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Obesity Disrupts H3K4me3-Mediated Lactate Accumulation and Efferocytosis in Hypoxic Macrophages

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ABSTRACT

Dysregulated macrophage function drives the development of obesity-associated pathologies. While macrophages adapt to their surrounding environment to maintain tissue homeostasis, the impact of obesity on macrophage adaptation to low oxygen levels remains elusive. Here, we show that hypoxia rapidly increases histone 3 lysine-4 trimethylation (H3K4me3) in bone marrow-derived macrophages (BMDMs) and that this response is impaired in BMDMs from high-fat diet (HFD)-induced obese mice, which significantly affected the expression of genes involved in metabolic pathways, resulting in decreased lactate accumulation, histone lactylation, and expression of genes involved in the maintenance of metabolic homeostasis. Moreover, altered adaptation to hypoxia in BMDMs from HFD mice led to a decreased efferocytosis capacity under hypoxia, which was reversed by supplementation with glucose or lactate. Serial bone marrow transplantation indicated that the maladapted hypoxia response for efferocytosis was imprinted in macrophage precursors in the bone marrow of HFD mice. In BMDMs, genetic disruption of the H3K4me3 demethylase KDM5A further enhances hypoxia-induced H3K4me3 and gene expression, along with lactate accumulation. In a dorsal skin biopsy model, while extracellular lactate levels decreased immediately after wounding but sharply increased in the early phase in normal mice, whereas lactate levels remained low in HFD mice, resulting in delayed wound healing. Our findings suggest that metabolic adaptation to hypoxia involves H3K4me3 and lactate accumulation in macrophages to perform efferocytosis under hypoxic conditions. Diet-induced obesity disrupts this pathway, resulting in impaired efferocytosis and delayed healing, with implications for altered macrophage functions in pathologies associated with obesity.

1 | Introduction

Obesity is a major health problem that leads to chronic diseases, such as type 2 diabetes mellitus and coronary heart disease [1, 2]. These obesity-associated complications are driven not only by excess adiposity but also by additional pathogenic mechanisms, including chronic low-grade inflammation and altered macrophage function. In obesity, macrophages exhibit an accumulation of pro-inflammatory phenotypes in adipose tissue

[3, 4] and reduced capacity of phagocytosis and efferocytosis in wound tissue macrophages [5], contributing to impaired resolution of inflammation and delayed tissue repair.

Macrophages are highly plastic cells that normally adapt their phenotype and function in response to local environmental cues. Hypoxia is a key regulator of important physiological and pathological processes [6]. Under homeostatic conditions, cells in most organs reside in an environment with lower oxygen

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levels (5%–12% O₂) [7] than ambient atmospheric conditions (21% O₂) [8, 9]. During pathological states such as ischemia or chronic inflammation, cells are exposed to hypoxia (1% O₂) due to vascular disruption and increased oxygen consumption. Dysregulated hypoxia sensing pathways in macrophages have been found in obesity [10] and other obesity-associated conditions, such as atherosclerosis [11], suggesting impaired adaptation to hypoxia microenvironments.

Cellular responses to hypoxia are primarily mediated by hypoxia-inducible factors (HIFs), transcription factors activated through stabilization of oxygen-sensitive enzymes that regulate gene programs enabling adaptation to low-oxygen environments [12, 13]. Recent studies have identified that hypoxia-responsive transcriptional programs are influenced by oxygen-sensitive histone modifications [14–17]. In particular, hypoxia induces histone hypermethylation and upregulates gene expression through increased histone 3-lysine 4 trimethylation (H3K4me3) [18]. However, whether hypoxia-associated H3K4me3 contributes to macrophage hypoxic responses remains unknown. Notably, H3K4me3 has also been implicated in long-term innate immune memory in a mouse model of hypercholesterolemia, in which epigenetic dysregulation spans from macrophage precursor cells in the bone marrow to differentiated macrophages [19].

The clearance of apoptotic cells by macrophages, or efferocytosis, is a key phagocytic process that promotes inflammation resolution and tissue repair [20]. In obesity, impaired efferocytosis in wound macrophages contributes to persistent non-resolution [5]. Prolonged hypoxia primes macrophages to activate glycolytic gene programs associated with enhanced efferocytosis capacity [21], as glycolysis and lactate production are essential for maintaining efficient efferocytotic function [22]. Lactate further promotes the proliferation of efferocytotic macrophages to amplify pro-resolution programs [22, 23], and hypoxia-induced lactate drives histone lactylation of pro-resolution genes, providing an epigenetic mechanism linking metabolism to macrophage functional adaptation [24].

Here, we studied the role of H3K4me3 in bone marrow-derived macrophages (BMDMs) using a diet-induced obesity mouse model fed a high-fat diet (HFD), which exhibits significant obesity, insulin resistance, and impaired wound healing characterized by non-resolution [25]. Our results identify H3K4me3 as a key regulator of macrophage adaptation to hypoxic environments, promoting lactate accumulation, histone lactylation, and efferocytosis. Diet-induced obesity disrupts this H3K4me3-mediated hypoxia response, resulting in impaired lactate accumulation and delayed wound healing.

2 | Materials and Methods

2.1 | Animals

Animal protocols used for experiments were approved by the Institutional Animal Care and Use Committees of Upstate Medical University and University of Illinois at Chicago (UIC), and animals were cared for according to the National Institutes of Health (NIH) guidelines for the care and use of laboratory

animals under protocol numbers #468 and #474 at Upstate and #17–161 at UIC. All animals were group-housed in a temperature-controlled facility with a 12-h light/dark cycle. C57BL/6J male mice (strain #:000664) at the age 3–4 weeks were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and fed a high-fat diet (HFD, 60% kcal fat, Research Diets Inc., New Brunswick, NJ, USA, D12492) or low-fat control diet (LFD, 10% kcal fat, Research Diet Inc., D12450B) with the same protein content as the HFD for 16–20 weeks. EGFP mice (C57BL/6-Tg(CAG-EGFP)131Osb/LeySopJ, Strain #:006567) were purchased from the Jackson Laboratory, and house-bred male mice of similar age (within 1 month) fed HFD or LFD for 20 weeks were used as donors for bone marrow transplantation. All comparisons between HFD- and LFD-fed mice were made strictly within the same cohort of animals. Eight-week-old male C57BL/6J mice were used as recipients for bone marrow transplantation. Tamoxifen-inducible *Kdm5a* knockout mice, cross-bred B6.129S6(Cg)-Kdm5a^{tm1Kael}/J (RRID:IMSR_JAX:008571) with B6.129-Gt(ROSA)26Sor^{tm1(cre/ERT2)Tyj}/J (RRID:IMSR_JAX:008463), were house bred from breeders given by the laboratory of Dr. William Kaelin Jr. Gene knockout was induced by intraperitoneal tamoxifen (75 mg/kg body weight) once every 24 h for five consecutive days. Genotyping was performed by Transnetyx. All comparisons between Cre + and Cre- mice were made strictly within the littermates.

2.2 | Cells

Bone marrow-derived macrophages (BMDMs) were isolated and cultured as previously described [26], with minor modifications. Briefly, bone marrow (BM) cells were collected from the femur and tibia bones and centrifuged at 10000×g for 15 s. After resuspension in culture media, cells were seeded in plates and cultured in RPMI medium with 10% heat-inactivated FBS (ThermoFisher, Waltham MA, USA) and 20 ng/mL recombinant mouse macrophage colony-stimulating factor (M-CSF, PeproTech, Cranbury, NJ, USA, 315–02). After 3 days of incubation at 37°C with 5% CO₂, the medium was replaced with fresh medium containing M-CSF. Cells were allowed to differentiate into macrophages for a total of 7 days and then harvested by removing the supernatant. BMDMs were exposed to 1% hypoxia in a hypoxia chamber (BioSpherix, C-174 and ProOx C21, Parish, NY, USA) for 1 or 24 h or cultured in a normoxic environment before harvesting or performing assays.

Bone marrow-derived dendritic cells were generated by culturing bone marrow progenitors in RPMI medium supplemented with 10% FBS and 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF, PeproTech) for 10 days.

2.3 | Immunofluorescence Imaging

For immunofluorescence imaging, 1.2×10⁵ BMDMs were cultured on a chamber slide (Thermo Fisher Scientific) for 24 h and then exposed to hypoxia for Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 10 min, permeabilized with 0.2% Triton X-100 (Millipore Sigma, Burlington, MA, USA) for 10 min, blocked with 1% donkey serum (Jackson ImmunoResearch Inc., West Grove, PA, USA)

for 30 min, and incubated with rabbit anti-H3K4me3 antibody (Abcam, ab8580, Cambridge, UK) overnight. The cells were then washed with PBS and incubated with 1:1000 anti-rabbit Alexa Fluor 647-conjugated secondary antibody (Thermo Fisher Scientific) for 30 min. After washing with PBS, the cells were stained with Hoechst 33258 (Millipore Sigma). Images were obtained using an automated cell imaging system (ImageXpress Pico, Molecular Devices, San Jose, CA, USA), and high-content imaging analysis was performed to count the cells and quantify the signal intensity using CellReporterXpress (Molecular Devices).

2.4 | ChIP-Seq Library Preparation and Sequencing

ChIP-seq experiments were performed using a rabbit anti-H3K4me3 antibody (Abcam) in BMDMs derived from HFD or LFD mice. We prepared BMDMs from at least three mice, and BMDMs from each mouse were divided into hypoxia or normoxia groups to assess the response to hypoxia in BMDMs derived from the identical mouse. After incubation in 1% hypoxia or normoxia for 1 h, the BMDMs were crosslinked with 0.4% paraformaldehyde for 10 min at room temperature (RT) and immediately quenched with 0.125 M glycine (Millipore Sigma) for 5 min at RT. For the hypoxia treatment group, samples were processed in a large hypoxia chamber (BioSpherix, C-Shuttle and ProOx P360) to maintain the hypoxic environment during the procedures. Then, the cells were quickly washed with PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF, Millipore Sigma) and cOmplete EDTA-free protease inhibitor cocktail (Millipore Sigma) and were resuspended in 500 μ L chilled nuclei prep buffer and incubated on ice for 5 min. 2×10^6 BMDMs were sonicated using a Covaris M220 sonicator (Covaris, Woburn, MA, USA) at a high-power setting (peak power: 150, duty factor: 20, cycles: 200) for 330 s, the setting we had determined in preliminary experiments to yield a modal fragment size of 150 bp. After incubation with 1 μ g H3K4me3 antibody at 4°C overnight, ChIP-DNA was prepared using ZymoMag Protein A beads (Zymo Research), and immunoprecipitated chromatin samples were prepared using the Zymo-Spin ChIP kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Input samples were prepared using 10% chromatin samples. ChIP and input libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). Libraries were sequenced on an Illumina NextSeq500 (Illumina, San Diego, CA, USA) with a High Output NextSeq kit (Illumina) using 2×75 bp paired-end reads.

2.5 | ChIP-Seq Data Processing

The FASTQ files were processed using PartekFlow (Partek Inc., St. Louis, MO, USA). Briefly, the raw reads were aligned to the mouse genome annotation mm10 using Bowtie2. Peaks were called using the MACS2 peak caller in narrow peak mode, with the IP sample bam files set as treatment and the corresponding input bam files set as control [27]. The called peaks were quantified to regions and normalized, and the normalized peaks were then subjected to statistical analysis using the Partek

gene-specific analysis (GSA) algorithm. Differential H3K4me3 peaks were identified using thresholds ($p < 0.05$) and screened based on size (least square [LS] mean > 20). Promoter peaks were defined as ± 3 kb from the nearest transcription start site (TSS), intergenic peaks were defined as > 3 kb upstream of the nearest TSS and > 3 kb of the nearest transcription end site (TES), and gene body peaks were defined as all peaks not identified as promoter or intergenic peaks.

To visualize the coverage tracks, bam files were merged using Samtools merge, and the merged bam files were normalized with counts per million (CPM), followed by conversion to bed-graph files with bamCoverage. Merged bedgraph files were visualized using the IGV genome browser.

Pathway enrichment analysis was performed using the Molecular Signatures Database (MSigDB) online tool provided by Gene Set Enrichment Analysis (GSEA) with hallmark genes and < 0.05 p -value cut-off [28, 29]. ChIP-sequencing data were deposited under the NCBI GEO accession number GSE231991.

2.6 | RNA Isolation

Cells were lysed with TRI Reagent (Zymo Research), and total RNA was extracted using the Direct-zol RNA Miniprep Kit (Zymo Research) according to the manufacturer's instructions. RNA quantity was evaluated using QubitFlex and Qubit broad-range RNA assay kits (ThermoFisher). The quality of the RNA samples was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies), and all samples showed an RNA integrity number of > 9 .

2.7 | RNA-Seq

RNA sequencing libraries were prepared using the Illumina Stranded Total RNA Prep with Ribo-Zero Plus (Illumina, triplicate per condition). Once prepared, the indexed cDNA libraries were pooled in equimolar amounts and sequenced with single-end 75 bp reads on an Illumina NextSeq500.

The FASTQ files obtained from RNA-seq and GSE192969 were processed using PartekFlow (Partek Inc., St. Louis, MO, USA). In brief, the raw reads were aligned using STAR and the aligned reads were quantified to the annotation model through a quantification algorithm, Partek E/M. Normalization was performed using the median ratio through PartekFlow. The normalized counts were subjected to statistical analyses using GSA. Differentially expressed genes (DEGs) were identified using the following thresholds ($p < 0.05$, fold change < -2.0 or $2.0 < \text{fold change}$) [28, 30]. Gene ontology (GO) analysis for differential peaks and differentially expressed genes was performed using the Molecular Signatures Database (MSigDB, <https://www.gsea-msigdb.org/gsea/index.jsp>) [22, 23] and Ingenuity Pathway Analysis (QIAGEN, Germantown MD, US). Statistical significance was set at $p < 0.05$. Additional analysis and visualization of extended figures were performed using nf-core/rnaseq on Pluto (<https://pluto.bio>). Part of the data were analyzed and visualized using RNA-sequencing data deposited under NCBI GEO accession GSE231992.

2.8 | Hematopoietic Stem and Progenitor Cell Isolation

Bones from LFD or HFD mice or EGFP (C57BL/6-Tg(CAG-EGFP)131Osb/LeySopJ, Jackson Laboratory) were dissected, including femur, tibia, iliac, and brachial bones from the legs and arms. BM cells were collected either by centrifugation at $10000 \times g$ for 15 s or by flushing with FACS buffer and purified by Ficoll separation with Histopaque-1119 (Millipore Sigma). Cells were stained with unconjugated rat anti-mouse lineage-specific antibodies (Ter-119, Mac1, Gr-1, B220, CD5, CD3, CD4, CD8, and CD127; BioLegend, San Diego, CA, USA), followed by incubation with Dynabeads anti-rat IgG (Thermo Fisher). Unbound cells, including HSPCs, were collected after magnetic sorting. Cells were then stained with goat anti-rat PE-Cy5 secondary antibody (Thermo Fisher) for lineage-specific primary antibodies and c-kit-APCeFluor780 (Thermo Fisher), Sca1-PB (BioLegend), and CD150-PE (BioLegend) antibodies. Cell sorting was performed using a FACS AriaIII cell sorter (BD Biosciences). Data analysis was performed using the FlowJo v10.8 software (BD). In the cyclosporin A-treated group, HSPCs were isolated using buffers containing $50 \mu\text{g/mL}$. The sorted cells were collected in RPMI supplemented with 30% FBS for further analysis and downstream application.

2.9 | Quantitative RT-PCR

RNA (1000 ng) was reverse transcribed using iScript (BioRad, Hercules, CA, USA), and qPCR was performed in duplicate using iTaq Universal SYBR Green Supermix (BioRad). Ct values were obtained using a CFX Opus 384 Real-Time PCR System (BioRad), and data were generated using the comparative threshold cycle (Delta CT) method by normalizing to hypoxanthine phosphoribosyltransferase (*Hprt*).

2.10 | Immunoblots

BMDMs were lysed with M-PER Mammalian Protein Extraction Reagent (Thermo Fisher) containing cOmplete Mini Protease Inhibitor Cocktail (Millipore Sigma) according to the manufacturer's instructions. The lysate was centrifuged for 10 min at $14000 \times g$ and 4°C , and the supernatant was collected. For histone extraction, BMDMs were washed with pre-warmed serum-free RPMI and lysed with ice-cold extraction buffer (Active Motif, Carlsbad, CA, USA) with a cOmplete mini protease inhibitor cocktail (Millipore Sigma). Cells were homogenized by vigorous pipetting, and the cell lysate was centrifuged to obtain crude histones. A neutralization buffer (Active Motif) containing 0.1 M DTT (Millipore Sigma) and a Halt protease inhibitor cocktail (Thermo Fisher) was added to the crude histones. Protein concentration was quantified using Qubit Protein and Protein Broad Range (BR) Assay Kits (Thermo Fisher). $25 \mu\text{g}$ of protein lysates were denatured at 95°C for 5 min in $4\times$ Laemmli Sample Buffer (BioRad) containing β -Mercaptoethanol (Millipore Sigma). Protein samples were separated on 4%–15% SDS-PAGE gels (BioRad) at 100 V and electro-transferred to $0.45 \mu\text{m}$ nitrocellulose membranes (BioRad) at 50 V for 85 min (for cytoplasmic protein) or to

$0.2 \mu\text{m}$ nitrocellulose membranes (BioRad) at 30 V for 70 min (for histones). The membranes were blocked with EveryBlot Blocking Buffer (BioRad) for 5 min and incubated for 1 h at room temperature with primary antibodies. The appropriate HRP-conjugated secondary antibodies were used for chemiluminescent detection of proteins using Clarity Max Western ECL Substrate (BioRad). Membranes were scanned with a ChemiDoc imaging system (BioRad) and quantified using Image Lab 6.1.0 software (BioRad). The following primary antibodies were used for immunoblotting: Hif1 α (36169, Cell Signaling), Arg1 (GTX109242, GeneTex), β -actin (3700, Cell Signaling), H3K4me3 (ab8580, Abcam), H3K9me3 (13969, Cell Signaling), H3K27me3 (9733, Cell Signaling), H3K18la (PTM-1406, PTM BIOLABS), and total H3 (3638, Cell Signaling). The following secondary antibodies were used for immunoblotting: anti-rabbit HRP-linked IgG antibody (5196–2504, BioRad) and anti-mouse HRP-linked IgG antibody (7076, Cell Signaling).

2.11 | Induction of Apoptosis in Neutrophils and Jurkat Cells

Murine neutrophils were isolated using a density gradient centrifugation-based protocol, as previously described [31]. Briefly, bone marrow cells harvested from mouse femurs and tibias were centrifuged using Histopaque 1119 (Millipore Sigma) and Histopaque 1077 (Millipore Sigma). Neutrophils were collected at the interface of Histopaque 1119 and 1077. A mixture of early/late apoptosis and necrotic death was induced by irradiating Jurkat cells and isolated neutrophils under a UV lamp (254 nm, Stratalinker UV 1800, Stratagene Corporation, La Jolla, CA, USA) for 1 and 15 min, respectively. The cells were incubated in a CO_2 incubator for 3 h. This method routinely yielded greater than 80% Annexin V-positive cells, including early- and late-stage apoptosis.

2.12 | Efferocytosis Assay

BMDMs were plated in a 96-well plate at a density of 0.4×10^5 cells per well and exposed to hypoxia or normoxia for 24 h prior to the assay. A mixture of early and late apoptotic neutrophils or Jurkat cells was stained with CellTracker Red CMTX Dye (ThermoFisher) by incubation for 20 min, and BMDMs were stained with carboxyfluorescein succinimidyl ester (CFSE, ThermoFisher) by incubation for 20 min. Labeled dying cells were added to BMDMs at a 5:1 ratio and co-cultured for 1 h, followed by vigorous washing three times with $1\times$ PBS to remove dying cells. Cells were fixed with 1% paraformaldehyde (Electron Microscopy Sciences) and imaged using an automated cell imaging system (ImageXpress Pico; Molecular Devices). Analysis was performed to count CellTrackerRed⁺/CFSE⁺ BMDMs using CellReporterXpress software (Molecular Devices).

For live cell imaging, apoptotic cells were stained with $1.3 \mu\text{M}$ pHrodo Green STP Ester (Thermo Fisher) or CypHer5e NHS ester (Cyntiva) by incubating for 45 min at 37°C after apoptosis induction. Labeled dying cells were added to BMDMs at a 5:1 ratio, and cells were imaged using Image Xpress Pico in the

environmental control unit, which introduces normoxia or 1% hypoxia at 37°C and 5% CO₂ environment. Images were captured every 10 min for up to 6 h using ImageXpress Pico. The signal intensity was measured using CellReporterXpress.

For the continual efferocytosis assay, the first round of dying cells was labeled with CellVue Claret Far Red (Millipore Sigma) and co-cultured with macrophages at a 1:1 ratio for 45 min. Apoptotic cells were then washed away using cold assay media. Macrophages were subsequently rested in a CO₂ incubator for 2 h prior to co-culture with a second round of dying cells that were labeled with pHrodo Green. Continual efferocytosis was assessed by live cell imaging as CellVueClaret⁺ (1st dying cell uptake) and CellVueClaret⁺ pHrodoGreen⁺ (2nd dying cell uptake) in BMDMs. Images were captured every 10 min for up to 2 h using ImageXpress Pico. The signal intensity was measured using CellReporterXpress.

2.13 | Skin Biopsy

We used male adult mice (8–12 week-old) fed a normal chow diet or HFD (D12492, Research Diet) and L-NAME treatment was carried out for 15 weeks according to a previous study. L-NAME was purchased from Sigma-Aldrich or Cayman Chemical and dissolved in drinking water (0.5 g/L). At the end of the study, the mice were subjected to dorsal skin biopsies. Dorsal skin biopsies were performed as previously described [32, 33]. Two 8 mm diameter full-thickness excisional skin wounds were created on the back of each mouse using a dermal biopsy punch under anesthesia and analgesia. The wounds were covered with Tegaderm (3 M) to keep them moist until day 7 after wounding. At indicated time points, mice were euthanized by isoflurane asphyxiation and bilateral thoracotomy, and full-thickness wound tissues were collected from mice at specified time points for downstream analysis. For histology, the samples were fixed in 10% neutral buffered formalin and processed using standard protocols for paraffin embedding. Sections were cut at 0.7 µm thickness and mounted on glass slides for histological analysis. Three staining methods were employed: Hematoxylin and Eosin (H&E) for general tissue morphology, Masson's trichrome for collagen and connective tissue visualization, and Azan-Mallory for detailed differentiation of connective tissue components. Following staining, the slides were examined under a light microscope to assess wound healing parameters, including granulation tissue formation and collagen deposition. All staining procedures were performed in parallel with the appropriate controls to ensure reproducibility and specificity.

2.14 | Lactate Measurement

Intracellular lactate concentration was quantified using Lactate-Glo Assay (Promega, Madison, WI, USA) after lysing 50000 BMDMs with 0.6 N HCl (Millipore Sigma) containing 0.16% Dodecyl trimethylammonium bromide (Millipore Sigma) according to the manufacturer's instructions. Luminescence was measured using a Synergy H1 plate reader (Agilent Technologies). For wounds, tissue was excised using a punch biopsy stick and non-enzymatically dissociated at 4°C. The

finely minced tissue in DPBS by scissors was ground by one cycle of the soft tissue protocol on TissueGrinder (Fast Forward Discoveries). Cells and solid tissue were removed by centrifugation, and the cell-free supernatant was collected for lactate measurement using the Lactate-Glo Assay (Promega).

2.15 | Analysis of Cellular Metabolic Activity

BMDMs were seeded into XFe96 Microplates (Agilent Technologies, Santa Clara, CA, USA). After 24-h culture, the medium was changed to Seahorse XF base medium (Agilent Technologies) supplemented with 10 mM glucose, 2 mM glutamine, and 1 mM sodium pyruvate, and the BMDMs were incubated for 1 h in a no CO₂ incubator. The extracellular acidification rate (ECAR) was measured using a Seahorse XF96e analyzer (Agilent Technologies) under basal conditions and after the administration of mitochondrial inhibitors (0.5 µM rotenone/antimycin A, Agilent Technologies) and 2-deoxy-d-glucose (50 mM 2-DG, Agilent Technologies) as a glycolysis inhibitor. The glycolytic proton efflux rate (PER) was calculated using WAVE software (Agilent Technologies). The oxygen consumption rate (OCR) was measured under basal conditions and after the administration of mitochondrial inhibitors 1.5 µM oligomycin, 1.0 µM FCCP, and 0.5 µM rotenone/antimycin A (Agilent Technologies).

2.16 | Cytokine Production Measurement

To measure cytokine production from BMDMs, cell culture media was collected and centrifuged at 800 × g for 10 min followed by cytokine quantification of the supernatant by Luminex Discovery Assay (Bio-Techne). Data was acquired on a MAGPIX instrument (ThermoFisher), and cytokine concentrations were calculated using xPONENT software (Diasorin).

2.17 | Competitive Bone Marrow Transplantation

For competitive transplantation assays, 8–12-week-old C57BL/6 recipient mice were lethally irradiated (9 Gy in one dose) and retroorbitally injected with 5000 purified SLAM HSPCs (EGFP⁺) and 100000 whole BM cells obtained from C57BL/6 mice (EGFP[−]) as competitors. Peripheral blood was collected by retroorbital bleeding at 8 and 16 weeks after transplantation and stained with specific antibodies for flow cytometry analysis. For serial transplantation, HSPCs were isolated 16 weeks after the first transplantation. Lethally irradiated 8–12-week-old C57BL/6 recipient mice were injected with 3000 HSPCs (EGFP⁺) together with 100000 whole BM cells obtained from C57BL/6 mice (EGFP[−]) as competitors. BMDMs were isolated 16 weeks after 2nd transplantation, and a dying cell clearance assay was performed with the pH-sensitive dye CypHer5E NHS Ester (1 µM, Cytiva, Marlborough, MA, USA) or without hypoxia treatment.

2.18 | Data Availability

The GEO accession number for ChIP-seq of BMDMs is GSE231991, and that for RNA-seq of BMDMs is GSE231992.

The GEO accession number for RNA-seq of immortalized macrophages has been published previously (GSE192969) [21].

3 | Results

3.1 | Diet-Induced Obesity Disrupts the Rapid Increase in H3K4me3 in Macrophages in Response to Hypoxia

Histone methylation can be induced by hypoxia in an acute manner in multiple cell types [16]. To test whether macrophages also experience hypoxia-induced histone methylation, we used primary murine macrophages differentiated from macrophage precursors in the bone marrow under macrophage colony-stimulating factor (M-CSF). These fully differentiated BMDMs were subjected to brief (1 h) exposure to hypoxia (1% O₂) and analyzed for global levels of trimethylation on lysine 4 of histone H3 (H3K4me3), lysine 9 of histone H3 (H3K9me3), and lysine 27 of histone 3 (H3K27me3). In response to hypoxia, control BMDMs obtained from mice fed a control low-fat diet (LFD-BMDMs) showed a significant increase in the global levels of these methylation marks (Figure 1a). An increase in global H3K4me3 levels was confirmed using immunofluorescence high-content imaging (Figure 1b). In BMDMs obtained from mice fed a high-fat diet (HFD-BMDMs), hypoxia failed to induce H3K4me3 enrichment, whereas H3K9me3 and H3K27me3 enrichment were comparable to those in LFD-BMDMs (Figure 1a). High-content imaging also recapitulated the impaired H3K4me3 response to hypoxia in HFD-BMDMs (Figure 1c). Importantly, the levels of HIF-1 α were similarly increased in both LFD- and HFD-BMDMs (Figure 1d).

3.2 | Hypoxia-Increased H3K4me3 Peaks Mark a Subset of Metabolic Genes During Hypoxic Adaptation in Macrophages

We performed ChIP-seq to identify the genomic locations of H3K4me3 modified by hypoxia. Among the total of H3K4me3 25247 peaks in LFD-BMDMs (Figure S1), we found significant changes in ChIP-seq signal at 698 peaks ($p < 0.05$, LSmean ≥ 20): 448 peaks (64.2%) were increased after hypoxia treatment, and 250 peaks (35.8%) were decreased (Figure 2a and Table S1). Consistent with a previous report in a different cell type, HeLa cells [16], more than 80% of the H3K4me3 peaks that increased in hypoxia were located at gene promoter regions, with the rest of the peaks located at either intergenic regions or gene body regions (Figure 2a). To link H3K4me3 levels to biological processes and pathways, we assigned a promoter-associated peak to the gene whose transcription start site (TSS) was closest to the peak and then performed Gene Set Enrichment Analysis (GSEA). An increase in H3K4me3 at promoter regions (398 peaks in total) was found in genes involved in multiple metabolic pathways, including glycolysis, oxidative phosphorylation, and fatty acid metabolism (Figure 2b and Table S2).

Next, we investigated the genomic location of H3K4me3 peaks in HFD-BMDMs exposed to acute hypoxia compared to normoxia. We found hypoxia-induced changes in H3K4me3

enrichment at 958 peaks, including 330 increased and 628 decreased peaks (Figure 2c). Thus, in comparison to LFD-BMDMs, HFD-BMDMs had 118 fewer increased peaks and 378 more decreased peaks. We also found a lower proportion of increased H3K4me3 peaks at the promoter regions in HFD-BMDMs than in LFD-BMDMs (69% vs. 89%), while the difference in hypoxia-increased peaks was found near TSS (Figure S2). Moreover, in contrast to LFD-BMDMs, the H3K4me3 peaks increased in HFD-BMDMs in response to hypoxia were not associated with any metabolic pathway (Figure 2d). Indeed, LFD-BMDMs and HFD-BMDMs shared only 24 H3K4me3 peaks increased during hypoxia (Figure 2e). Thus, diet-induced obesity disrupts acute H3K4me3 changes in hypoxia in terms of its total levels and genome locations.

We next examined the impact of H3K4me3 changes in hypoxia on gene expression. First, RNA-seq data from our cultured BMDMs identified 54 upregulated and 20 downregulated genes (Adj. $p < 0.01$) at 1 h of hypoxia in LFD-BMDMs (Table S3 and Figures S3a and S4). None of these 54 genes acutely upregulated after hypoxia were marked by hypoxia-increased H3K4me3 (Figures S3a and S4), exhibiting a stark contrast with HeLa cells, in which increased H3K4me3 is associated with immediate gene activation [16]. Only 13 upregulated and 5 downregulated genes (Adj. $p < 0.01$) at 1 h of hypoxia were found in HFD-BMDMs (Table S4; Figures S3b and S5), indicating a correlation between blunted H3K4me3 changes and acute gene activation. Pathway analysis by GSEA showed that hypoxia and glycolysis were similarly enriched in both LFD- and HFD-BMDMs after 1 h of hypoxia (Figure S3c), suggesting that acutely induced hypoxia- and glycolysis-related genes were comparable. To assess the delayed effect of H3K4me3 changes by hypoxia, we first utilized a publicly available RNA-seq dataset of bone marrow-derived immortalized macrophages exposed to hypoxia (GSE192969) [21]. The number of genes induced by hypoxia increased over time, from 391 genes at 3 h to 934 genes at 7 days (prolonged) of hypoxia exposure. Remarkably, the same processes, hypoxia, fatty acid metabolism, and glycolysis, were enriched among the genes with increased H3K4me3 peaks as well as among the upregulated genes (Figure S3d). Among the 22 genes overlapping with the H3K4me3 peak, 12 genes, including *Aldoa*, *Adm*, and *Depdc1a*, exhibited increased H3K4me3 levels at their promoters (Figure 2g,h and Figure S3e). RT-qPCR confirmed the upregulated expression of *Aldoa*, *Adm*, and *Depdc1a* in LFD-BMDMs after 24 h of hypoxia but not in HFD-BMDMs (Figure 2i), while the *Depdc1a* gene response remained intact. These findings suggest that H3K4me3 changes induced by hypoxia at gene promoters have a delayed impact on the transcript levels of genes associated with metabolic pathways in LFD-BMDMs, which are dysregulated in HFD-BMDMs.

3.3 | Lactate Accumulation and Histone Lactylation During Hypoxia Are Inhibited in HFD-BMDMs

As a hypoxia-induced metabolic shift is typically represented by increased glycolysis, we measured lactate, one of the end products of glycolysis, in BMDMs exposed to hypoxia compared to those kept in normoxia. As expected, LFD-BMDMs

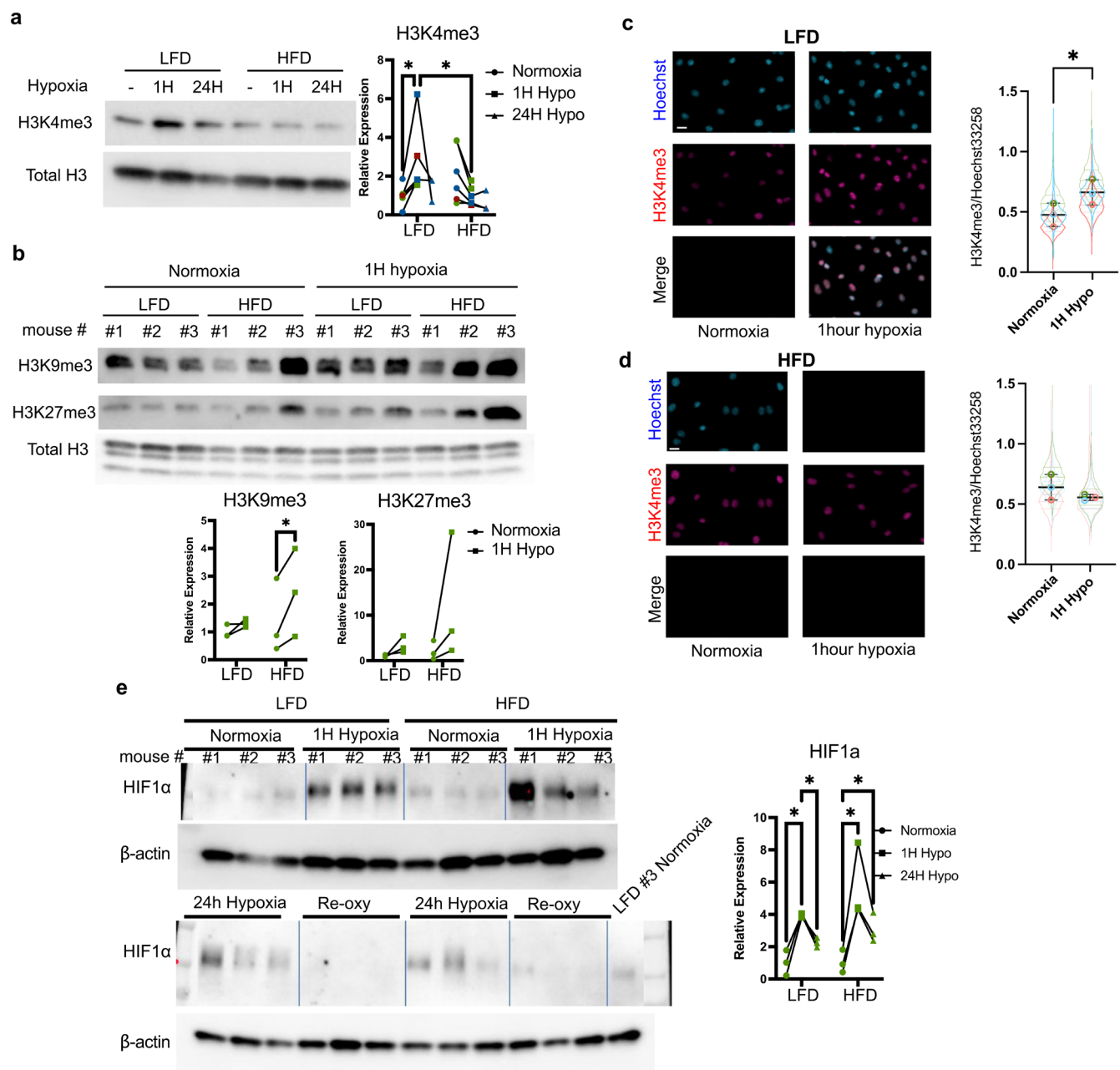


FIGURE 1 | Obesity impairs hypoxia-inducible histone methylation in bone marrow-derived macrophages (BMDMs). (a and b) Western blot analysis of histone methylation after acute (1 h) hypoxia of BMDMs from low-fat diet (LFD) or high-fat diet (HFD) mice. Hypoxia-induced changes in histone methylation were quantified as fold-changes vs. normoxia for each cell source of the mouse ($n = 3$ -5 biologically independent samples, and mouse # and connected line represent each mouse source of the cells). (c) High-content immunofluorescent imaging of H3K4me3 in BMDMs after acute hypoxia in LFD-BMDMs. Hoechst33258 (Blue, top) for nuclei, H3K4me3 antibody (Red, middle) and merged (Bottom) are shown. Scale bar indicates 100 μ m. The ratio of H3K4me3/Hoechst33258 intensity was analyzed in a total of $n = 7000$ –8000 cells from three biological replicates. (d) High-content imaging of H3K4me3 in BMDMs after acute hypoxia in HFD-BMDMs. The ratio of H3K4me3/Hoechst33258 intensity was analyzed in a total of $n = 7000$ –8000 cells from three biological replicates. (e) Western blot analysis of hypoxia inducible factor (HIF)-1 α in total cell lysates of LFD- and HFD-BMDMs after hypoxia. Densitometry analysis vs. β -Actin ($n = 3$ biologically independent samples). For all panels, data are shown as the mean $\pm$ s.e.m. Statistical significance was determined using unpaired two-tailed Student's t -test (a–c) and one-way ANOVA with Tukey's multiple comparison test (d) * $p < 0.05$.

cultured in hypoxia for 24 h had increased intracellular lactate levels (Figure 3a). Disrupted H3K4me3 changes and failure to induce genes, such as *Aldoa* and *Adm*, led to reduced lactate accumulation in HFD-BMDMs (Figure 3a). As glycolytic capacity was identical in normoxia between LFD- and HFD-BMDMs (Figure S6), our data suggest that maladaptation to hypoxia

is responsible for less lactate accumulation in HFD-BMDMs. We further assessed histone lactylation because lactate accumulation may induce an alternative activation program in macrophages via this histone modification [24]. As expected, histone 3-lysine 18 lactylation (H3K18la) was increased in LFD-BMDMs but not in HFD-BMDMs (Figure 3b). Among the genes

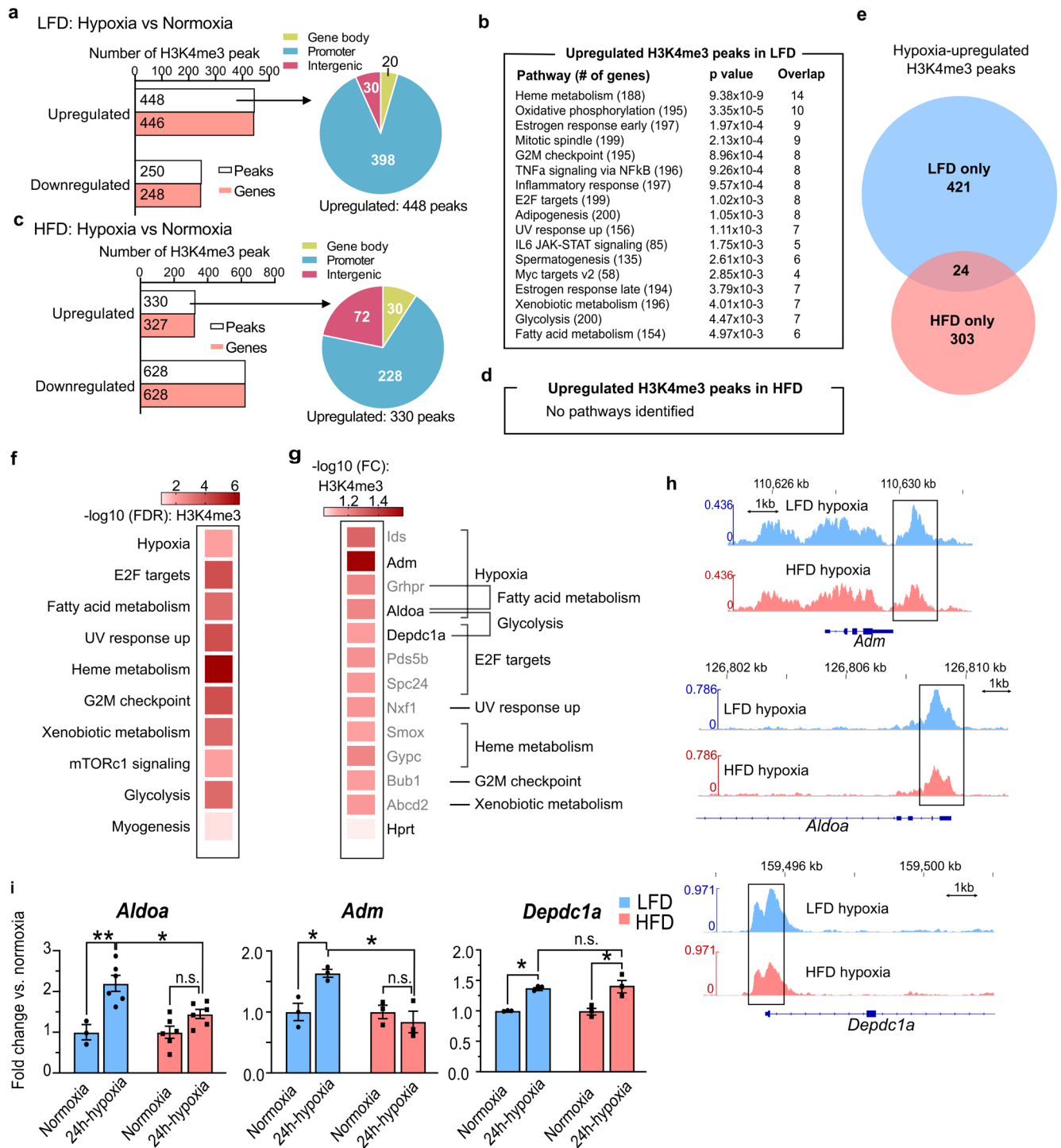


FIGURE 2 | Characterization of hypoxia-inducible H3K4me3 peaks in LFD-BMDMs and HFD-BMDMs. Chromatin immunoprecipitation followed by sequence (ChIP-seq) for H3K4me3 was performed in LFD- and HFD-BMDMs with or without acute hypoxia ($n = 3$ biological replicates). (a) Number and distribution of hypoxia-increased H3K4me3 peaks after hypoxia in LFD-BMDMs. (b) Gene set enrichment analysis (GSEA) of hypoxia-increased H3K4me3 peaks in LFD-BMDMs revealed multiple metabolic pathways (FDR (adj. p) < 0.005). (c) Number and distribution of hypoxia-increased H3K4me3 peaks after hypoxia in HFD-BMDMs. (d) GSEA of hypoxia-increased H3K4me3 peaks in HFD-BMDMs showed no significant pathways (FDR < 0.005). (e) Venn diagram of hypoxia-increased H3K4me3 peaks between LFD- and HFD-BMDMs. (f) Pathways found in hypoxia-increased H3K4me peaks were compared with RNA-seq data after 1 h (own data, $n = 3$ biological replicates). (g) Individual genes found in hypoxia-increased H3K4me peaks were compared with RNA-seq data after 1 h. (h) Hypoxia-increased H3K4me3 peak at the promoter regions of *Aldoa*, *Adm*, and *Depdc1a* in LFD-BMDMs and their lack of upregulation in HFD-BMDMs. (i) q-PCR analysis of *Aldoa*, *Adm*, and *Depdc1a* mRNA after 24 h of hypoxia compared to normoxia ($n = 3$ –6 biologically independent samples). Data are shown as the mean $\pm$ s.e.m. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparison test. * $p < 0.05$.

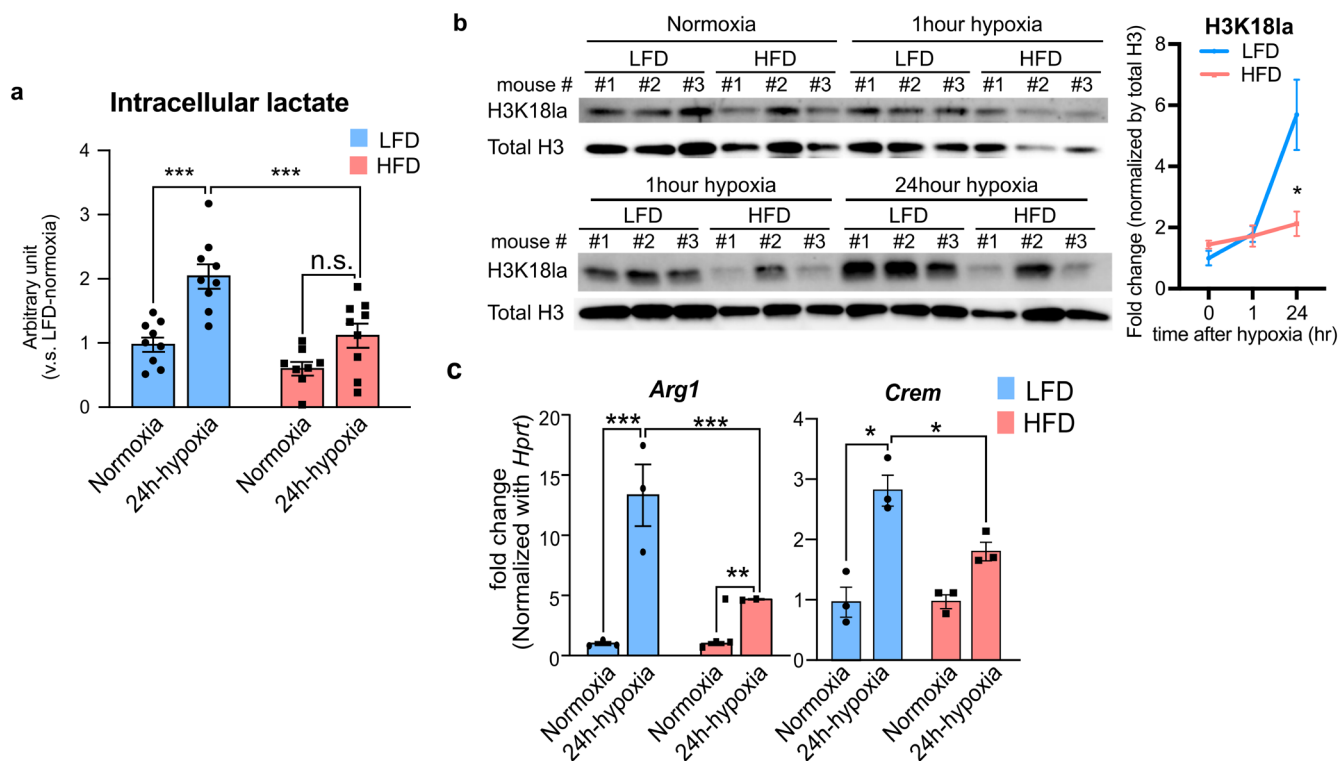


FIGURE 3 | Acutely increased H3K4me3 levels by hypoxia are linked with *Alda* and *Adm* gene induction and lactate accumulation 24 h after hypoxia. (a) Intracellular accumulation of lactate 24 h after hypoxia in LFD- and HFD-BMDMs, compared to 24 h normoxia ($n = 6-9$ biologically independent samples). Concentrations are shown as linear scale arbitrary units vs. control. (b) Western blot analysis of histone 3-lysine 18 lactylation (H3K18la) in isolated histone fractions from LFD- and HFD-BMDMs cultured under normoxia and for the indicated duration of hypoxia ($n = 3$ mice). Fold-change of densitometry (H3K18la/total H3) compared to LFD-normoxia for top and LFD-1 h hypoxia for bottom indicates relative H3K18la expression. (c) H3K18la targets *Arg1* and *Crem* gene expression by quantitative PCR ($n = 3$ mice). For all panels, data are shown as the mean $\pm$ s.e.m. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparison test. * $p < 0.05$; *** $p < 0.001$; n.s. not significant.

associated with the alternative activation of macrophages, *Arg1* and *Crem* were upregulated under hypoxia in LFD-BMDMs, whereas the hypoxia-induced increases in these genes were reduced in HFD-BMDMs (Figure 3c). These results suggest that lactate accumulation and histone lactylation are impaired in HFD-BMDMs under hypoxic conditions.

3.4 | Efferocytosis Under Hypoxia in HFD-BMDMs Is Inhibited in a Glucose and Lactate-Dependent Manner and Is Imprinted in Their Precursors in HFD Mice

To determine the macrophage function linked to impaired histone modifications and lactate accumulation under hypoxia in HFD-BMDMs, we first examined inflammatory gene expression and found no differences in inflammatory genes *Il1b* and *Tnfa* between LFD- and HFD-BMDMs (Figure S7a,b) exposed to hypoxia. No differences in lipopolysaccharide (LPS)-induced *Il1b*, *Il6*, and *Tnfa* (Figure S7c,d) were found in HFD-BMDMs under hypoxia compared to LFD-BMDMs under hypoxia.

Efferocytosis, a phagocytic process involving the engulfment of apoptotic cells and digestion of the engulfed cellular material, depends on intricate metabolic state transitions, such as glycolysis in the early engulfment stage [20, 22, 34] and fatty acid synthesis in the later digestion stage [35]. To assess

overall efferocytosis, we used apoptotic Jurkat T cells labeled with PKH26 dye. We found that when cultured under hypoxic conditions, HFD-BMDMs had a lower capacity for efferocytosis than LFD-BMDMs (Figure 4a). Next, Jurkat T cells were loaded with a pH-sensitive dye that fluorescently labels the components of apoptotic cells in low-pH phagolysosomes. Live cell imaging confirmed the lower efferocytosis in HFD-BMDMs under hypoxia (Figure 4b). Note that hypoxia-increased efferocytosis in LFD-BMDMs was only observed when PKH26-dye stained apoptotic cells were used but not pH-sensitive dye (data not shown), suggesting that the engulfment of apoptosis rather than the entire process of efferocytosis through digestion was increased by hypoxia in our system and that the effect of hypoxia on efferocytosis may be inconsistent at 24 h in parallel of the previous study [21]. Nonetheless, we consistently observed impaired efferocytosis in HFD-BMDMs under hypoxia compared with LFD-BMDMs, and high-glucose and lactate supplementation under hypoxia was sufficient to rescue the lower efferocytosis in HFD-BMDMs (Figure 4b). When the digestion of cellular materials was assessed, HFD-BMDMs exhibited significantly higher non-fragmented signals and lower fragmented signals than LFD-BMDMs (Figure 4c), suggesting slower digestion of engulfed apoptotic cells in HFD-BMDMs. We also found that HFD-BMDMs had lower continual efferocytosis, in which lactate is a key promoter of the process [22], compared with LFD-BMDMs (Figure 4d). We also tested efferocytosis in dendritic cells (DCs) grown from the BM and did not observe differences

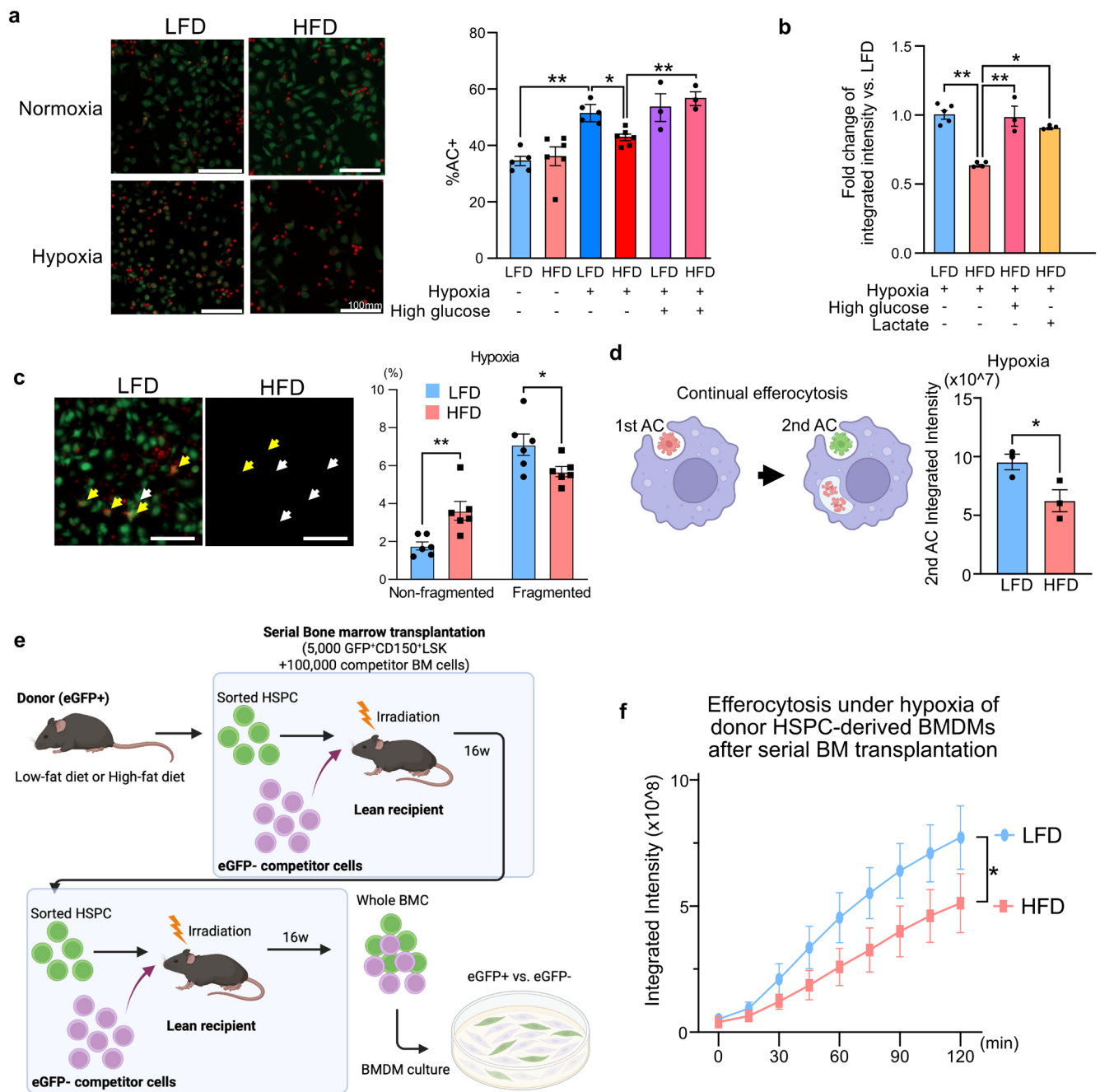


FIGURE 4 | Diet-induced obesity impairs efferocytosis under hypoxic conditions. Efferocytosis was assessed in BMDMs preconditioned with hypoxia for 24 h compared with normoxia for 24 h. (a) BMDMs preconditioned in hypoxia/normoxia with or without high glucose (25 mM) for 24 h were labeled with CFSE (green) and co-cultured with CellTracker Red-labeled apoptotic neutrophils (AC: Red) at a 5:1 ratio and co-cultured for 1 h ($n = 3-5$ biological replicates). The scale bar indicates 100 μ m. (b) BMDMs preconditioned with hypoxia with or without high glucose (25 mM) or lactate (25 mM) for 24 h were co-cultured with pHrodo Green-labeled apoptotic Jurkat T cells ($n = 3-5$ mice). (c) Non-fragmented/fragmented CellTracker Red-labeled apoptotic cells (red) in the CFSE-labeled BMDMs (green). White arrows indicate non-fragmented apoptotic cells in BMDMs, and yellow arrows indicate fragmented dying cells in BMDMs. Scale bars indicate 100 μ m. (d) Continual apoptotic cell clearance was assessed after co-culture of BMDMs and CellVue Claret Far Red (red) labeled apoptotic Jurkat cells for 45 min. After rinsing and a 2 h resting period, 2nd incubation of apoptotic Jurkat cells were labeled with pHrodo Green (green). Maturation of continual apoptotic cell clearance was evaluated by the signal intensity of pHrodo Green in pHrodo Green⁺CellVue Claret⁺ BMDMs ($n = 3$ mice). (e) Schematic representation of the serial competitive bone marrow transplantation assay. EGFP⁺ CD150⁺LSK cells sorted from LFD- and HFD-fed EGFP mice were intravenously injected into lean (EGFP⁻) mice that were lethally irradiated (9.5 Gy) along with (EGFP⁻) competitor bone marrow cells, and a second transplantation was performed. BMDMs were grown from bone marrow cells isolated 16 weeks after 2nd transplantation. (f) Efferocytosis of EGFP⁺BMDMs ($n = 5$ biological replicates) using Jurkat T cells labeled with CyPhers dye. For all panels, data are shown as the mean $\pm$ s.e.m. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparison test in (a-c); Student's *t*-test in (d); and two-way ANOVA in (f). * $p < 0.05$; ** $p < 0.01$.

in efferocytosis capacity between LFD- and HFD-derived DCs under normoxia and hypoxia (Figure S8), suggesting that HFD-impaired efferocytosis is specific to BMDMs under hypoxia in a normal culture environment.

We further tested whether impaired efferocytosis in HFD-BMDMs was caused by long-term immune memory in hematopoietic stem progenitor cells (HSPCs) in HFD mice. To do this, we performed two rounds of serial bone marrow transplantation using HSPCs sorted from eGFP mice fed with LFD or HFD and then analyzed the efferocytosis of donor-derived eGFP⁺BMDMs using live cell imaging (Figure 4e). Consistent with the potential role of H3K4me3 in innate immune memory [36–38] and dysregulation of H3K4me3 revealed in this study, we found that BMDMs derived from donor HSPCs from HFD mice exhibited a lower capacity for efferocytosis under hypoxia, even after serial transplantation (Figure 4f), suggesting that efferocytosis under hypoxia in HFD-BMDMs is imprinted in HSPCs in HFD mice.

3.5 | KDM5A Regulates Hypoxia-Increased H3K4me3, Lactate Accumulation and Efferocytosis in BMDMs

Rapid H3K4me3 induction can be mediated by the inhibition of H3K4 demethylase, KDM5A, which is known to have reduced demethylase activity in a low-oxygen environment [16]. To test the role of KDM5A in the hypoxic response of BMDMs, we generated *Kdm5a* knockout (KDM5A-KO) BMDMs using a tamoxifen-inducible Cre system (Figure 5a,b). While KDM5A-KO did not alter H3K4me3 levels without hypoxia, it did increase the levels of H3K4me3 and its sustainability in hypoxia (Figure 5c), independently of HIF-1 α (Figure 5d). These findings confirm that KDM5A functions as a negative regulator of hypoxia-induced H3K4me3 in a HIF-1 α -independent manner in mouse BMDMs. As the genes associated with a rapid increase in H3K4me3 were disrupted in HFD-BMDMs, KDM5A-KO showed overlapping gene dysregulation with HFD-BMDMs under hypoxia; *Aldoa* and *Adm* were not upregulated by hypoxia, whereas *Depdc1a* change remained intact in KDM5A-KO BMDMs (Figure 5e), which is similar to the gene dysregulations in HFD-BMDMs (Figure 2i). These changes correlated with an increase in total (intracellular and extracellular) lactate accumulation, whereas intracellular lactate levels remained unchanged (Figure 5f), suggesting that enhanced lactate generation by KDM5A-KO. Collectively, hypoxia-increased H3K4me3, which is negatively regulated by KDM5A, controls *Aldoa* and *Adm* gene enrichment and lactate generation.

We next examined efferocytosis and found impaired efferocytosis in KDM5A-KO BMDMs under normoxic conditions (Figure 5g). Under hypoxia, however, efferocytosis of KDM5A-KO BMDMs was identical to that of KDM5A-intact BMDMs (Figure 5h), suggesting that the augmentation of H3K4me3 changes and lactate accumulation in KDM5A-KO BMDMs enhanced efferocytosis under hypoxia. To clarify the role of KDM5A in efferocytosis, we treated BMDMs with KDM5A catalytic inhibitors. YUKA1 [39] and CPI455 [40]. These inhibitors increased efferocytosis under both normoxia and hypoxia (Figure 5i), suggesting that inhibiting KDM5A demethylation activity, similar to the effect of hypoxia, promotes efferocytosis, whereas KDM5A plays a role

in efferocytosis under normoxic conditions through its catalytic-independent function [41].

3.6 | Low Lactate Levels in the Early Phase of Healing Delay Wound Healing in Diet-Induced Obesity

Lactate accumulation and efferocytosis can promote wound healing, especially the early phase of skin wound healing [23, 34, 42]. Moreover, efferocytosis can induce lactate release into the extracellular space [20]. Therefore, we next examined the in vivo relevance of reduced lactate accumulation and efferocytosis in HFD-BMDMs under hypoxia using a dorsal skin biopsy model with non-enzymatic tissue dissection, which allowed us to measure extracellular lactate levels in wound tissues. We found that lactate levels on day 1 were lower than those in unwound skin and gradually increased over time in normal wild-type mice (Figure 6a). Lactate accumulation in the early phase of wound healing on day 3 was significantly lower, along with impaired wound closure, in HFD-induced obese mice than in control mice (Figure 6b). This reduction in lactate levels on day 3 was associated with HFD-induced impairment of skin wound closure (Figure 6c).

3.7 | KDM5A Deficiency Alone Is Insufficient to Promote Skin Wound Healing

As BMDMs from KDM5A-KO mice exhibited enhanced lactate accumulation, we performed a dorsal skin biopsy in KDM5A-KO mice to determine the in vivo consequences of KDM5A deficiency. KDM5A-KO mice did not show significant impairment in wound closure in both male and female mice, while we found a tendency to promote healing in KDM5A-KO males (Figure 7a). We found an increase in connective tissue formation at the wound edge area of the wound in KDM5A-KO mice in the early stage (day 3) (Figure 7b), but this was not accompanied by a change in tissue lactate levels (Figure 7c) and did not change the extent of connective tissue formation in the later stage (day 21) (Figure 7d). Next, tamoxifen-inducible KDM5A-KO mice were fed an HFD for 12 weeks and subjected to dorsal skin biopsy two weeks after the initial tamoxifen injection. We did not find a significant influence of KDM5A-KO on wound closure in the obese environment (Figure 7e). Thus, the promoting effect of KDM5A-KO on wound healing was limited to the early stage of skin wound healing and did not affect overall wound closure in both normal and HFD-induced obesity.

4 | Discussion

Hypoxia-inducible signaling is important for homeostasis, and its dysregulation has been found in macrophages in obesity [10, 43]. In this study, we identified a disruption of the hypoxia-induced H3K4me3 pathway in BMDMs from HFD-induced obesity. The hypoxia-induced increase in H3K4me3 in BMDMs, which we found to potentially define the metabolic adaptation of macrophages to hypoxia, notably through lactate accumulation and histone lactylation, was further linked with the alternative activation of macrophages. Disruption of this pathway

is associated with reduced efferocytosis and impaired healing response in the early stages of wound healing.

Our data showed that H3K4me3-increased genes under hypoxia involve a wide range of metabolic pathways, including glycolysis, heme metabolism, and fatty acid metabolism. However, our

bioinformatics analysis primarily focused on gene promoters only revealed the link between H3K4me3 increases and gene activation at the delayed time point in BMDMs rather than immediate gene activation as shown in HeLa cells [16]. There was little overlap in the increased H3K4me3 peaks between murine BMDMs and human HeLa cells (Figure S9 and Table S5),

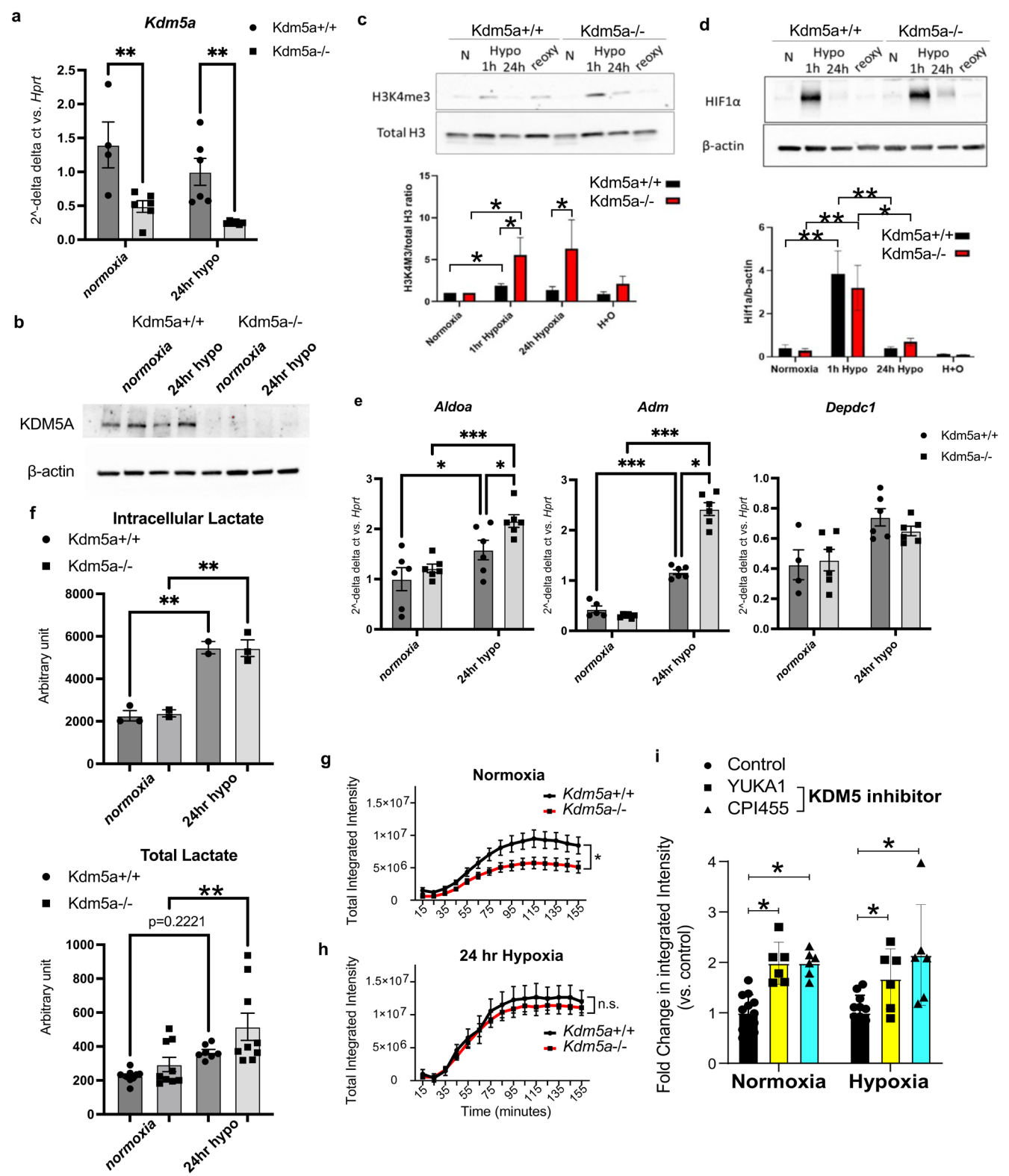


FIGURE 5 | Legend on next page.

FIGURE 5 | KDM5A demethylase is involved in hypoxia-induced H3K4me3 and lactate accumulation. (a, b), KDM5A knockdown of BMDMs from in vivo tamoxifen-inducible *Kdm5a*^{-/-} (Cre-ERT2⁺) and *Kdm5a*^{+/+} (Cre-ERT2⁻) mice. q-PCR (a) and western blotting (b) show *Kdm5a* gene and KDM5A protein expression 24 h after hypoxia in *Kdm5a*^{+/+} (Cre-) and *Kdm5a*^{-/-} (Cre+) BMDMs compared to normoxia ($n=4-6$ biologically independent samples). (c) Western blot analysis of H3K4me3 in the nuclear fraction of *Kdm5a*^{-/-} and *Kdm5a*^{+/+}-BMDMs after hypoxia and reoxygenation (reox or H + O) ($n=3$ mice). (d) Western blot analysis of HIF-1 α in the nuclear fraction of *Kdm5a*^{-/-} and *Kdm5a*^{+/+}-BMDMs after hypoxia and reoxygenation (reox) ($n=3$ mice). (e) *Aldoa*, *Adm*, and *Depdc1a* gene induction 24 h after hypoxia in *Kdm5a*^{+/+} and *Kdm5a*^{-/-}-BMDMs, compared to 24 h normoxia ($n=6$ biologically independent samples). (f) Intracellular and total (intracellular + extracellular) accumulation of lactate 24 h after hypoxia in *Kdm5a*^{+/+} and *Kdm5a*^{-/-}-BMDMs, compared to 24 h normoxia ($n=3-9$ biologically independent samples). The concentrations are shown as linear-scale arbitrary units. (g, h) Efferocytosis of pHrodoRed-stained Jurkat cells by BMDMs from *Kdm5a*^{+/+} and *Kdm5a*^{-/-} mice. BMDMs were preconditioned under normoxia (g) or hypoxia (h) for 24 h ($n=3$ mice). (i) Efferocytosis of pHrodoRed-stained Jurkat cells by BMDMs from wild-type mice pretreated with KDM5 inhibitors, YUKA1 (10 μ M) or CPI455 (50 nM), for 72 h ($n=6-9$ biological replicates). For all panels, data are shown as the mean $\pm$ s.e.m. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparison test in (a-f, i); two-way ANOVA in (g, h). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

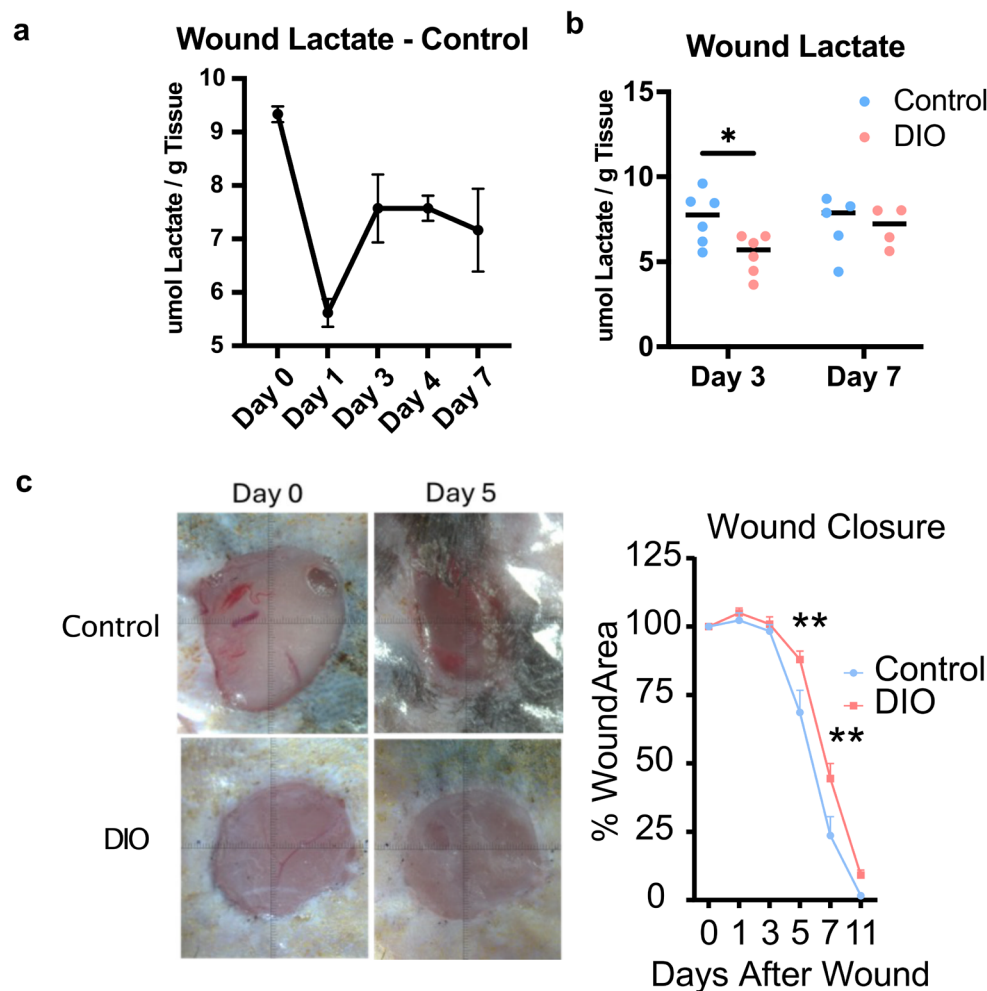


FIGURE 6 | Diet-induced obesity lowers lactate accumulation in the early phase of healing and delays wound healing. (a) Mice fed a normal chow diet were subjected to a dorsal skin biopsy with an 8 mm diameter. Lactate levels in the tissue supernatant were measured using an enzymatic assay ($n=2-6$ per time point). (b) mice fed a normal chow diet or high-fat diet with L-NAME (DIO) were subjected to dorsal skin biopsy with a diameter of 8 mm. Lactate levels in the tissue supernatant were measured using an enzymatic assay ($n=4-6$ per time point). (c) Wound closure was measured using photographs taken under a stereomicroscope. For all panels, data are shown as the mean $\pm$ s.e.m. Statistical significance was determined using the Student's *t*-test in (b) and two-way ANOVA with Tukey's multiple comparison test in (c). * $p < 0.05$; ** $p < 0.01$.

suggesting that the downstream effect of hypoxia-dependent H3K4me3 on transcriptional regulation is likely highly cell type- or context-dependent. Furthermore, we demonstrated in bone marrow transplantation (Figure 4e) that the maladapted

to hypoxia response for efferocytosis is imprinted in the bone marrow precursor of macrophages in HFD mice, aligning with the potential role of H3K4me3 in diet-induced innate immune memory originating from hematopoietic progenitor cells [37].

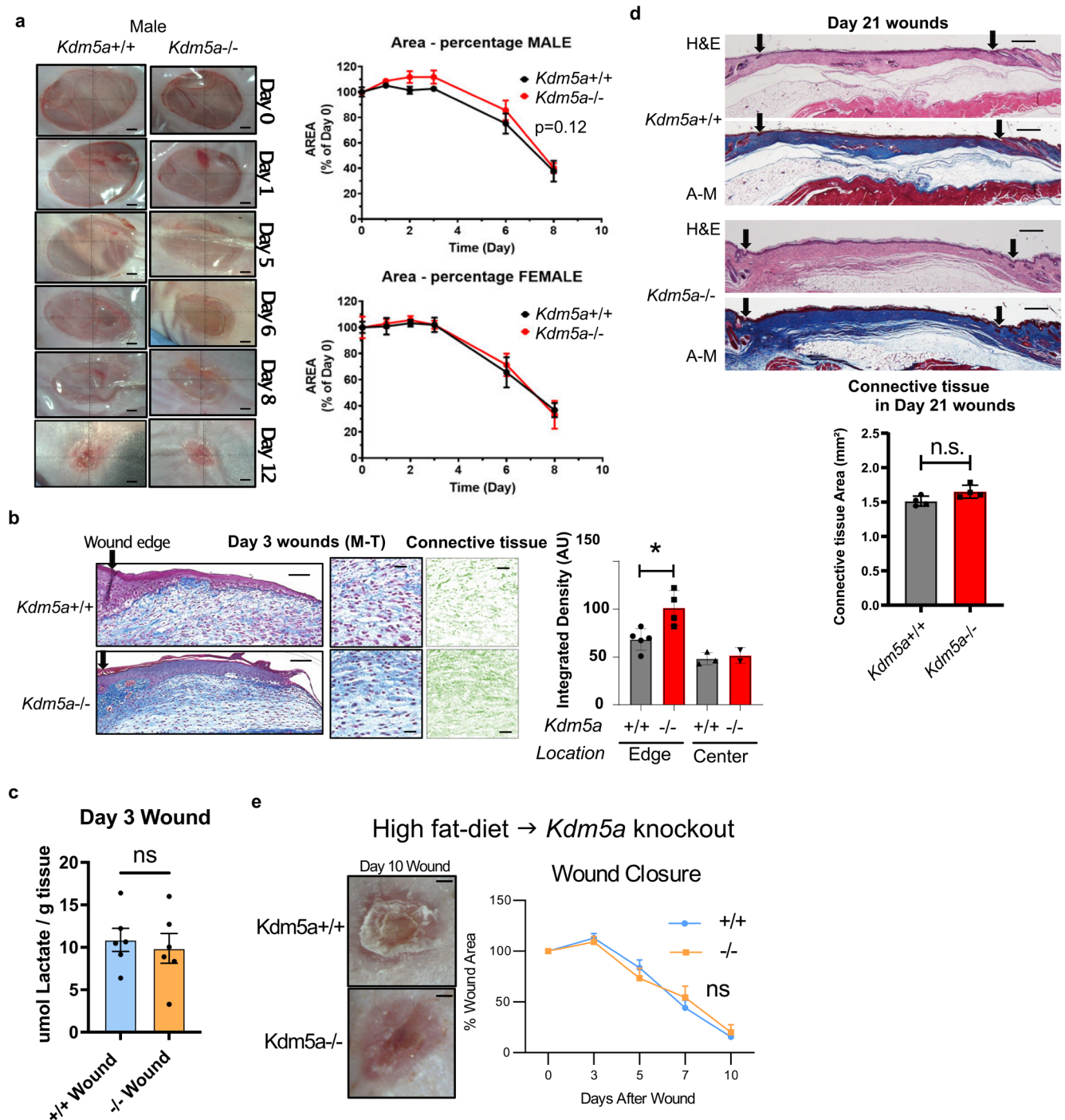


FIGURE 7 | KDM5A knockout has a limited impact on skin wound healing in mice. (a) Tamoxifen-driven Cre-inducible KDM5A knockout was induced in male and female *Kdm5a*^{fl/fl}-CreERT2 mice 2 weeks before dorsal skin biopsy of 8 mm in diameter. Wound closure was measured at the indicated time points ($n=6$ mice per group). Bar in images = 1 mm. (b) Masson-Trichrome staining sections from wounds on day 3 were analyzed to quantify the integrated intensity of the blue staining, reflecting the density of connective tissue ($n=5$ mice with exclusions). The wound edge area was defined as the tissue below the epithelial tongue, and the central area was defined as not covered by the newly formed epithelium. The extracted blue staining is shown on the right. Bar = 100 μ m (left) and 20 μ m (middle and right). (c) Lactate levels in the tissue supernatant on day 3 were measured using an enzymatic assay ($n=6$ mice). (d) Azan-Mallory staining sections from wounds on day 21 were analyzed to quantify the integrated intensity of the blue staining, reflecting the density of connective tissue ($n=4$ mice). (e) Male *Kdm5a*^{fl/fl}-CreERT2 mice were fed a high-fat diet for 15 weeks, followed by tamoxifen-driven Cre-inducible KDM5A knockout. A dorsal skin biopsy with a diameter of 8 mm was performed after 2 weeks. Wound closure was measured at the indicated time points ($n=6$ mice per group). Bar in images = 1 mm. For all panels, data are shown as the mean $\pm$ s.e.m. Statistical significance was determined using two-way ANOVA with Tukey's multiple comparison test in panels a and e, and the Student's *t*-test in (b–d). * $p < 0.05$; n.s., not significant.

Considering the complex roles of H3K4me3 in transcriptional regulation [44], further investigation will elucidate the hypoxia-induced and obesity-dysregulated H3K4me3 linked gene regulation that is responsible for lactate accumulation in macrophages.

We showed that histone lactylation is downstream of H3K4me3-dependent metabolic changes, as we demonstrated that known histone lactylation target genes *Arg1* and *Crem* [24] were dysregulated in HFD-BMDMs. A recent study further indicated that histone lactylation is linked to histone acetylation in the context of β -glucan-induced training in human and murine macrophages [45], suggesting a complex interaction of multiple histone modifications regulating hypoxia-induced and lactate-mediated macrophage phenotypic switch, in addition to other mechanisms such as lactate-activating mTORC1 and HIF-2 α in lysosomes [46]; a pathway involving mitochondrial antiviral-signaling protein (MAVS) and RIG-I [47]; and lactate-induced extracellular acidosis that activates the transcriptional repressor [48]. Thus, HFD-dysregulated lactate can influence macrophage function through mechanisms beyond histone lactylation.

We show that the disrupted H3K4me3-lactate axis in HFD-BMDMs under hypoxia is linked to impaired efferocytosis capacity, which is dependent on glycolysis and lactate accumulation [22, 34]. Efferocytosis requires orchestrated metabolic programming involving fatty acid oxidation and amino acid utilization [35, 49–51]. Our data suggest that an increase in H3K4me3 levels in hypoxia may be a prerequisite for metabolic programming to promote efferocytosis. When macrophages are exposed to hypoxia for a long period, such as 7 days, metabolic remodeling at the transcriptional and translational levels promotes efferocytosis [21]. While we found a mild overlap of such transcriptional changes after prolonged hypoxia with our gene set of hypoxia-increased H3K4me3 (Extended Figure 3e), further studies are needed to determine whether H3K4me3 plays a direct role in the metabolic adaptation for efferocytosis. Interestingly, RNA elongation through RNA polymerase II pause-release, which is positively regulated by H3K4me3 [52], is a crucial mechanism of continual efferocytosis [53].

Using genetic deletion of KDM5A as a tool to manipulate H3K4me3 in the hypoxic response [16], we demonstrated that a lack of this demethylase enhanced H3K4me3 and lactate accumulation in BMDMs under hypoxia. At least a handful of hypoxia-increased H3K4me3-marked genes, *Aldoa*, *Adm*, and *Depdc1* followed the same trend as we found in the hypoxia-induced changes in LFD-BMDMs, suggesting the involvement of KDM5A in hypoxia response in macrophages. However, KDM5A knockout failed to promote efferocytosis under hypoxia, partly due to its efferocytosis-inhibiting effect in a hypoxia-independent manner. This was reflected in the insignificant effect of KDM5A knockout on lactate accumulation and showed a modest pro-healing effect during the early phase of wound healing. We did not observe significant changes in wound closure in either non-obese or obese conditions following KDM5A-KO, possibly because efferocytosis under hypoxia remained unaltered. As pharmacological inhibition of

KDM5 was sufficient to increase efferocytosis in vitro, it is necessary to determine whether such an approach activates the H3K4me3-lactate axis in vivo to promote wound healing.

Enhanced efferocytosis during the early phase of skin wound healing accelerates the repair process [34]. In line with the hypoxic conditions typically present in early wound environments, we observed reduced tissue lactate levels in diet-induced obesity (Figure 6b), which may reflect both low lactate accumulation under hypoxia, as shown in this study, and efferocytosis-induced lactate release by macrophages [20] in wound tissue. Our findings support the notion that lactate acts as a key pro-healing metabolite in skin wound repair [42]. However, its potential as a biomarker may be limited to sterile wounds, as elevated lactate levels are also associated with infection-related delays in healing [54]. Collectively, we demonstrated that efferocytosis in the early phase of healing is a key event in wound healing and may be impaired in obesity-associated pathology. Our findings underscore the importance of the H3K4me3-lactate axis in supporting macrophage function in hypoxic tissues and suggest a potential epigenetic mechanism underlying altered macrophage function in obesity-associated pathologies.

Author Contributions

K.T., J.D., J.L., J.R.J., and N.U. designed and performed experiments using macrophage culture models in hypoxic environments. N.U. and K.T. performed the bioinformatics analyses. J.L., J.R.J., and N.U. designed and performed the experiments using the skin biopsy model. M.T., E.V.B., and N.U. established the knockout mouse model. M.B. designed and performed additional western analysis. M.F., M.T., T.P., and G.G. helped modify workflows and obtained preliminary observations of cultured macrophages or histology. Y.F. and Y.A. performed the histological analyses. G.F. and T.J.K. provided guidance and metabolic profiles of diet-induced obese animals. E.V.B., G.F., and T.J.K. contributed to the initial conceptualization of the study. E.V.B. helped interpret the KDM5A and histone modification data. K.T., J.L., G.F., E.V.B., T.J.K., and N.U. wrote the manuscript. N.U. supervised the project and provided final approval.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Stored in repository.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** H3K4me3 ChIP-seq analysis. (a) Number of peaks called in each condition, 3/3 represents peaks called in all three replicates (high stringency peaks) and merged represents the merged data from all three replicates. (b) High stringency peaks and genes (genes with peaks) were called in each condition. (c) Heatmap of CPM-normalized read counts at Transcription Start Sites (TSS) of protein-coding genes for each condition. **Figure S2:** Relationship of fold change and peak height in H3K4me3 ChIP-seq. (a) Relationship between fold change (y-axis) and LSmean (x-axis) in H3K4me3 peaks in response to hypoxia in LFD-BMDMs (left) and HFD-BMDMs (right). (b) Relationship between fold change (y-axis) and number of peaks (x-axis) in H3K4me3 peaks in response to hypoxia in LFD-BMDMs (left) and HFD-BMDMs (right). **Figure S3:** Hypoxia-induced genes in LFD- and HFD-BMDM. (a) Volcano plot showing differentially expressed genes after acute hypoxia (1 h) in LFD-BMDMs. After hypoxia treatment, 71 and 33 genes were upregulated and downregulated (Adj. $p < 0.05$), respectively. The numerical data are presented in Table S1. (b) Volcano plot showing differentially expressed genes after acute hypoxia (1 h) in HFD-BMDMs. After hypoxia treatment, 21 and 9 genes were upregulated and downregulated (Adj. $p < 0.05$), respectively. The numerical data are presented in Table S2. (c) Gene set enrichment analysis (GSEA) was performed using the fgsea R package and the fgseaMultilevel() function 1. The sign ($\log_2$ fold change) * $-\log_{10}$ (p -value) from the 1 h hypoxia vs. normoxia differential expression comparison was used to rank genes. The Hallmark gene set collection from the Molecular Signatures Database (MSigDB) was curated using the msigdb R package4. Prior to running GSEA, the list of gene sets was filtered to include only those with between 5 and 1000 genes. The barplot shows the GSEA results for each tested gene set with a p -value ≤ 0.01 . The y-axis shows the normalized enrichment score (NES) from GSEA, which represents the magnitude and direction of

enrichment. A positive NES indicates greater enrichment in the hypoxia group. (d) Pathways found in hypoxia-increased H3K4me peaks were compared with RNA-seq data after 1 h (own data, $n = 3$ biological replicates), 3 h, and 7 days (GSE192969). (e) Individual genes found in hypoxia-increased H3K4me peaks were compared with RNA-seq data after 1 h (own data), 3 h, and 7 days (GSE192969). **Figure S4:** Hypoxia-induced genes in LFD-BMDMs. Clustering analysis was performed for all samples using differentially abundant features in the comparison of Fat Diet 1 h hypoxia vs. Low Fat Diet basal. Features were filtered using an adjusted p -value ≤ 0.01 and a log2 fold change threshold of 0.5 (showing features that were both positive and negative). Prior to plotting, the data were counts per million (CPM)-normalized, log2-transformed, and z -score-transformed. The heatmap shows counts per million (CPM)-normalized, log2-transformed, and z -score-transformed values. The dendrograms show clustering by the Euclidean distance for samples and targets. The analysis was performed using Pluto (<https://pluto.bio>). **Figure S5:** Hypoxia-induced genes in HFD-BMDMs. Clustering analysis was performed for all samples using differentially abundant features in the comparison of Fat Diet 1 h hypoxia vs. High Fat Diet basal. Features were filtered using an adjusted p -value ≤ 0.01 and a log2 fold change threshold of 0.5 (showing features that were both positive and negative). Prior to plotting, the data were counts per million (CPM)-normalized, log2-transformed, and z -score-transformed. The heatmap shows counts per million (CPM)-normalized, log2-transformed, and z -score-transformed values. The dendrograms show clustering by the Euclidean distance for samples and targets. The analysis was performed using Pluto (<https://pluto.bio>). **Figure S6:** Extended Data Figure 6: No changes in glycolytic capacity in normoxic HFD-BMDMs. (a) The glycolytic proton efflux rate (PER) of BMDMs was analyzed using a Seahorse XFe96 analyzer. Rotenone and antimycin A were added to determine compensatory PER following the basal measurement of PER., followed by the addition of 2-deoxy-D-glucose to ensure that the observed PER was caused by glycolysis. (b) The basal and compensatory glycolytic PER were comparable between LFD- and HFD-BMDMs. (c) The oxygen consumption rate (OCR) of BMDMs was analyzed using a Seahorse XFe96 analyzer. Injection of oligomycin, FCCP, and rotenone/antimycin A was utilized to determine the spare, maximal, and non-mitochondrial respiratory capacity. (d) The basal and maximal respiratory capacity was higher in HFD BMDMs compared to LFD. All values are means $\pm$ SEM; n.s., not significant. $n = 3$ biological replicates; statistical significance was determined using Student's t -test. **Figure S7:** Extended Data Figure 7: Inflammatory gene expression after hypoxia in LFD- and HFD-BMDMs. (a) *Il1b* expression 24 h after hypoxia compared with normoxia control ($n = 6$ –9 biological replicates). (b) *Tnfa* expression 24 h after hypoxia compared with normoxia control ($n = 6$ biological replicates). (c) *Il1b*, *Il6*, and *Tnfa* expressions 24 h after hypoxia compared with normoxia control followed by 10 ng/mL LPS stimulation for 4 h ($n = 3$ biological replicates). (d) *Il1b*, *Il6*, and *Tnfa* expressions 24 h after hypoxia compared with normoxia control followed by 10 ng/mL LPS stimulation for 24 h ($n = 3$ biological replicates). (e–g) TNF- α , IL-6, and IL-10 cytokine measured in the cell culture media after 24 h hypoxia compared with normoxia control ($n = 3$ biological replicates). (h–j) Expression of cell surface markers CD86, MHCII, and CD206 after 24 h hypoxia compared with normoxia control measured by flow cytometry ($n = 3$ biological replicates). Relative expression levels vs. LFD controls were calculated using the $\Delta\Delta C_t$ method compared with *hprt* levels. All values are means $\pm$ SEM; n.s., not significant. Statistical significance was determined using two-way ANOVA with Tukey's HSD post hoc test for (a–d) and (e–j). For figures (e–g), statistical significance was determined by one-way ANOVA with Sidak's multiple comparison test. **Figure S8:** Efferocytosis of GM-CSF-differentiated bone marrow-derived DCs from LFD and HFD mice. (a) LFD- and HFD-dendritic cells preconditioned with 1% O₂ for 24 h were tested for efferocytosis. Apoptotic Jurkat cells were stained with pHrodo Green, and the integrated signal intensity was measured using live cell imaging in an environmental chamber (1% O₂) ($n = 3$ mice and at least duplicate cultures). (b) LFD- and HFD-treated dendritic cells without hypoxia were tested for efferocytosis. Apoptotic Jurkat cells were stained with pHrodo Green, and the integrated signal intensity was measured using live cell imaging in an environmental chamber (21% O₂) ($n = 3$ mice and

at least duplicate cultures). Statistical significance was determined using two-way ANOVA with Tukey's HSD post hoc test. All values are means $\pm$ SEM; n.s., not significant. **Figure S9:** Overlap of hypoxia-upregulated genes between BMDMs and HeLa cells. (a) Overlap of hypoxia-upregulated genes between BMDMs and HeLa cells illustrated with a Venn diagram. (b) The gene expression levels in BMDMs of immediately upregulated genes in HeLa cells were determined by qPCR. $n = 3$ biological replicates; statistical significance was determined using two-way ANOVA with Tukey's HSD post hoc test. All values are expressed as mean $\pm$ SEM; * $p < 0.05$. n.s., not significant. **Table S1:** Differentially expressed H3K4me3 peaks after 1 h of hypoxia in LFD-BMDMs. **Table S2:** Gene ontology analysis of differentially expressed H3K4me3 peaks after 1 h of hypoxia in LFD-BMDMs. **Table S3:** RNA-seq of LFD-BMDMs after 1 h hypoxia. **Table S4:** RNA-seq of HFD-BMDMs after 1 h hypoxia. **Table S5:** Peak list of H3K4me3 increases after hypoxia in HeLa cells from Batie et al.