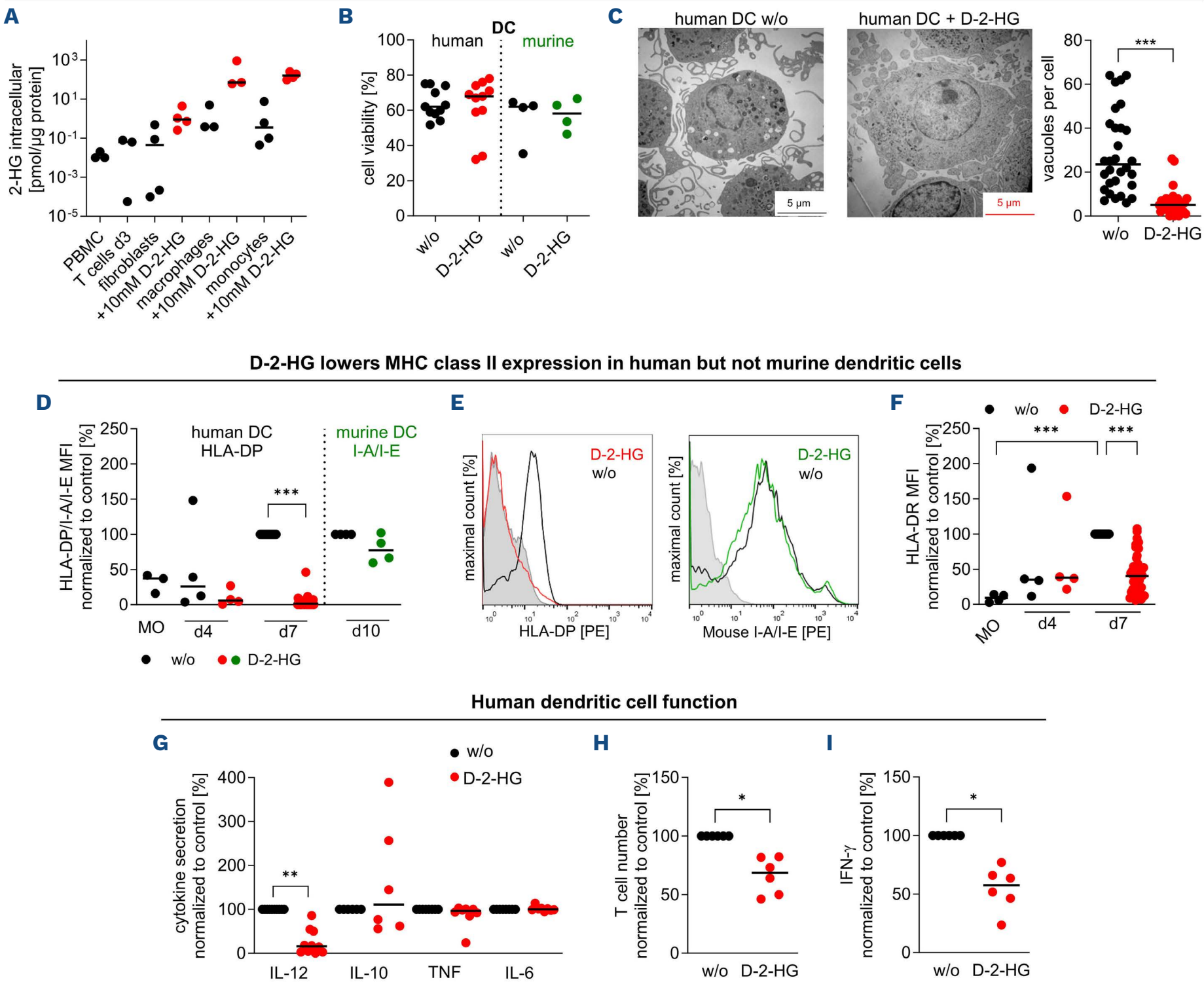

Figure 1. D-2-hydroxyglutarate inhibits human dendritic cell differentiation. (A) Intracellular levels of 2-hydroxyglutarate (2-HG) were analyzed by liquid chromatography tandem mass spectrometry in peripheral blood mononuclear cells (N=3), T cells (after 3 days of culture, N=3), fibroblasts (N=4), monocytes (N=4) and macrophages (N=3 after 7 days) from healthy donors incubated with or without 10 mM D-2-HG (20 h). (B) Viability of dendritic cells (DC) from humans (N=11 donors) and mice (N=4 mice) were analyzed after 7 (human DC) or 10 (murine DC) days of culture. (C) Transmission electron microscopy of DC cultured for 7 days in the presence or absence of 10 mM D-2-HG. One representative experiment out of four is shown at a magnification of 10,000x. Vacuoles of ten cells per donor and condition (N=40 for control and D-2-HG treatment) were counted. (D) Untreated human monocytes (N=3) and monocyte-derived DC cultured with or without 20 mM D-2-HG were analyzed on day 4 (N=4) and 7 (N=31). Murine DC (N=4) were cultured for 10 days with or without 20 mM D-2-HG. HLA-DP (human) or I-A/I-E (murine) cell surface expression was analyzed by flow cytometry and normalized to the expression of the control (monocyte-derived DC at day 7 of culture, without D-2-HG). (E) Representative histogram of HLA-DP (human) or I-A/I-E (murine) expression analyzed by flow cytometry. (F) HLA-DR surface expression on untreated monocytes (N=4) before the start of culture in comparison to the expression on DC on days 4 (N=3) and 7 (N=46) of culture (with and without 20 mM D-2-HG) as determined by flow cytometry. (G) Levels of cytokines (interleukin-12 [N=10]), interleukin-10 [N=6], tumor necrosis factor [N=8] and interleukin-6 [N=8]) were determined by enzyme-linked immunosorbent assay (ELISA) in supernatants of DC stimulated with lipopolysaccharide (100 ng/mL) for 24 h. (H) Human CD4 T cells (10^5) were stimulated with DC (10^4) from an allogeneic donor differentiated in the presence or absence of 20 mM D-2-HG. On day 7 of the mixed lymphocyte reaction, T-cell proliferation (N=6) was measured by cell counting. (I) Interferon-γ secretion of T cells (N=6) was analyzed in supernatants of day 5 cultures by ELISA and normalized to control. Symbols represent individual donors analyzed in independent experiments and horizontal bars mark median values. For two-group comparisons a Mann-Whitney test or Wilcoxon test was used. For multiple-group comparisons the Kruskal-Wallis and *post-hoc* Dunn test were performed. *P*<0.05 was considered statistically significant (**P*<0.05, ***P<0.01, ****P*<0.001). PBMC: peripheral blood mononuclear cells; w/o: without; MO: monocytes; MFI: mean fluorescence intensity; IL: interleukin; TNF: tumor necrosis factor; IFN: interferon.

field microscopic analysis of murine bone marrow-derived DC revealed no changes in cell morphology between cells treated with D-2-HG or untreated controls (*Online Supplementary Figure S1B*).

To assess whether D-2-HG could also affect early myeloid progenitor cells, we analyzed the impact of D-2-HG on differentiation of human CD34$^+$ hematopoietic stem and progenitor cells in colony-forming cell (CFC) assays. After plating defined numbers of CD34$^+$ cells, burst-forming units and colony-forming units (CFU) were scored on day 14. D-2-HG treatment resulted in a considerably lower number of CFC when compared to control (median 5.0 *vs*. 14.5 CFC/100 CD34$^+$ cells; *P*=0.057) and colonies were smaller in size (*Online Supplementary Figure S1C, D*). Of note, D-2-HG resulted in a marked reduction of CFU-granulocyte-macrophage myeloid progenitors (3.8% *vs*. 33.9%), while the proportion of CFU-erythroid (45.5% *vs*. 89.1%) increased (*Online Supplementary Figure S1C, D*).

D-2-hydroxyglutarate limits MHC class II expression in human but not murine dendritic cells and impairs T-cell stimulation

To further evaluate the effects of D-2-HG on human and murine DC differentiation, we analyzed cell surface expression of the MHC class II antigen HLA-DP and its murine equivalent in immature human and murine DC. Upregulation of HLA-DP during human monocyte to DC differentiation was significantly blocked in D-2-HG-treated cells (*P*<0.001) (Figure 1D, E). In contrast to human DC, surface expression of the HLA-DP analog I-A/I-E was not significantly impaired on murine bone marrow-derived DC (*P*=0.25) (Figure 1D, E). Based on these results we analyzed the expression of other MHC class II molecules on human DC. In line with the findings for HLA-DP, the expression of HLA-DQ and -DR was also significantly reduced on day 7 after D-2-HG treatment (Figure 1F, *Online Supplementary Figure S1E*). Surface expression of MHC class I molecules also decreased during D-2-HG treatment but the decrease did not reach statistical significance (*Online Supplementary Figure S1F*). Furthermore, D-2-HG prevented upregulation of other DC markers such as CD1a and DC-SIGN (*Online Supplementary Figure S1G, H*). CD14, a monocyte marker that is downregulated during DC differentiation, was lost after 4 days even in the presence of D-2-HG (*Online Supplementary Figure S1I*). Next, we analyzed the cytokine profile of D-2-HG-treated DC. DC were activated with lipopolysaccharide for 24 h and cytokine levels were measured in culture supernatants. Similar to the already described short-term effects of D-2-HG,[22] interleukin-12 production was significantly reduced by D-2-HG after 7 days (84%, *P*=0.002), whereas interleukin-10 secretion was increased in three out of six donors. Tumor necrosis factor and interleukin-6 production was not influenced by D-2-HG treatment (Figure 1G). Interestingly, in line with low interleukin-12 secretion and MHC class II expression, D-2-HG-treated DC were less efficient stimulators in an allogeneic mixed lymphocyte reaction with CD4 T cells, resulting in significantly lower T-cell proliferation (70% that of controls) and interferon-γ secretion (reduced by approximately 45%) (Figure 1H, I). Overall, D-2-HG affected the morphology and maturation of human DC, correlating with an impaired ability to stimulate T cells.

D-2-hydroxyglutarate reprograms metabolism and DNA demethylation during dendritic cell differentiation

Metabolic processes are crucial for activation and differentiation of immune cells and metabolic changes also occur during DC differentiation. DC exhibit a marked increase in the number of mitochondria and respiration compared to monocytes, whereas oxidative phosphorylation (OXPHOS) is reduced when DC become activated during maturation.[23] We therefore hypothesized that D-2-HG might block DC differentiation and function via metabolic alterations. To verify this, we measured the levels of amino acids, glucose, pyruvate, and lactate in cell culture supernatants of DC differentiated in the presence or absence of D-2-HG. Treatment with D-2-HG resulted in accelerated glucose metabolism with significantly lower glucose (median 7.5 mM *vs*. 0.77 mM) and higher lactate (median 4.4 mM *vs*. 16.2 mM) levels in DC culture supernatants (Figure 2A). Thus, D-2-HG might contribute to the recently reported strong glycolytic activity of myeloid cells in the tumor environment.[24] In addition, D-2-HG-treated DC produced significantly less alanine (median 473.1 μM *vs*. 392.5 μM) and consumed more arginine (median 707.6 μM *vs*. 606.6 μM) (Figure 2A). The concentrations of essential amino acids were not altered by D-2-HG treatment (*Online Supplementary Figure S2A*).

In line with higher lactate levels, the expression of LDH subunit A (LDHA) was increased (Figure 2B, C). LDH is transcriptionally regulated by MYC[25] and MYC expression increased in the presence of D-2-HG during DC culture and was significantly upregulated on day 7 (Figure 2B, D). This was accompanied by a significant drop in pH after 3-5 days of culture in the presence of D-2-HG (Figure 2E). A similar trend was observed with the L-enantiomer L-2-HG. At the end of the 2-HG-treated DC culture, acidification was comparable to that of DC cultures supplemented with 10 mM lactic acid (Figure 2E). In addition, D-2-HG and L-2-HG did not change oxygen consumption (*Online Supplementary Figure S2B*).

As D-2-HG is produced by mutated IDH, we analyzed DC expressing mutated IDH1 R132H and IDH2 R140Q upon RNA transfection by high-resolution respirometry. Basic oxygen consumption (ROUTINE respiration) of DC expressing mutated IDH1 and IDH2 was increased compared to that of DC expressing wild-type IDH1 and IDH2 (*Online Supplementary Figure S2C*). To compare the effects of D-2-HG produced endogenously or added exogenously, we also analyzed D-2-HG-treated DC by high-resolution respirometry. ROUTINE respiration was significantly higher in DC treated for 7 days with D-2-HG (*P*=0.001) (*Online Supplementary Figure S2D*). Higher mitochondrial activity can result in elevated produc-

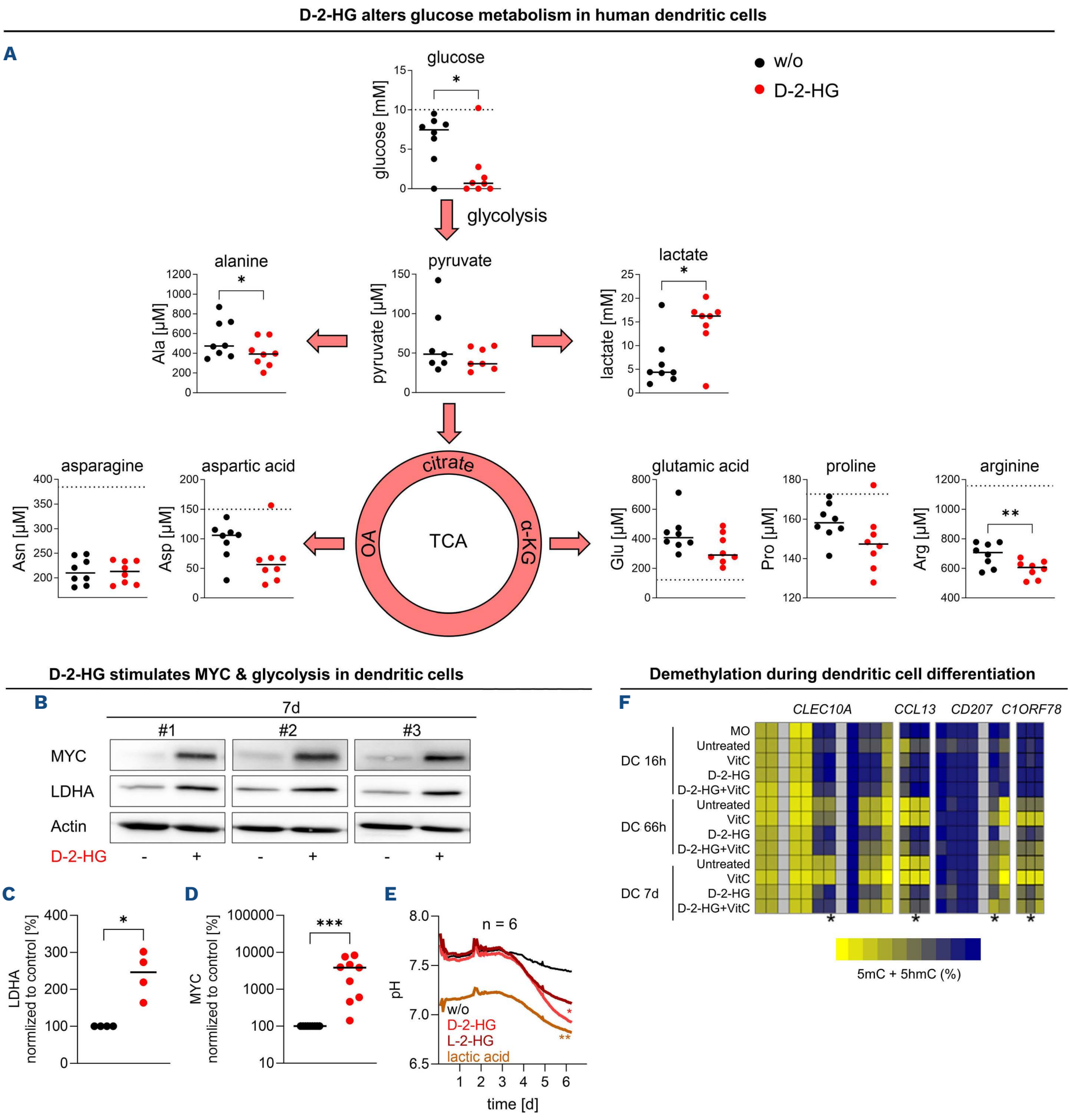

Figure 2. D-2-hydroxyglutarate induced changes in amino acid and glucose metabolism. (A) Concentrations of metabolites in supernatants of dendritic cells (DC, N=8) cultured with or without 20 mM D-2-hydroxyglutarate (D-2-HG) measured on day 7 of culture. Dashed lines indicate the concentrations in RPMI medium. (B) Western blot analysis of lactate dehydrogenase A (LDHA) and MYC in DC cultured with or without 20 mM D-2-HG for 7 days. (C, D) Scatter plots showing the summary and quantification of actin-normalized LDHA (C) and MYC (D) signals relative to the control. (E) pH values were monitored in the absence or presence of 20 mM D-2-HG or L-2-HG or 10 mM lactic acid every 5 minutes for 7 days by PreSens technology; the mean values of six independent experiments are shown. (F) Bisulfite-converted DNA of DC was analyzed by MassARRAY Epityper analysis. Four loci showing active DNA demethylation during monocyte-derived DC differentiation (*CLEC10A, CCL13, CD207, C10ORF78*) were analyzed. Data represent the mean of three different donors and are presented as heatmaps. The methylation ratio (including 5-methylcytosine and 5-hydroxymethylcytosine, which cannot be distinguished after bisulfite treatment) at single CpG dinucleotides (individual boxes) is indicated by shades of yellow to blue (yellow: no methylation, dark blue: 100% methylation). Gray boxes indicate CpG that were not

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detected by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. A bar plot presentation of representative CpG (marked with asterisks) indicating active demethylation is shown in *Online Supplementary Figure S2H* and methylation ratios of all single CpG units for individual donors are provided in *Online Supplementary Table S1*. (A-E) Symbols represent individual donors analyzed in independent experiments and horizontal bars mark median values. For two-group comparisons, the Mann-Whitney test was used; in (E) two-way analysis of variance and a *post-hoc* Tukey test were performed. $P<0.05$ was considered statistically significant (*$P<0.05$, ***P<0.01, ***$P<0.001$). w/o: without; Ala: alanine; Asn: asparagine; Asp: aspartic acid; OA: oxaloacetate; TCA: tricarboxylic acid cycle; α-KG: α-ketoglutarate; Glu: glutamic acid; Pro: proline; Arg: arginine; DC: dendritic cells; MO: monocytes; VitC: vitamin C; 5mC: 5-methylcytosine; 5hmC: 5-hydroxymethylcytosine.

tion of reactive oxygen species. However, no significant change in the formation of reactive oxygen species was observed (*Online Supplementary Figure S2E*). In summary, D-2-HG treatment led to higher glycolytic and respiratory activity of DC.

Mitochondria are involved in epigenetic modulation, as they generate acetyl-CoA and sustain S-adenosylmethionine production used for the acetylation and methylation of histones and DNA, respectively. Loss of DNA methylation occurs during differentiation of human monocytes into DC and is linked to the expression of TET2.[21] It is known that D-2-HG blocks key enzymes of DNA demethylation such as TET-family 5-methylcytosine hydroxylases.[8] We therefore investigated the impact of D-2-HG on DNA demethylation during DC differentiation in the absence or presence of vitamin C, a cofactor of TET2 known to increase its activity. We observed a pattern of delayed demethylation of D-2-HG-treated DC at loci that normally become demethylated during DC differentiation, such as *CLEC10A, CCL13, CD207* and *C10ORF78* (Figure 2F). Vitamin C accelerated demethylation of all genes analyzed and partially counteracted the inhibitory effect of D-2-HG on methylation and ROUTINE respiration (Figure 2F, *Online Supplementary Figure S2F*). As expected, the methylation status of two loci that were not changed during differentiation (*MMP7* and *HOXB1*) was also not modulated by D-2-HG (*Online Supplementary Figure S2G, H*). These data indicate that D-2-HG delays DNA demethylation during monocyte to DC differentiation, which may contribute to its strong impact on differentiation.

Modulating dendritic cell metabolism with vitamin C and lactate dehydrogenase inhibitors

Culture medium of DC treated with either D-2-HG or L-2-HG exhibited a lowered pH and we therefore compared lactate levels in the corresponding supernatants. Lactate was elevated in culture supernatants of DC treated with both enantiomers (Figure 3A) and both enantiomers lowered HLA-DR and HLA-DP expression of DC (Figure 3B, C). To clarify a possible role of lactic acid in the regulation of MHC class II molecules, we supplemented DC cultures with 10 mM lactic acid, which significantly reduced HLA-DR and HLA-DP expression (Figure 3B, C) in line with a comparable extracellular pH in DC cultures supplemented with either 2-HG or lactic acid (Figure 2E).

As vitamin C partially rescued the D-2-HG-induced delay in demethylation during DC differentiation, we investigated whether vitamin C treatment would be able to rescue the metabolic phenotype and DC marker expression. Vitamin C reduced the elevated ROUTINE respiration in HG-treated DC cultures (*Online Supplementary Figure S2F*) but lactate levels in supernatants were still higher than those in the DC control (Figure 3A). Nevertheless, DC marker expression was partially restored (Figure 3D-K).

Next we tried to counteract the suppressive effect of D-2-HG by treating DC with the selective LDHA inhibitor GNE140 (1 μM).[26] Combined treatment with GNE140 and vitamin C resulted in a significant decrease in lactate levels (Figure 3A). The viability of DC was diminished by vitamin C treatment, but not by D-2-HG and GNE140 (*Online Supplementary Figure S3A*). GNE140 or vitamin C alone increased the D-2-HG-impaired expression of MHC class II, CD1a and DC-SIGN and combined treatment with vitamin C and GNE140 raised the expression levels almost back to control levels for HLA-DR (94%) and CD1a (89%) (Figure 3H-K). HLA-DP and DC-SIGN expression was improved and no longer significantly decreased, but did not reach control levels. Similar effects were found with the pyrazole-based LDHA/B inhibitor NCI-737 (0.1 μM) in combination with vitamin C. NCI-737 lowered lactate secretion Figure3A and partially reverted MHC class II expression (*Online Supplementary Figure S3B-E*). Overall, LDH inhibition was able to strengthen the effects of vitamin C and led to a consistent improvement of MHC class II expression in human DC.

Exogenous D-2-hydroxyglutarate treatment inversely regulates lactate production and MHC class II expression in primary IDH wild-type acute myeloid leukemia cells

As D-2-HG strongly affected the metabolism of monocyte-derived DC, we asked whether this also held true for primary AML blasts. We, therefore, measured amino acid and glucose metabolite levels in the supernatants of IDH wild-type AML cells cultured in the presence of D-2-HG. D-2-HG significantly elevated glucose metabolism and increased lactate levels in AML cells (lactate 8.99 mM *vs*. 14.1 mM), in line with the effects observed in DC (Figure 4A). Induction of lactate production and secretion in AML blasts was not as pronounced as in DC, likely a consequence of the already much higher basal supernatant levels of lactate of AML cells compared to DC. In contrast to DC, we observed no alterations in amino acid levels (Figure 4A, *Online Supplementary Figure S4A*). The effects of D-2-HG on DC and AML cells are summarized in the heatmap in *Online Supplementary Figure S4B*; metabolite levels were

normalized to medium concentration (1 equals the medium concentration). Cell count and cell viability were not affected by D-2-HG treatment (Figure 4B, C). Again, lactate levels in supernatants of AML cells treated with D-2-HG for 7 days were significantly higher (Figure 4D). As lactic acid has been reported to alter cell differentiation and function predominantly at concentrations higher than 10 mM,[27] we analyzed HLA-DP (Figure 4E, F) and HLA-DR expression (*Online Supplementary Figure S4C, D*) relative to the lactate level in AML supernatants. Here, MHC class II expression

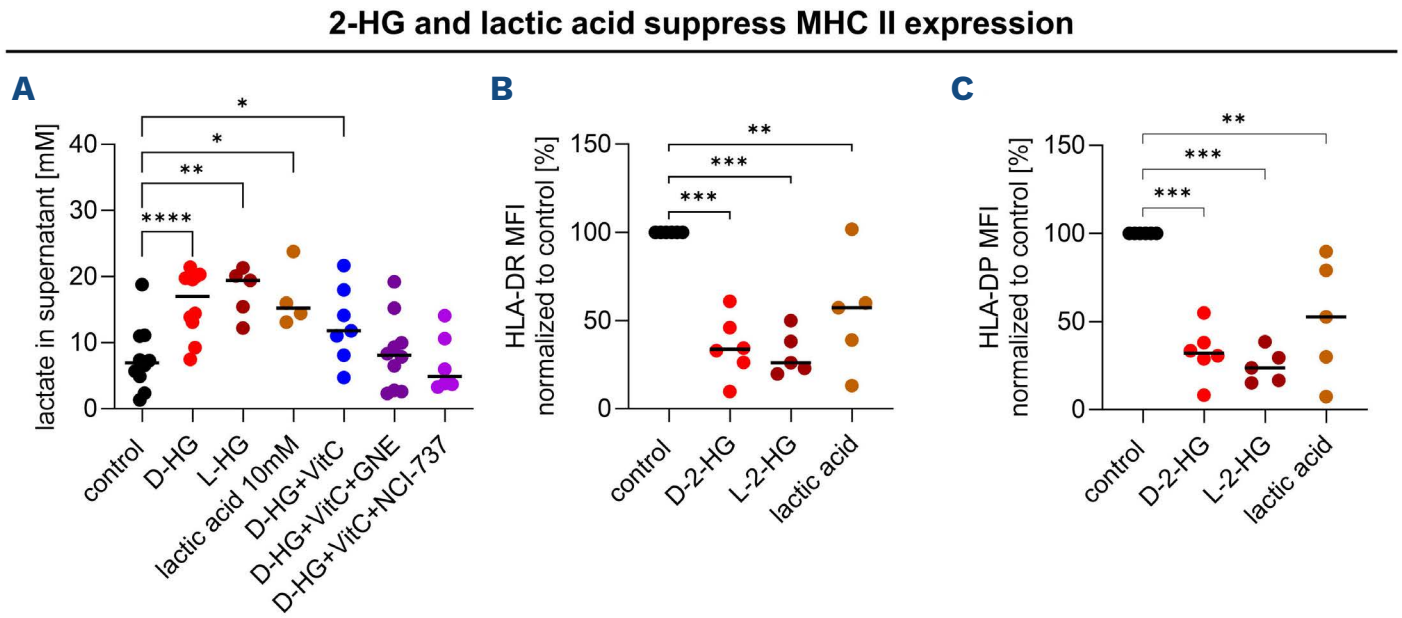

Figure 3. Vitamin C and lactate dehydrogenase inhibitor treatment counteract D-2-hydroxyglutarate-induced effects. (A) Scatter plot showing lactate level in supernatants of dendritic cells (DC) treated with 20 mM D-2-hydroxyglutarate (D-2-HG) or L-2-hydroxyglutarate (L-2-HG) or 10 mM lactic acid (started on day 0), 1 mM vitamin C (VitC, started on day 0) plus the lactate dehydrogenase inhibitor GNE140 (1 μM, started on day 2), and 1 mM VitC plus the lactate dehydrogenase inhibitor NCI-737 (0.1 μM, started on day 2) for 7 days. (B) HLA-DR and (C) HLA-DP expression on DC treated for 7 days with D-2-HG, L-2-HG or 10 mM lactic acid was analyzed by flow cytometry. (D-G) Representative histograms of HLA-DP expression in DC treated with D-2-HG and inhibitors (1 mM Vit C, 1 μM GNE140) as indicated in the Figure. Numbers indicate median fluorescence intensity values. (H) HLA-DP (N=5), (I) HLA-DR (N=5), (J) CD1a (N=4) and (K) DC-SIGN (N=4) surface expression on DC cultured with D-2-HG and inhibitors (1 mM VitC, 1 μM GNE140) as indicated in the Figure and analyzed by flow cytometry. Symbols represent individual donors analyzed in independent experiments and horizontal bars mark median values. For multiple-group comparisons, one way analysis of variance and a *post-hoc* Dunnett, Friedman or Dunn test were performed. $P<0.05$ was considered statistically significant (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$). MFI: mean fluorescence intensity; GNE: GNE140.

was significantly lower in samples with lactate concentrations above 10 mM than in samples with lower lactate levels, irrespectively of D-2-HG treatment and culture time (median HLA-DP 315 *vs*. 728, HLA-DR 3,175 *vs*. 7,299, respectively) (Figure 4E, *Online Supplementary Figure S4C*). Additionally higher lactate concentrations strongly correlated with lower HLA-DP (r = -0.6105, *P*=0.0055) and HLA-DR (r = -0.7068, *P*=0.0005) expression (Figure 4F, *Online Supplementary Figure S4D*).

In summary, D-2-HG is a strong inducer of glycolytic ac-

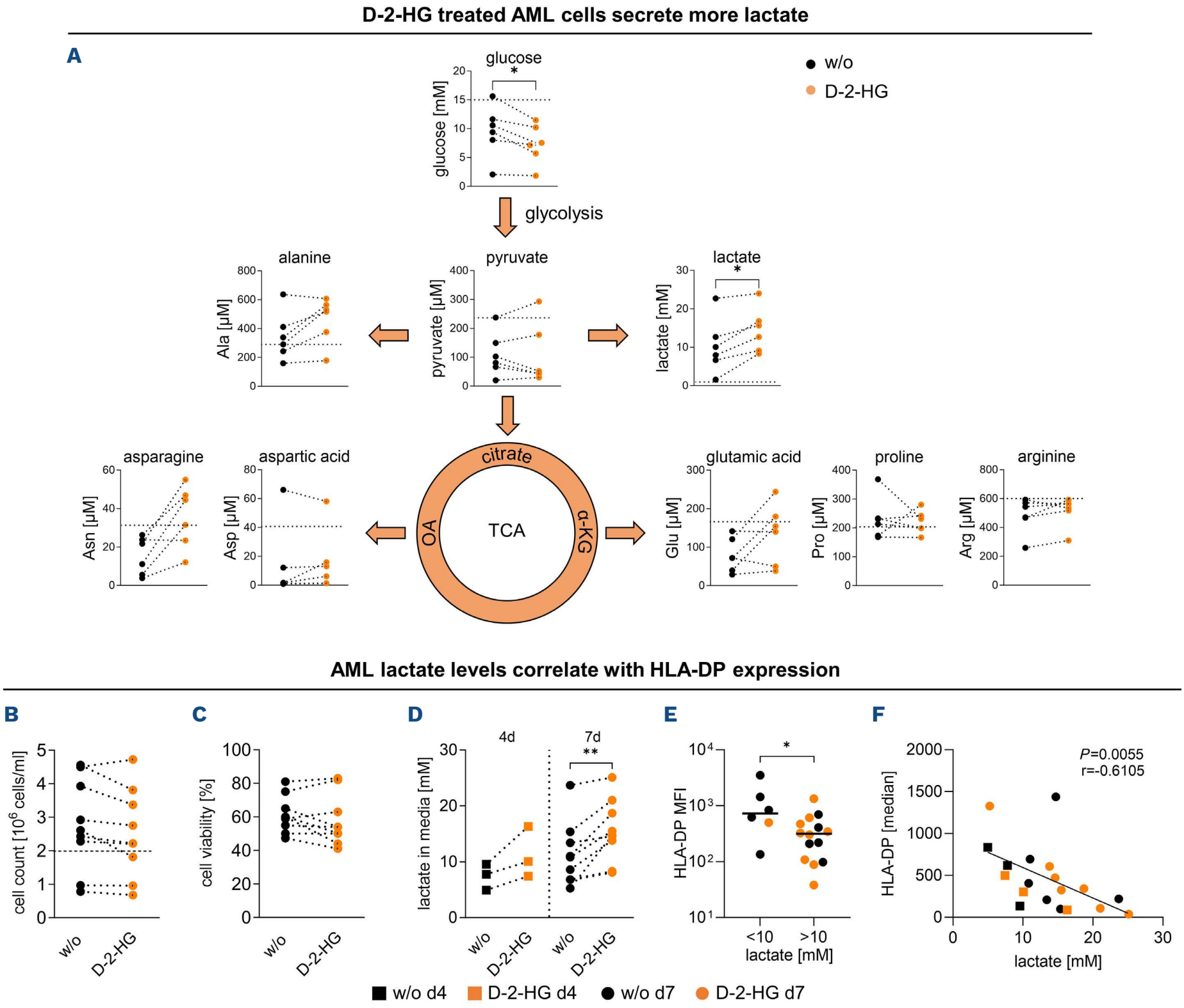

Figure 4. Primary acute myeloid leukemia blasts treated with exogenous D-2-hydroxyglutarate show altered metabolism and MHC class II expression. (A) Scatter plots showing concentrations of metabolites in supernatants of acute myeloid leukemia (AML) cells cultured with or without 20 mM D-2-hydroxyglutarate (D-2-HG) measured on day 7 of culture (N=6). Dashed lines indicate the respective concentrations in the culture medium without cells. (B) Cell yield (dashed line indicates starting cell counts) and (C) viability of AML blasts (N=9) after 7 days of culture with or without 20 mM D-2-HG. (D) Lactate levels (4 days, N=3; 7 days, N=9) of isocitrate dehydrogenase (IDH) wild-type AML cells cultured with or without 20 mM D-2-HG. (E) HLA-DP surface expression of IDH wild-type AML blasts cultured for either 4 or 7 days separated by lactate concentrations below and above 10 mM. (F) Correlations of lactate concentration and HLA-DP expression are shown. AML samples cultured without D-2-HG are displayed in black, samples treated with D-2-HG are shown in orange; samples after 4 days are shown as squares and those after 7 days as dots (<10 mM N=6, >10 mM N=14). For two-group comparisons a Wilcoxon test or Mann-Whitney U test was performed. *P*<0.05 was considered statistically significant (**P*<0.05, ***P*<0.01). (F) Spearman rank correlation coefficient was calculated. w/o: without; Ala: alanine; Asn: asparagine; Asp: aspartic acid; OA: oxaloacetate; TCA: tricarboxylic acid cycle; α-KG: α-ketoglutarate; Glu: glutamic acid; Pro: proline; Arg: arginine; MFI: mean fluorescence intensity.

tivity and lactate production not only in non-malignant myeloid cells but also in primary AML cells and it suppresses MHC class II expression.

IDH-mutated acute myeloid leukemia blasts accumulate 2-hydroxyglutarate, express lower HLA-DP levels and are less susceptible to T-cell-mediated lysis

To evaluate the impact of endogenously produced D-2-HG on MHC class II expression, we analyzed primary AML blasts expressing mutated or wild-type IDH. First, we determined the endogenous levels of 2-HG in patients' AML cells. As expected, 2-HG levels were significantly higher in IDH1- and IDH2-mutated blasts than in wild-type cells (wild-type 0.001 *vs*. IDH1-mutated 8.67 *vs*. IDH2-mutated 13.43) (Figure 5A). Analysis of The Cancer Genome Atlas RNA sequencing data revealed no significant differences between IDH-mutated and wild-type AML blasts regarding *HLA-DP, -DQ* and *-DR* α and β gene expression or the central regulator of *CIITA* (Figure 5B, C). However, on the protein level, expression of HLA-DP (10.5 *vs*. 1.0) was significantly lower in IDH-mutated blasts, while that of HLA-DQ (4 *vs*. 5.5) and HLA-DR (10 *vs*. 3.5) was not altered (Figure 5D). In line with the lower levels of surface HLA-DP, overall HLA-DP protein expression was diminished in AML blasts as analyzed by western blot (Figure 5E).

HLA-DP is an important allo-antigen that stimulates graft-*versus*-leukemia effects.[28] Frequent HLA-DP mismatches between patients and donors make HLA-DP targeted therapy clinically attractive for patients undergoing hematopoietic stem cell transplantation and we previously showed that HLA-DPB1-specific T cells are able to specifically target allogeneic mismatched antigens on AML blasts.[29] Hence, we performed a T-cell-mediated AML killing assay to study the immune escape of IDH-mutated HLA-DPB1*04:01$^+$ AML blasts using T cells expressing an HLA-DPB1*04:01-specific T-cell receptor upon RNA electroporation.[30] In line with the significantly decreased HLA-DP protein expression, HLA-DP-specific lysis of IDH mutated AML blasts by T-cell receptor-modified T cells was clearly reduced when compared to that of IDH wild-type AML blasts (Figure 5F, G).

Vitamin C impairs the survival of acute myeloid leukemia cells and a combination of vitamin C and GNE140 upregulates MHC class II expression

As GNE140 and vitamin C treatment partially rescued MHC class II expression on D-2-HG-treated DC, we analyzed whether GNE140 and vitamin C treatment would also increase MHC class II expression on primary AML cells. Culture of AML cells for 4 to 7 days, even without treatment, reduced cell survival, but vitamin C and co-treatment with GNE140 further diminished the number of living cells, especially in IDH-mutated AML (Figure 6A-C, *Online Supplementary Figure S5A-C*). HLA-DP expression of cultured living AML cells tended to be higher, and HLA-DR was significantly increased after 7 days of treatment with GNE140/vitamin C (Figure 6D, E). These data suggest that MHC class II expression is linked to glucose metabolism and lactate levels not only in non-malignant DC but also in AML cells.

Discussion

Mutated IDH1 and IDH2 enzymes convert α-KG to the structurally similar D-2-HG, which competitively inhibits several ketoglutarate-dependent dioxygenases and, thereby, limits histone and DNA demethylation, which is known to impair hematopoietic differentiation.[9,11] DNA methylation analysis during the differentiation of human monocytes to DC also revealed a delayed demethylation pattern in DC in the presence of D-2-HG. Vitamin C, a co-factor of Fe^{2+} and α-KG-dependent dioxygenases, is known to improve TET2 activity[31] and Gerecke *et al*. combined an IDH1 inhibitor with vitamin C to increase demethylation in HCT116 cells carrying a heterozygote IDH1 R132H mutation.[32] In our experiments, vitamin C could only partially revert the effect of D-2-HG on DC, which might be related to the D-2-HG-induced and persistent glycolytic phenotype with elevated MYC expression and lactate levels.

Beyond epigenetic modifications, several studies have demonstrated that exogenous or endogenous D-2-HG or L-2-HG has a strong impact on (tumor) cell metabolism. Both 2-HG enantiomers inhibited ATP synthase (complex V) in human glioblastoma and other neuronal cells.[33,34] In addition, primary AML blasts with *IDH1/2* mutations exhibited lower cytochrome *c* oxidase activity (complex IV).[35] In contrast, our own data showed that overexpression of IDH1 R132H and IDH2 R140Q resulted in a higher oxygen consumption rate. Furthermore, exogenous D-2-HG treatment also increased basal respiration of monocyte-derived DC. Similar results have been reported for primary human T cells, where 2-HG treatment shifted metabolism from aerobic glycolysis towards respiration,[36] indicating that non-transformed cells might react differently from malignant hematopoietic cells with an already altered metabolism. However, in a recently published study, *IDH1*-mutated AML cells also showed higher OXPHOS,[37] which has been described for other tumor entities harboring an *IDH1* mutation.[38] Besides OXPHOS, decreased glycolysis and downregulation of LDHB were demonstrated in the NOMO-1 leukemia cell line and patients' IDH wild-type AML cells after incubation with 2-HG, whereas in NB4 leukemia cells glycolysis was supported.[39] In glioma, LDHA seems to be silenced by promoter methylation in IDH-mutated tumors which in turn should result in limited glycolysis.[40] These results suggest that effects of 2-HG on OXPHOS and glycolysis are both context- and cell type-dependent.

Our own data show that exogenous D-2-HG promotes not only OXPHOS but also glycolysis. Accelerated glycolysis in an early phase of differentiation disturbed development from monocytes to DC and limited MHC class II expression. The metabolic shift to glycolysis and the related production of

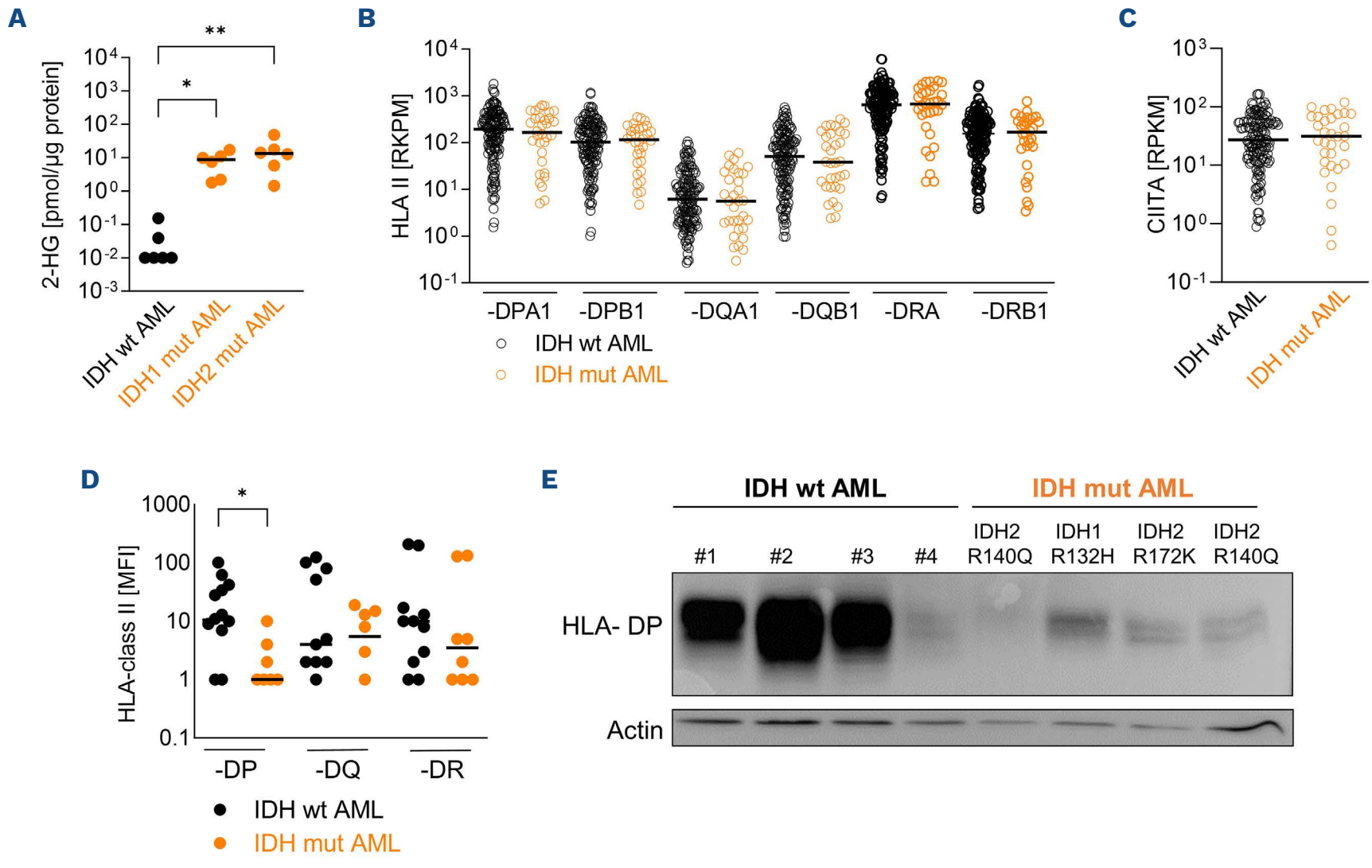

Figure 5. Endogenous D-2-hydroxyglutarate produced by mutated IDH reduces MHC class II protein expression in primary acute myeloid leukemia blasts. (A) Intracellular levels of 2-hydroxyglutarate (2-HG) were analyzed by liquid chromatography tandem mass spectrometry in acute myeloid leukemia (AML) blasts with wild-type isocitrate dehydrogenase (IDH) (N=6), mutated IDH1 (N=6) or mutated IDH2 (N=6). (B) Transcription levels of MHC class II α and β chain genes (*HLA-DP, -DQ* and *-DR*) of AML blasts expressing wild-type or mutated *IDH* were analyzed in RNA-sequencing data from The Cancer Genome Atlas (TCGA), including wild-type (N=155) and IDH-mutated (N=35) AML blasts from different patients at primary AML diagnosis. (C) *CIITA* expression data of AML exported from TCGA (for numbers see above). (D) Surface expression of HLA class II molecules (HLA-DP [wild-type N=14, mutated N=8], -DQ [wild-type N=11, mutated N=8] and –DR [wild-type N=11, mutated N=8]) on primary AML blasts expressing mutated or wild-type IDH analyzed by flow cytometry. (E) Total HLA-DP expression of AML blasts expressing mutated (N=4) or wild-type (N=4) IDH was evaluated by western blot analysis. Specific IDH mutations are depicted. (F) Primary HLA-DPB1*04:01^{+} AML blasts expressing wild-type (black symbols) or mutated IDH (orange symbols) were analyzed for their recognition by CD8 T cells expressing an HLA-DPB1*04:01-specific T-cell receptor upon RNA electroporation in a standard 5 h [^{51}Cr] release assay at

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the indicated effector (T cell)-to-target (AML blast) ratios. Specific lysis of IDH wild-type (N=3) and IDH2 mutated (N=3) AML blasts is shown. (G) As a control, CD8 T cells were electroporated without RNA (mock). (A-D) Symbols represent individual donors analyzed in independent experiments and horizontal bars mark median values. For two-group comparisons a Mann-Whitney test was used and for multiple-group comparisons a Kruskal-Wallis and *post-hoc* Dunn test were performed. *P*<0.05 was considered statistically significant (**P*<0.05, ***P*<0.01). wt: wild-type; mut: mutated; RKPM: reads per kilo base per million mapped reads; TCR: T-cell receptor; E:T: effector-to-target cell ratio.

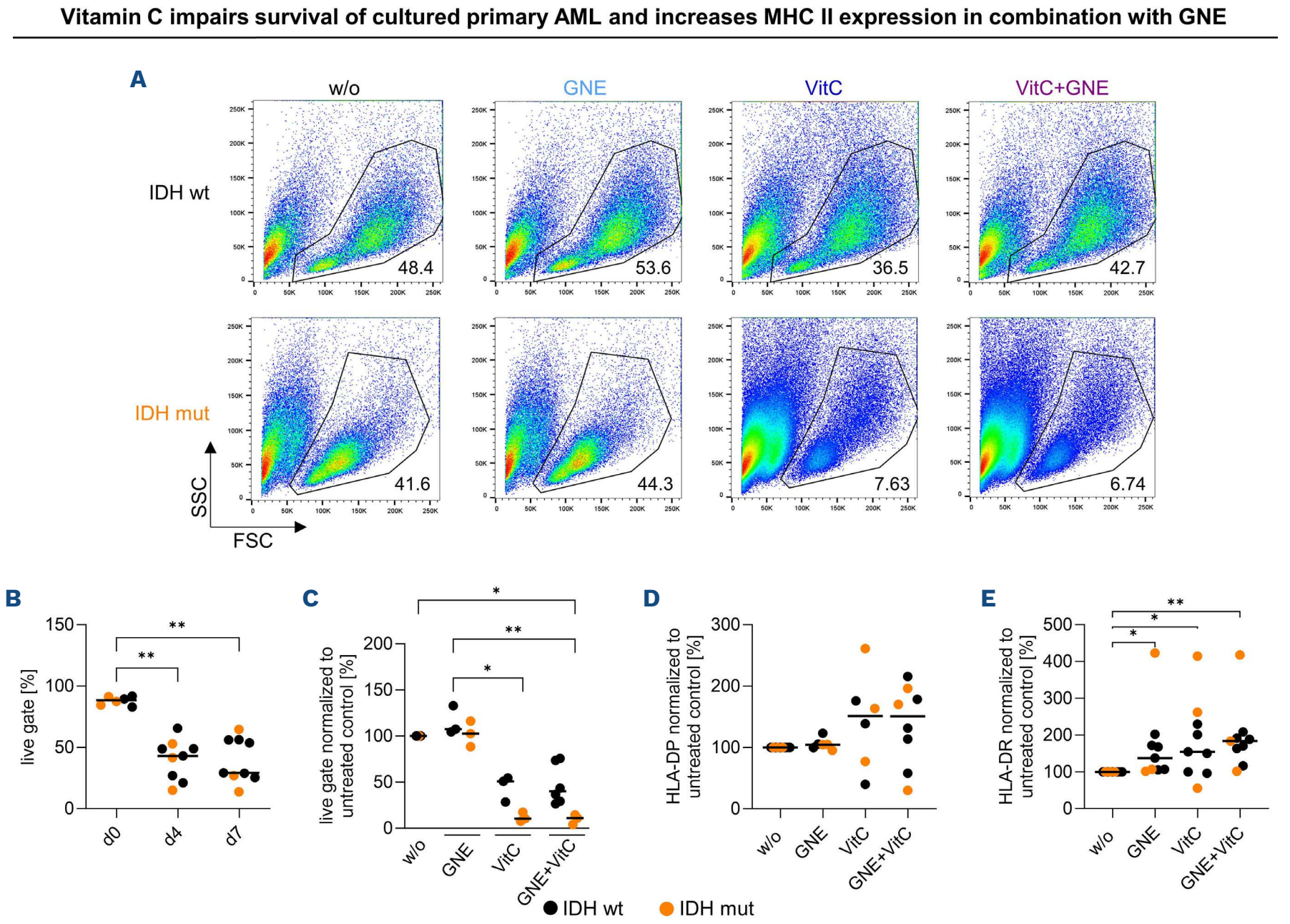

Figure 6. Vitamin C impairs survival of cultured primary acute myeloid leukemia cells and increases MHC class II expression in combination with GNE140. Primary cultured AML cells expressing wild-type (black) and mutant isocitrate dehydrogenase (IDH) (orange) were analyzed before (d0) and after 4 and 7 days of culture in the absence or presence of 2 mM vitamin C (VitC) and 1 μM GNE140, a lactate dehydrogenase inhibitor. (A) Representative FACS plots with live gating on primary cultured AML with or without treatment on day 7. (B) Summarized data on viability kinetics of untreated AML cells expressing wild-type (black) and mutant IDH (orange). (C) Percentage of living cells and (D) HLA-DP and (E) HLA-DR levels in the presence of 2 mM VitC and 1 μM GNE140 on day 7, normalized to the percentage of living, untreated cells. For multiple-group comparisons, a Kruskal-Wallis or Friedman test and *post-hoc* Dunn test were performed. *P*<0.05 was considered statistically significant (**P*<0.05, ***P*<0.01). wt: wild-type; mut: mutated; w/o: without; GNE: GNE140; VitC: vitamin C.

lactic acid could inhibit DC in a paracrine fashion, as lactic acid directly blocks DC differentiation.[27] However, in a later phase of DC differentiation, glycolysis seems to be of crucial importance and is a prerequisite for DC migration.[41] The balance between OXPHOS and glycolysis is under the control of transcription factors such as MYC or hypoxia inducible factor-1α (HIF-1α).[42] Interestingly, previous papers have already linked single nucleotide polymorphisms at 8q24.21 with MYC deregulation and greater risk of IDH-mutant glioma formation.[43]

O'Neill and colleagues found that endogenous 2-HG levels increased in lipopolysaccharide-activated murine macrophages and supported a highly glycolytic metabolic state through activation of the transcription factor HIF-1α.[44] In our experiments, DC expressed almost no HIF-1α (*data not shown*), but upregulated MYC after exposure to exogenous

D-2-HG, which was also accompanied by high glycolytic activity and low MHC class II expression. In line with this, tolerogenic DC induced by dexamethasone treatment showed higher MYC expression associated with elevated interleukin-10 secretion.[45] An interplay between MYC and 2-HG metabolism has been reported by Qiu and colleagues. Here, MYC induced the transcription of L/D-2-hydroxyglutarate dehydrogenase, which can degrade 2-HG.[46] Thus, it is tempting to speculate that MYC is increased by D-2-HG to reduce intracellular 2-HG levels and, thereby, limit the negative effects of 2-HG on DC. D-2-HG-treated DC upregulated MYC expression around day 4, suggesting that MYC could be responsible for LDHA induction and the observed accelerated glycolytic activity, as LDHA is a known c-MYC-responsive gene.[25] On the other hand, LDHA activity itself and concomitant acidification might induce MYC, given that a low pH value stabilizes the deubiquitinase ubiquitin carboxyl-terminal hydrolase 28 (USP28), which leads to deubiquitination of MYC and MYC protein stabilization.[47] However, applying different types of glycolytic inhibitors we could not consistently block the upregulation of MYC (*data not shown*).

The higher MYC levels in D-2-HG-treated DC may also partially explain the lower MHC class II expression, since MYC-overexpressing cells exhibit lower MHC class II expression, which also reduces immune recognition of B-cell lymphomas.[48] The relevance of 2-HG for regulation of MHC class II expression is underlined by our finding that AML blasts with mutated IDH and high endogenous levels of 2-HG exhibit low MHC class II expression. It is well known that solid tumors, as well as AML blasts, induce immune escape by downregulation of their MHC molecules[49] and expression of MHC class II molecules is lower in relapsed AML.[50] Our data may in part explain the downregulation of MHC class II expression in IDH-mutated AML blasts and other malignancies with D-2-HG accumulation, resulting in decreased T-cell recognition and immune control. In line, T-cell infiltration was improved by an IDH1 inhibitor in a glioma mouse model.[51] Recently, Friedrich *et al*. described D-2-HG-dependent activation of the kynurenine pathway and tryptophan degradation leading to re-education of tumor-infiltrating macrophages.[14] In lipopolysaccharide/interferon-stimulated human macrophages D-2-HG also reduced HLA-DR expression and an IDH inhibitor partially reversed low HLA-DR expression on macrophages in a murine model. Interestingly, similar results were obtained by Kadiyala *et al*. in an IDH1 R132H-mutated glioma mouse model in which IDH inhibitor treatment resulted in higher DC infiltration and upregulation of MHC class II molecules.[52] IDH-related D-2-HG production seems to represent a metabolic strategy to lower MHC class II expression and suppress the anti-tumor response.

The expression of MHC class II molecules is not only important for recognition of solid tumors, but also necessary for immune control of AML. Previous studies analyzing the impact of HLA-DP mismatch constellations between patients and donors demonstrated an important role of HLA-DP for the graft-*versus*-leukemia effect but also its role in immune escape.[28,29,50,53,54]

When testing allo-HLA-DP β chain-specific T cells with primary AML blasts, we observed low HLA-DP expression and poor T-cell recognition in a group of patients with mutated IDH. Further studies revealed that exogenous D-2-HG also stimulated glycolysis and reduced MHC class II expression in wild-type AML cells. However, in contrast to monocytes and monocyte-derived DC, the basal glycolytic activity was much higher in AML blasts. Enhanced glycolysis is a common feature in AML and a predictor of poor prognosis.[55] Glycolysis also leads to leukemia-derived lactic acid secretion, which interferes with T-cell activity and proliferation.[56] Therefore, targeting glycolysis and acidification is discussed as a novel strategy not only for AML, as altered glucose metabolism is closely associated with therapeutic resistance.

We tested whether glycolytic inhibition could counteract the effects of D-2-HG and rescue MHC class II expression during differentiation of monocytes to DC and in AML blasts. As expected, the LDH inhibitor GNE140 reduced lactate secretion and supported MHC class II, CD1a and DC-SIGN expression in DC and MHC class II expression in primary AML blasts. Hence, the effect of D-2-HG on the epigenetic landscape of differentiating monocytes likely contributes to the altered phenotype observed. Epigenetic modulators, histone deacetylase inhibitors, and DNA methyltransferase inhibitors can increase MHC class II expression on ovarian cancer cells.[57] As vitamin C partly rescued the of D-2-HG effects on DNA methylation and normalized respiration, we combined vitamin C with the LDH inhibitor GNE140.

The co-treatment with vitamin C and GNE140 had positive effects on CD1a and MHC class II expression in DC, but did not fully normalize their levels of expression. Similar results were obtained with another LDH inhibitor (NCI-737) that targets both LDHA and LDHB in DC. In addition, vitamin C and GNE140 administration supported MHC class II expression in AML cells. However, cell count was severely diminished by vitamin C. Similar data have shown that pharmacological concentrations of vitamin C kill cancer cells but not normal cells.[58] DNA methyltransferase inhibitors might further support MHC class II expression, and thereby improve antitumor immunity in AML with *IDH* mutations. As IDH inhibitors have been shown to limit therapy response to radiotherapy, PARP inhibitors or chemotherapy, it might be a new treatment option to target 2-HG-induced metabolic alterations in IDH-mutated tumor cells instead of using IDH inhibition.[59,60]

In summary, exogenous D-2-HG inhibits the differentiation of DC and induces a tolerogenic phenotype with low MHC class II expression as a result of epigenetic and metabolic reprogramming. A similar phenotype was found in primary AML blasts with mutated *IDH* and endogenous D-2-HG production. The combination of LDH inhibitors with the antioxidant/epigenetic modulator vitamin C could partially rescue the effect of D-2-HG in DC and supported MHC class II expression in AML blasts. We suggest a combina-

tion of anti-glycolytic drugs with epigenetic modulators or chemotherapy for the treatment of both solid tumors and leukemia that accumulate D-2-HG in order to stimulate the immune response and prevent immune escape.

Disclosures

No conflicts of interest to disclose.

Contributions

MK, ST, MR, and KR conceived the study. KR was responsible for the methodology. KH, MA, FV, RS, NB, KM, ZECC, HS, SI, KD, RSB, and FE conducted the investigations. KH, NB, KM, SI, HS, SMD, and KR were responsible for the visualization. MK and ST acquired funds and MK was the project administrator. MK, KR, ST, MR, and KD supervised the study. MK, ST, and KH wrote the original draft of the paper. MK, NB, ST, MR, KR, KH, KM, ZECC, CB, KD, PJO, PJS, WH, and CM contributed to writing, revising and editing the manuscript.

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Data-sharing statement

Data are available on reasonable request. All data relevant to the study are included in the article or uploaded as Online Supplementary Information.

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