

# Deletion of the *Mint3*/*Apba3* Gene in Mice Abrogates Macrophage Functions and Increases Resistance to Lipopolysaccharide-induced Septic Shock<sup>\*[5]</sup>

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Toshiro Hara<sup>‡</sup>, Kouhei Mimura<sup>‡</sup>, Takaya Abe<sup>§</sup>, Go Shioi<sup>§</sup>, Motoharu Seiki<sup>‡1</sup>, and Takeharu Sakamoto<sup>‡</sup>

From the <sup>‡</sup>Division of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo, Japan and the <sup>§</sup>Laboratory for Animal Resources and Genetic Engineering, RIKEN Center for Developmental Biology, Minatogima Minami, Chuou-ku, Kobe, Japan

Two major metabolic systems are usually used to generate ATP: oxidative phosphorylation (OXPHOS) in the mitochondria and glycolysis. Most types of cells employ OXPHOS for ATP production during normoxia but then shift energy production from OXPHOS to glycolysis when exposed to hypoxia. Hypoxia-inducible factor-1 (HIF-1) is the master transcription factor regulating this metabolic shift. On the other hand, macrophages are unique in making use of glycolysis for ATP generation constitutively even during normoxia. We recently proposed that in macrophages, *Mint3*/*APBA3* inhibits factor inhibiting HIF-1 (FIH-1) during normoxia, which in turn releases the suppression of HIF-1 activity by FIH-1. To demonstrate the physiological function of *APBA3* in macrophages, we established *Apba3*<sup>-/-</sup> mice. The mutant mice presented no apparent gross phenotype but exhibited significant resistance against LPS-induced septic shock. The level of ATP in macrophages obtained from the mutant mice was reduced to 60% of the level observed in wild type cells, which in turn led to reduced ATP-dependent activities such as glycolysis, cytokine production, and motility. We also generated mutant mice with the *Apba3* gene deleted specifically from cells of the myeloid lineage and confirmed that LPS-induced septic shock is mitigated significantly. Thus, we show cell type-specific regulation of energy production by *APBA3* in macrophages using genetically manipulated mice. The specific function of *APBA3* in macrophages might allow us to develop therapeutics to regulate aberrant macrophage function during infection and diseases.

Macrophages are major players in the innate immune system, which regulates early inflammatory responses against invading pathogens (1, 2). Macrophages also play pivotal roles in tissue homeostasis by communicating with cells in wounds.

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<sup>1</sup> To whom correspondence should be addressed: 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. Fax: 81-3-5449-5414; E-mail: mseiki@ims.u-tokyo.ac.jp.

During inflammation, macrophages produce cytokines, growth factors, and reactive oxygen molecules, thereby contributing to the pathology of various disease states (1–3). Inflammation often creates hypoxic conditions that are hazardous for cells. Exposure of cells to hypoxia leads to the induction of a survival program that either allows the cells to escape from such an environment or to adapt to it. In contrast, macrophages have to move into the inflamed tissue actively, despite the toxicity of hypoxia. For this purpose, macrophages employ an oxygen-independent energy production system unlike most other cells.

Although most cells produce ATP via oxidative phosphorylation (OXPHOS),<sup>2</sup> this process becomes inefficient when the oxygen supply is limited (4). To adapt to hypoxia, cells shift energy production from OXPHOS to glycolysis (anaerobic glycolysis), an oxygen-independent means of ATP production. Macrophages, however, are unique in that they make use of glycolysis for ATP production constitutively even during normoxia (aerobic glycolysis), and OXPHOS activity in macrophages is low (5, 6). The glucose analog 2-deoxyglucose (2-DG) inhibits glycolysis and ATP production in macrophages, whereas the OXPHOS inhibitor oligomycin, which inhibits ATP synthetase, does not (6). Reduced ATP production attenuates various macrophage functions such as motility, invasion, phagocytosis, and cytokine production (5, 6). Constitutive use of aerobic glycolysis for ATP enables macrophages to move into hypoxic inflammatory sites without the need for adaptation. Cellular glycolytic activity is correlated with the level of expression of the enzymes of the metabolic pathway, as well as of transporters of relevant substrates and metabolites. The transcription factor HIF-1 regulates the expression of a set of glycolysis-related genes and serves as a master regulator of the cellular adaptation program to hypoxia (7, 8). However, HIF-1 has been shown to be active in macrophages even during normoxia.

HIF-1 is a heterodimeric transcription factor comprising a regulatory  $\alpha$  and a nonregulatory  $\beta$  subunit (7–9). HIF-1 binds to hypoxia response elements localized within the promoter regions of target genes. The HIF-1 $\alpha$  gene is transcribed and

<sup>2</sup> The abbreviations used are: OXPHOS, oxidative phosphorylation; HIF-1, hypoxia-inducible factor-1; FIH-1, factor inhibiting HIF-1; 2-DG, 2-deoxyglucose; CBP, cAMP-responsive element-binding protein-binding protein; MT1-MMP, membrane type 1 matrix metalloproteinase; MEF, mouse embryonic fibroblast; BMDM, bone marrow-derived macrophage; GLUT-1, glucose transporter-1; PGK-1, phosphoglycerate kinase 1; NT, N-terminal.

translated nearly constitutively in many types of cells including macrophages, but the protein level is usually low during normoxia because of continuous degradation. Under oxygen-rich conditions, prolyl hydroxylases modify proline residues in the oxygen-dependent degradation domain of HIF-1 $\alpha$  and thereby generate recognition sites for the Von Hippel-Lindau protein. The Von Hippel-Lindau protein possesses E3 ubiquitin ligase activity and leads to proteasomal degradation of ubiquitinated HIF-1 $\alpha$ . Because prolyl hydroxylases require oxygen for hydroxylation (8), their activity is reduced during hypoxia, and degradation of HIF-1 $\alpha$  protein is prevented. The transcription stimulatory activity of residual HIF-1 $\alpha$  protein during normoxia is further suppressed by another oxygen sensor protein. HIF-1 $\alpha$  stimulates transcription via a C-terminal activation domain that binds p300/CBP. However, an asparagine residue (Asn-803) within the C-terminal activation domain can be hydroxylated by factor inhibiting HIF-1 (FIH-1) during oxygen-rich conditions, thereby preventing binding to p300/CBP (10, 11). During hypoxia, FIH-1 is inactive, and HIF-1 becomes active.

Despite the fact that HIF-1 $\alpha$  is inhibited in most types of cells by two independent mechanisms in an oxygen-sensitive manner, it nevertheless is able to drive aerobic glycolysis even during normoxia in macrophages. Indeed, genetic loss of HIF-1 $\alpha$  in macrophages reduces ATP production to 20% of wild type cells (5). This reduction in ATP content causes a severe defect in energy-dependent macrophage functions. However, the mechanism by which the activity of HIF-1 is maintained in macrophages during normoxia remained to be determined until recently. We proposed a mechanism whereby inactivation of FIH-1 by two key factors, membrane type 1 matrix metalloproteinase (MT1-MMP/MMP14) and APBA3/Mint3, leads to activation of HIF-1 during normoxia (12–14). MT1-MMP is an invasion-promoting membrane protease that degrades the extracellular matrix and is frequently expressed in malignant tumor cells and macrophages (15–17). The cytoplasmic tail of MT1-MMP binds FIH-1 and recruits cytoplasmic FIH-1 to the Golgi membrane where MT1-MMP resides until it is exported to the cell surface (14). APBA3/Mint3 was originally identified based on its ability to bind Alzheimer disease  $\beta$ -amyloid precursor protein (18, 19). APBA3/Mint3 localizes to the Golgi membrane by associating with membrane proteins such as  $\beta$ -amyloid precursor protein and furin (13, 20). In this study, we refer to APBA3 rather than Mint3, based on its gene name, *Apha3*. We showed previously that APBA3 can bind and inhibit FIH-1 in a stable complex (13). APBA3 competes with HIF-1 $\alpha$  for binding to FIH-1 and thereby inhibits the hydroxylation of HIF-1 $\alpha$  by the latter. Inhibition of FIH-1 by APBA3 is dependent upon the expression of MT1-MMP in macrophages (14). Therefore, inhibition of FIH-1 is thought to be accomplished by the following two steps. First, the cytoplasmic tail of MT1-MMP binds FIH-1 and recruits cytoplasmic FIH-1 to the Golgi. Second, FIH-1 is presented to APBA3, which is localized in close proximity to MT1-MMP.

We have already reported that MT1-MMP is essential to the maintenance of HIF-1 activity in macrophages, based on data generated using MT1-MMP-deficient mice (*Mmp14*<sup>-/-</sup>) (14, 17). Macrophage infiltration into acutely inflamed tissues was

abrogated in *Mmp14*<sup>-/-</sup> mice (17), whereas infiltration into more chronic sites of inflammation was unaffected (21). Macrophages isolated from the knock-out mice exhibited reduced levels of ATP (40% of wild type levels), lower glycolytic activity, expression of HIF-1 target genes, and increased FIH-1 activity (14). On the other hand, APBA3-deficient mice were generated by another group and reported to exhibit no apparent phenotype (22). Therefore, we established our own *Apha3*<sup>-/-</sup> mice to analyze the physiological significance of APBA3 for energy-dependent macrophage functions. We also generated a conditional knock-out of the *Apha3* gene in cells of the myeloid lineage so as to demonstrate that the resulting phenotype could be attributed to macrophages.

## EXPERIMENTAL PROCEDURES

**Generation of *Apha3* Mutant Mice**—Targeting vector was prepared, homologous recombinant embryonic stem cells were isolated, and *Apha3* mutant mice (accession number CDB0589K) were generated as described at the RIKEN Laboratory for Animal Resources and Genetic Engineering website. LysM-Cre and CAG-Cre transgenic mice were purchased from the Jackson Laboratory. CAG-FLPe transgenic mice (23) were purchased from RIKEN Bio Resource Center. The experiments were conducted according to the institutional ethical guidelines for animal experiments and the safety guidelines for gene manipulation experiments (The Institute of Medical Science, University of Tokyo).

**Genotyping**—The probe sequences used in Southern blot analysis and primers used in PCR analysis are listed in [supplemental Table S1](#).

**Cell Culture**—Bone marrow-derived macrophages were obtained as described previously (13, 14, 17). In brief, bone marrow was obtained from the tibia, femur, and humerus via flushing with PBS. The collected cells were then cultured in DMEM (Sigma) supplemented with 20% FBS and 30% L929-conditioned medium, after which they were grown at 37 °C in humidified 5% CO<sub>2</sub> for 7 days. The macrophages were rendered quiescent by culturing them overnight in medium lacking the L929-conditioned medium. Mouse embryonic fibroblasts (MEFs) were prepared as described previously (24) and cultured in DMEM supplemented with 10% FBS. Peritoneal macrophages and neutrophils were collected from mice intraperitoneally injected with 4.05% thioglycorate medium (Invitrogen) 4 h or 4 days after thioglycorate injection, respectively.

**Knockdown Experiments Using shRNAs**—shRNA sequences used for knockdown of target proteins were as follows: mouse FIH-1 sequence 1, 5'-caccggacctgaatacctgcaagacgaatctgcagg-tattcagggtccttt-3' and sequence 2, 5'-caccggaagattgtcatggactctc-gaaagaagtcctatgacaatcttcc-3'. These sequences were subcloned into the pENTR/U6 TOPO vector (Invitrogen) and then transferred by recombination into the lentivirus vector pLenti6 BLOCKiT. shRNA-expressing lentiviral vectors were generated and used according to the manufacturer's instructions.

**Preparation of Lentiviral Vectors**—Expression construct for APBA3-NT (encoding 1–214 amino acids) was prepared using a PCR-based method. A lentiviral vector carrying cDNA of APBA3-NT for expression was constructed using the ViraPower<sup>TM</sup> lentiviral expression system (Invitrogen). Lentiviruses in

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the culture medium were recovered from the cleared supernatant following centrifugation of the medium at  $1,400 \times g$ . Supernatants were passed through membrane filters (0.45- $\mu\text{m}$  pore size; Millipore), followed by two rounds of centrifugation at  $70,000 \times g$  for 2 h at 21 °C. The resulting pellet was resuspended in 200  $\mu\text{l}$  of DMEM, and the virus titer was measured using HeLa cells. Infected cells were selected by blasticidin resistance and counted. Lentivirus vectors were transduced into macrophages at a multiplicity of infection of 3.

**Invasion and Migration Assays**—Matrigel invasion and Transwell® migration assays were performed as described previously (17). Briefly, Transwells® with 8- $\mu\text{m}$  pore size filters (Corning) covered with or without Matrigel (Becton Dickinson) were inserted into 24-well plates. DMEM (500  $\mu\text{l}$ ) containing 10 ng  $\text{ml}^{-1}$  MCP-1 (R & D Systems) was added to the lower chamber, whereas a 200- $\mu\text{l}$  cell suspension ( $5 \times 10^4$  cells) was placed in the upper chamber. The plates were incubated at 37 °C in a 5%  $\text{CO}_2$  atmosphere for 2 and 6 h, for the migration and invasion assays, respectively. The cells in the lower chamber were then stained with Giemsa solution and counted. For the peritoneal exudation assay, the mice were injected intraperitoneally with 4.05% thioglycorate medium, and peritoneal exudate macrophages were counted 4 days after thioglycorate injection.

**Immunoblotting**—The cells were lysed with lysis buffer and centrifuged at  $20,000 \times g$  for 15 min at 4 °C. The supernatants were collected, and total protein content was measured using the Bradford assay (Bio-Rad). Lysates were separated by SDS-PAGE, transferred to membrane filters, and subjected to immunoblotting using an anti-APBA3/Mint3 mouse antibody (BD Biosciences), anti-FIH-1 goat antibody (Santa Cruz Biotechnology), and anti-actin mouse antibody (Millipore).

**Measurement of Lactic Acid Levels**—The cells were seeded onto 24-well plates ( $1 \times 10^5$ /well) in triplicate. Conditioned medium was collected after 6 h, and lactic acid was measured using a L-lactic acid kit (R-Biopharm), and the values were normalized to the protein concentrations determined using a Bradford assay kit (Bio-Rad).

**Measurement of ATP Concentrations**—The cells were cultured in the presence or absence of the glycolysis inhibitor 2-deoxyglucose (200  $\mu\text{g}/\text{ml}$ ; Sigma) or the OXPHOS inhibitor oligomycin (5  $\mu\text{g}/\text{ml}$ ; Sigma) for 2 h, and ATP levels were determined using the ATP bioluminescence assay kit CLS II (Roche Applied Science). ATP levels were normalized to the total protein concentration, determined using a Bradford assay kit (Bio-Rad).

**RNA Isolation, Reverse Transcription, and Real Time PCR**—Total RNA isolation, reverse transcription, and real time PCR were performed as previously described (13, 14). The following specific primers were used:  $\beta$ -actin sense, 5'-gccaacacagtgtctgtctgg-3';  $\beta$ -actin antisense, 5'-atctgctggaaggtggacag-3'; glucose transporter-1 (GLUT-1) sense, 5'-gggcatgtgtccagatgt-3'; GLUT-1 antisense, 5'-acgaggagcaccgtgaagat-3'; phosphoglycerate kinase 1 (PGK-1) sense, 5'-tgtcgttccaacaagctg-3'; and PGK-1 antisense, 5'-gggtgctcataaggacaacg-3'.

**LPS-responsive Cytokine Production Assay**—For *in vitro* analysis of cytokine production, macrophages were seeded ( $1 \times 10^5$ /well) into 96-well plates and stimulated with 100 ng  $\text{ml}^{-1}$

LPS. Supernatants of the macrophage cultures were collected at the indicated time, and TNF- $\alpha$  and IL-12 p70 levels were measured using the TNF- $\alpha$  ELISA kit (AssayPro) or the IL-12 p70 ELISA kit (Boster Biological Technology), respectively. For *in vivo* analysis, serum was obtained at the indicated time after LPS injection and subjected to the ELISA assay.

**LPS-induced Septic Shock Model**—Mice were injected intraperitoneally with LPS (15 mg  $\text{kg}^{-1}$  bodyweight) and their survival was analyzed. In some experiments, the mice were injected intraperitoneally with 2-DG (500 mg  $\text{kg}^{-1}$  bodyweight), or vehicle was injected 1 h before the LPS injection.

**Statistical Analysis**—We compared two subject groups by the two-sided *t* test or the logrank test.

## RESULTS

**Establishment of APBA3-deficient Mice**—APBA3-deficient mice were established as follows. First, we prepared embryonic stem cells with loxP sequences inserted into introns 1 and 3 of the *Apba3* allele and generated mice of the same genotype (Fig. 1A and supplemental Fig. S1). Next, we crossed these mice with CAG-Cre mice, which express Cre recombinase ubiquitously, so as to cause deletion of exons 2 and 3 of the *Apba3* allele. Among the resulting offspring, mice carrying the CAG-Cre transgene and deleted for the *Apba3* allele (CAG-Cre; *Apba3*<sup>+/-</sup>) were crossed with wild type mice so as to remove the CAG-Cre transgene. Then the *Apba3*<sup>+/-</sup> mice were crossed with wild type C57/BL6 mice for 12 generations before the experiments, and the WT and *Apba3*<sup>-/-</sup> littermates of *Apba3*<sup>+/-</sup> parents were used for the subsequent analyses. Deletion of the *Apba3* allele was confirmed by Southern blot analysis (Fig. 1B). We next examined expression of APBA3 in the brain, liver, kidney, and muscle tissues of *Apba3*<sup>+/+</sup> (WT) and *Apba3*<sup>-/-</sup> mice (Fig. 1C). APBA3 was expressed in all of the WT tissues tested but not in tissues obtained from the mutant mice. *Apba3*<sup>-/-</sup> mice were segregated according to the Mendelian ratio, and the KO mice showed no apparent gross phenotype, such as any defects in development, survival, or fertility, as has been reported previously (data not shown) (22).

**APBA3-deficient Mice Exhibit Resistance to LPS-induced Septic Shock**—Next, we analyzed the mutant mice by focusing on the function of macrophages. Macrophages are important for the host defense against pathogens (1, 2). However, hyperactivation of the cells sometimes causes a cytokine storm that results in septic shock (25). To examine the role of APBA3 in this process, we injected LPS intraperitoneally into WT and *Apba3*<sup>-/-</sup> mice, and we monitored the induction of septic shock. WT mice started to die 24 h after the injection and survival decreased thereafter to 10% at 96 h (Fig. 2A, WT). Meanwhile, survival of the mutant mice decreased to 50% at 120 h but did not decrease further after this point (Fig. 2A, *Apba3*<sup>-/-</sup>). Because TNF- $\alpha$  is a trigger for the cytokine storm (25), we measured its serum concentration and found that it increased sharply 1 h after LPS injection and then decreased gradually in WT mice (Fig. 2B). However, the peak levels of TNF- $\alpha$  in the mutant mice were ~50% of the values observed in WT mice (Fig. 2B). Next, we analyzed serum levels of a subunit of IL-12 (IL-12 p70), which is also a key factor for the induction of the cytokine storm and lethality of endotoxin shock (26). A spike in

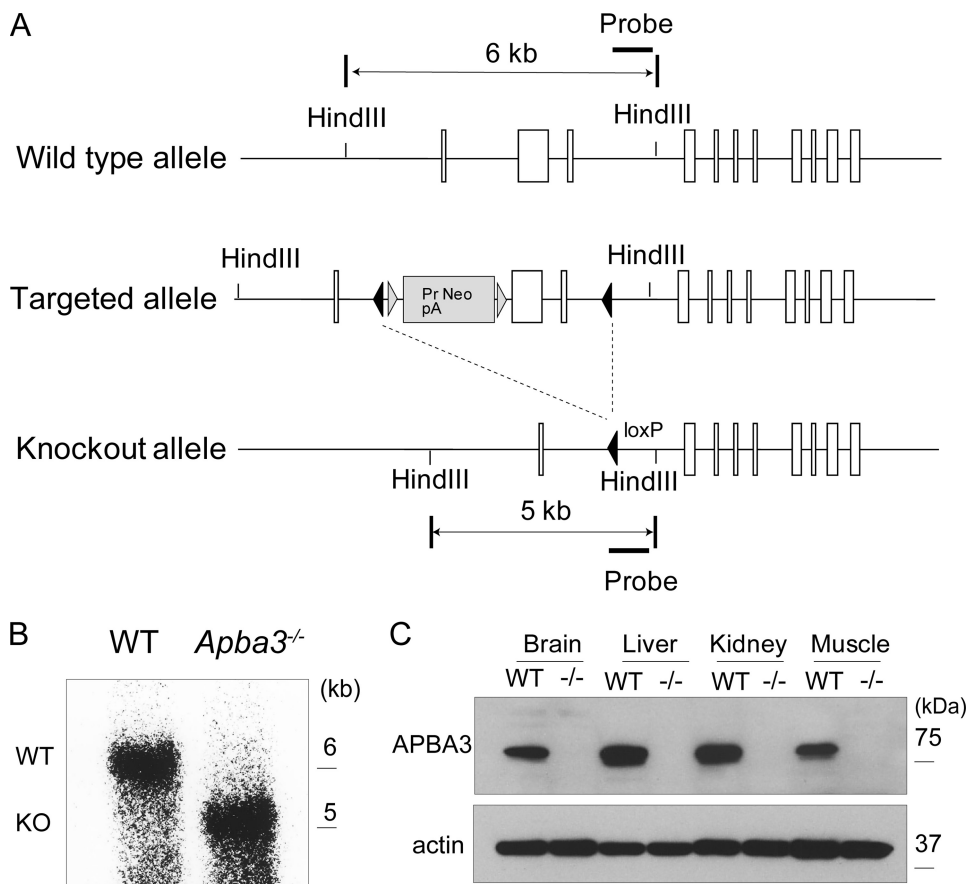


FIGURE 1. **Generation of *Apba3*<sup>-/-</sup> mice.** A, genomic structure of the murine *Apba3* locus and targeted allele before and after Cre-mediated excision of the Neo cassette and exons 2 and 3. B, Southern blot analysis of HindIII-digested genomic DNA isolated from the tail of wild type and *Apba3*<sup>-/-</sup> littermate mice. C, immunoblot analysis of lysates prepared from brain, liver, kidney, and muscle of wild type and *Apba3*<sup>-/-</sup> littermate mice.

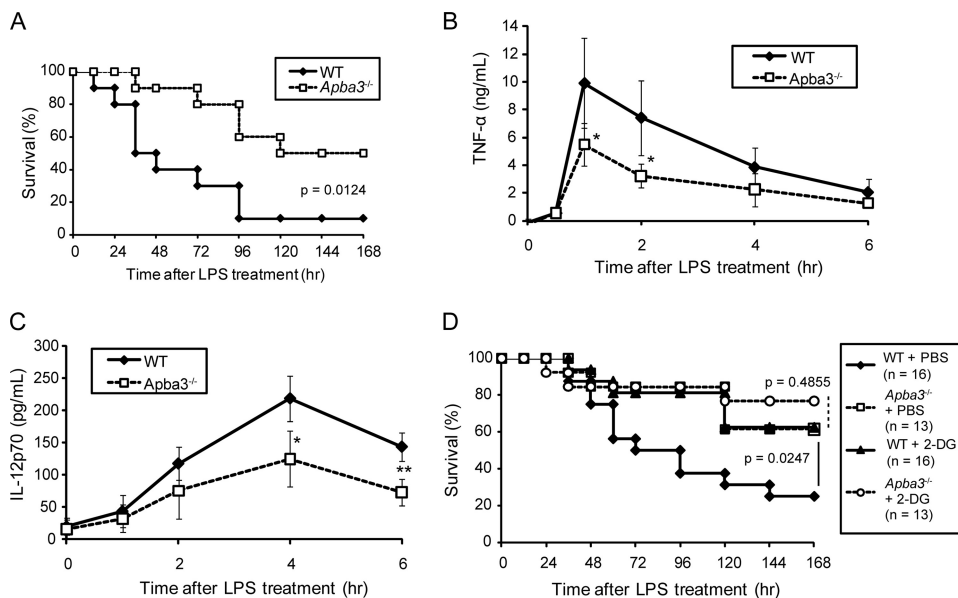


FIGURE 2. **APBA3 augments LPS-induced septic shock.** A, survival of WT ( $n = 10$ ) and *Apba3*<sup>-/-</sup> mice ( $n = 10$ ) after LPS injection. B and C, TNF- $\alpha$  (B) and IL-12 p70 (C) in serum from WT and *Apba3*<sup>-/-</sup> mice were measured at the indicated time after LPS injection. D, administration of 2-DG prior to LPS injection increases survival in WT mice ( $n = 16$ ) to a level similar to that observed in *Apba3*<sup>-/-</sup> mice ( $n = 13$ ). The data in A and D were analyzed using the logrank test. The data in B and C were analyzed using the Student's *t* test. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

IL-12 p70 levels was observed following the increase in TNF- $\alpha$  expression that reached a peak around 4 h after injection of LPS. However, the peak levels of IL-12 p70 in the serum of the

mutant mice reached only ~50% of that observed in the WT mice (Fig. 2C). These results indicate that compared with wild type mice, *Apba3*<sup>-/-</sup> mice are resistant to LPS-induced septic

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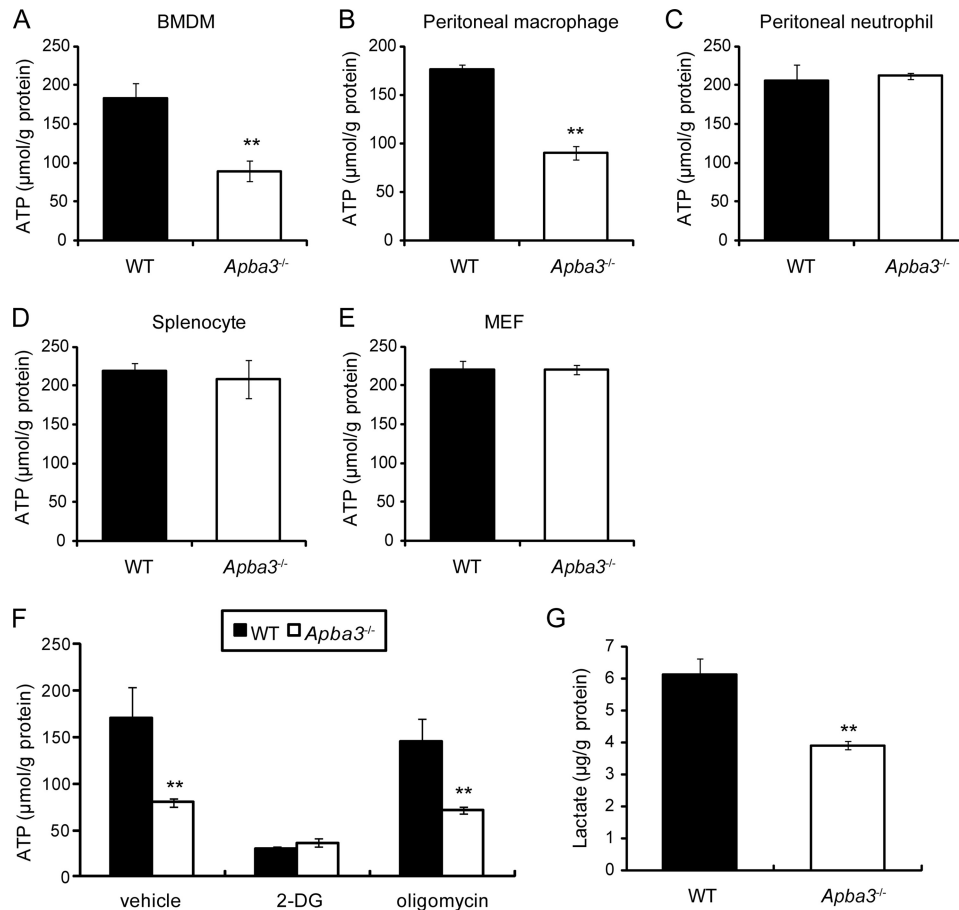


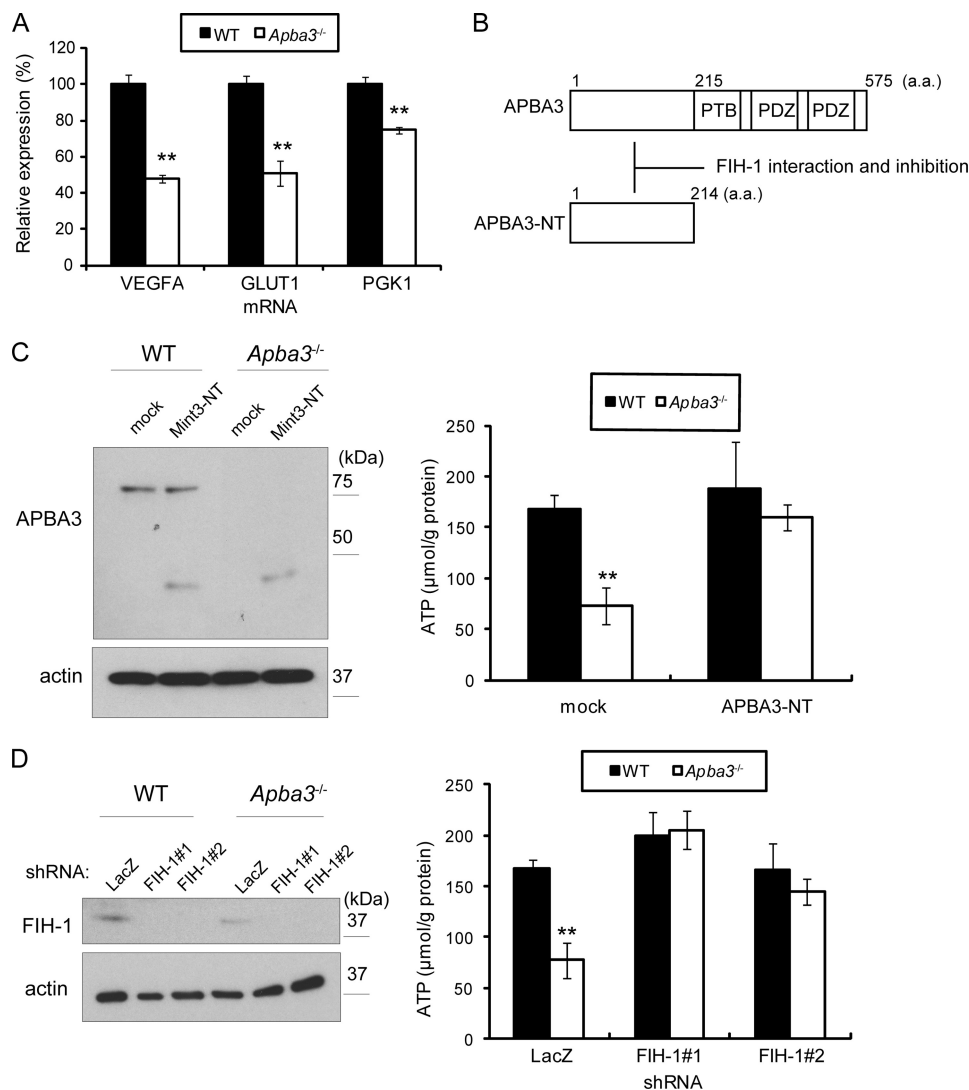
FIGURE 3. **Apba3<sup>-/-</sup> macrophages are defective in glycolysis.** A–E, ATP content of BMDMs (A), peritoneal macrophages (B), peritoneal neutrophils (C), splenocytes (D), and MEF (E) from WT and Apba3<sup>-/-</sup> mice. F, ATP contents of WT and Apba3<sup>-/-</sup> macrophages treated with vehicle, 2-DG, or oligomycin. G, lactate production in WT and Apba3<sup>-/-</sup> macrophages. The data in A–G were analyzed using the Student's *t* test. \*\*, *p* < 0.01.

shock, likely because of a defect in the production of the relevant cytokines. To examine whether the resistance to LPS-induced septic shock in the mutant mice is correlated to the glycolytic activity, we injected 2-DG intraperitoneally into the mice prior to LPS injection, and we monitored the lethality of the WT and mutant mice. In WT mice, the survival of the vehicle-treated group was 25%, and this effect was mitigated by 2-DG preadministration (61.5% survival; Fig. 2D). However, 2-DG preadministration had no effect on survival of the mutant mice. Therefore, induction of septic shock in WT mice is presumably mediated by cells that make use of APBA3-dependent glycolysis for ATP production. We observed that 2-DG treatment of WT mice decreased the TNF- $\alpha$  concentration in the serum (supplemental Fig. S2). Taken together, it is likely that the septic shock induced by LPS is mediated by cytokines produced by cells that make use of APBA3 to maintain glycolytic activity for energy production.

**Macrophages Derived from APBA3-deficient Mice Produce Less ATP**—The mutant mice showed a phenotype that appears to be attributable to defective macrophage function. To confirm this further, we analyzed macrophages and other types of cells isolated from the WT and mutant mice. Bone marrow-derived macrophages (BMDMs) and peritoneal macrophages from the mutant mice showed an ~40% reduction of ATP content compared with cells from WT mice (Fig. 3, A and B). How-

ever, no such difference in ATP levels was observed in peritoneal neutrophils, splenocytes, or MEFs (Fig. 3, C–E). Treatment of either mutant or WT BMDM with oligomycin had no effect upon ATP levels, whereas 2-DG treatment severely inhibited ATP production (Fig. 3F). The glycolytic activity of BMDMs was estimated by measurement of lactate production, with WT exhibiting a greater level of production than mutant cells (Fig. 3G). Thus, we have shown that APBA3 regulates ATP production in a cell type-specific fashion.

**FIH-1 Suppresses ATP Production in APBA3-deficient Macrophages**—As predicted by the proposed mechanism by which APBA3 inhibits FIH-1 and activates HIF-1 in WT macrophages (13), mutant macrophages lacking APBA3 expressed lower levels of HIF-1 target genes than did WT cells, based on an analysis of the mRNAs encoding VEGF, GLUT-1, and PGK-1 (Fig. 4A). APBA3 contains phosphotyrosine-binding and PDZ domains at the C terminus that mediate binding to membrane proteins such as  $\beta$ -amyloid precursor protein (Fig. 4B) (27). The N-terminal fragment of APBA3 (APBA3-NT) was previously demonstrated to be sufficient to inhibit FIH-1 (13). Therefore, we compared the effect of expression of APBA3-NT in WT and mutant macrophages (Fig. 4C). Expression of APBA3-NT in the mutant macrophages restored ATP content to the levels observed in WT cells (Fig. 4C). Consistent with the observation that FIH-1 is activated in APBA3-deficient macrophages,



**FIGURE 4. APBA3 regulates ATP production through an effect upon the FIH-1/HIF-1 axis.** *A*, Apba3<sup>-/-</sup> macrophages express lower levels of HIF-1 target genes. Expression of mRNAs encoding VEGFA, GLUT-1, and PGK-1 was measured by RT-PCR in both WT and Apba3<sup>-/-</sup> macrophages. *B*, domain structure of APBA3. APBA3 consists of an NT portion that binds FIH-1, a phosphotyrosine-binding (PTB) domain, and two PDZ domains. Amino acid positions are indicated. The NT portion is sufficient to inhibit FIH-1. *C*, forced expression of APBA3-NT stimulates ATP production in Apba3<sup>-/-</sup> macrophages to a level similar to that observed in WT cells. Protein expression was confirmed by Western blot (*left panel*). *D*, FIH-1 activity is reduced in WT macrophages compared with that observed in Apba3<sup>-/-</sup> cells. FIH-1 protein was detected by Western blot as in the *left panel*. Expression of FIH-1 was knocked down using two shRNA sequences (#1 and #2). Down-regulation of FIH-1 results in greater ATP production (*right panel*). Note that the level of ATP in WT cells is comparable with that observed in the FIH-1 knockdown cells. shRNA targeted to the LacZ mRNA (shLacZ) served as a negative control. The data in A–C were analyzed using the Student's *t* test. \*\*, *p* < 0.01.

depletion of FIH-1 in the mutant macrophages using targeted shRNAs gave rise to a restoration of ATP content similar to that observed following expression of APBA3-NT (Fig. 4D). Secretion of cytokines from macrophages also consumes ATP, and consistent with this, we observed that mutant macrophages stimulated with LPS released lower levels of TNF- $\alpha$  and IL-12 p70 than did the WT cells (Fig. 5).

**APBA3-deficient Macrophages Exhibit Reduced Motility and Invasiveness**—Reflecting the shortage of ATP, the mutant macrophages exhibited reduced motility in the Boyden chamber assay (Fig. 6A) and reduced invasion into Matrigel (Fig. 6B). Expression of APBA3-NT restored the motility of the mutant macrophages but had no effect upon WT macrophages (Fig. 6C). Even *in vivo*, ~50% fewer mutant than WT macrophages migrated into the peritoneal cavity following thioglycolate treatment (Fig. 6D).

**Myeloid Cell-specific APBA3 Deficiency in Mice Mitigates Septic Shock**—Finally, we evaluated whether the observed resistance to septic shock in the mutant mice could be attributed to defective macrophage functions. For this purpose, we deleted the *Apba3* gene specifically in the myeloid lineage. We crossed mice with a targeted allele in the *Apba3* locus (Fig. 7A, *Targeted allele*) with CAG-FLPe transgenic mice (23) so as to excise the Neo cassette flanked by FRT sequences (supplemental Fig. S3). The resulting mice carrying a floxed *Apba3* allele (Apba3<sup>+/<sup>fl</sup></sup>) (Fig. 7A, *Floxed allele*) and a CAG-FLPe transgene were crossed with WT mice to remove the CAG-FLPe transgene. Then the Apba3<sup>+/<sup>fl</sup></sup> mice were crossed with WT C57/BL6 mice over 12 generations for further experiments. Mice deficient for APBA3 in myeloid cells were prepared by crossing LysM-Cre transgenic mice with Apba3<sup>fl/fl</sup> mice. We confirmed the excision of the floxed *Apba3* allele in the macrophages but

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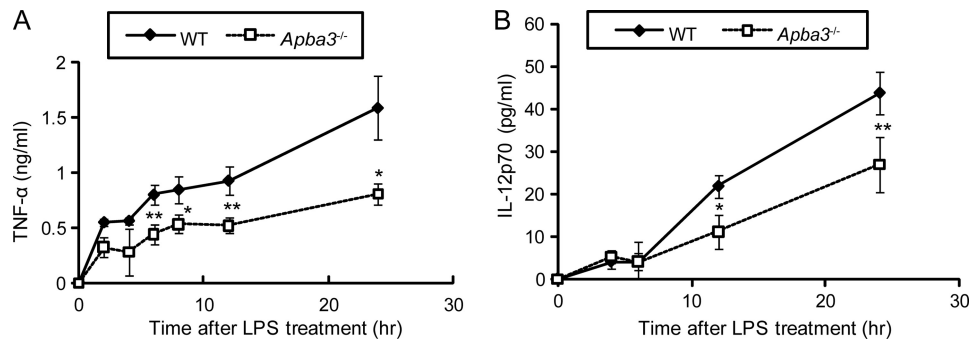


FIGURE 5. **APBA3 regulates LPS-responsive cytokine production in macrophages.** Apba3<sup>-/-</sup> macrophages are defective in cytokine production in response to LPS. TNF-α (A) and IL12p70 (B) production in WT and Apba3<sup>-/-</sup> macrophages was measured at the indicated time after LPS stimulation. The data in A and B were analyzed using the Student's *t* test. \*, *p* < 0.05; \*\*, *p* < 0.01.

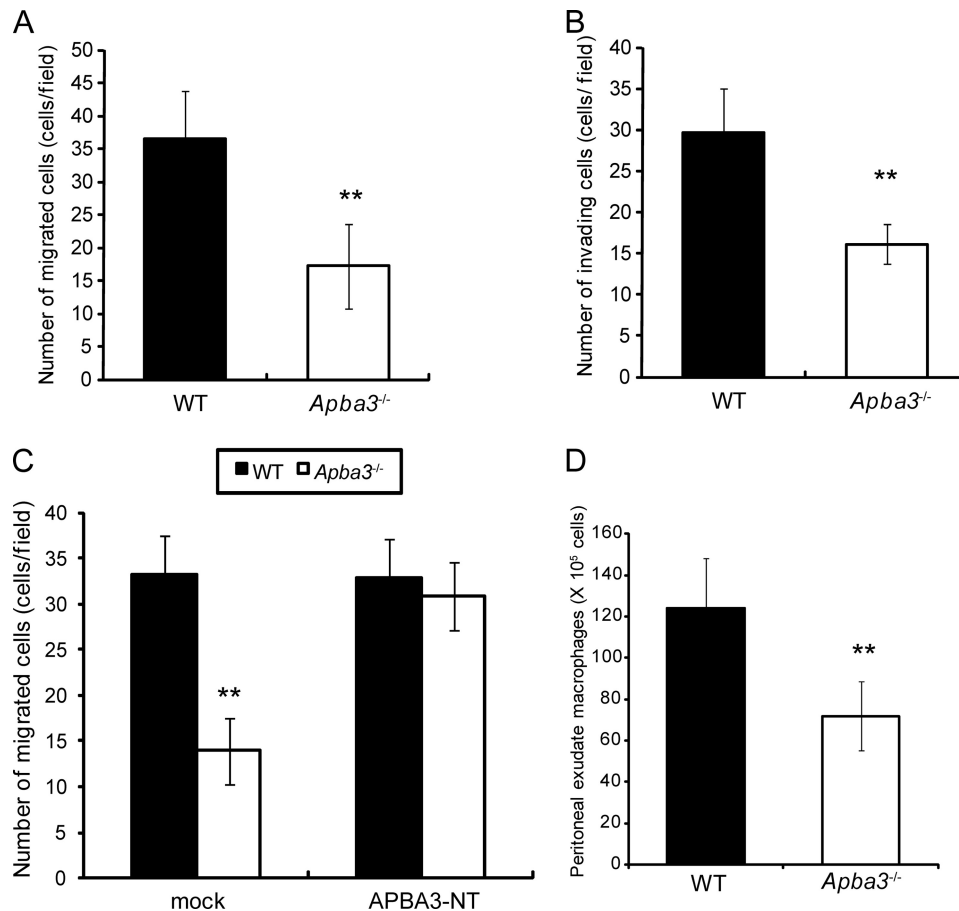


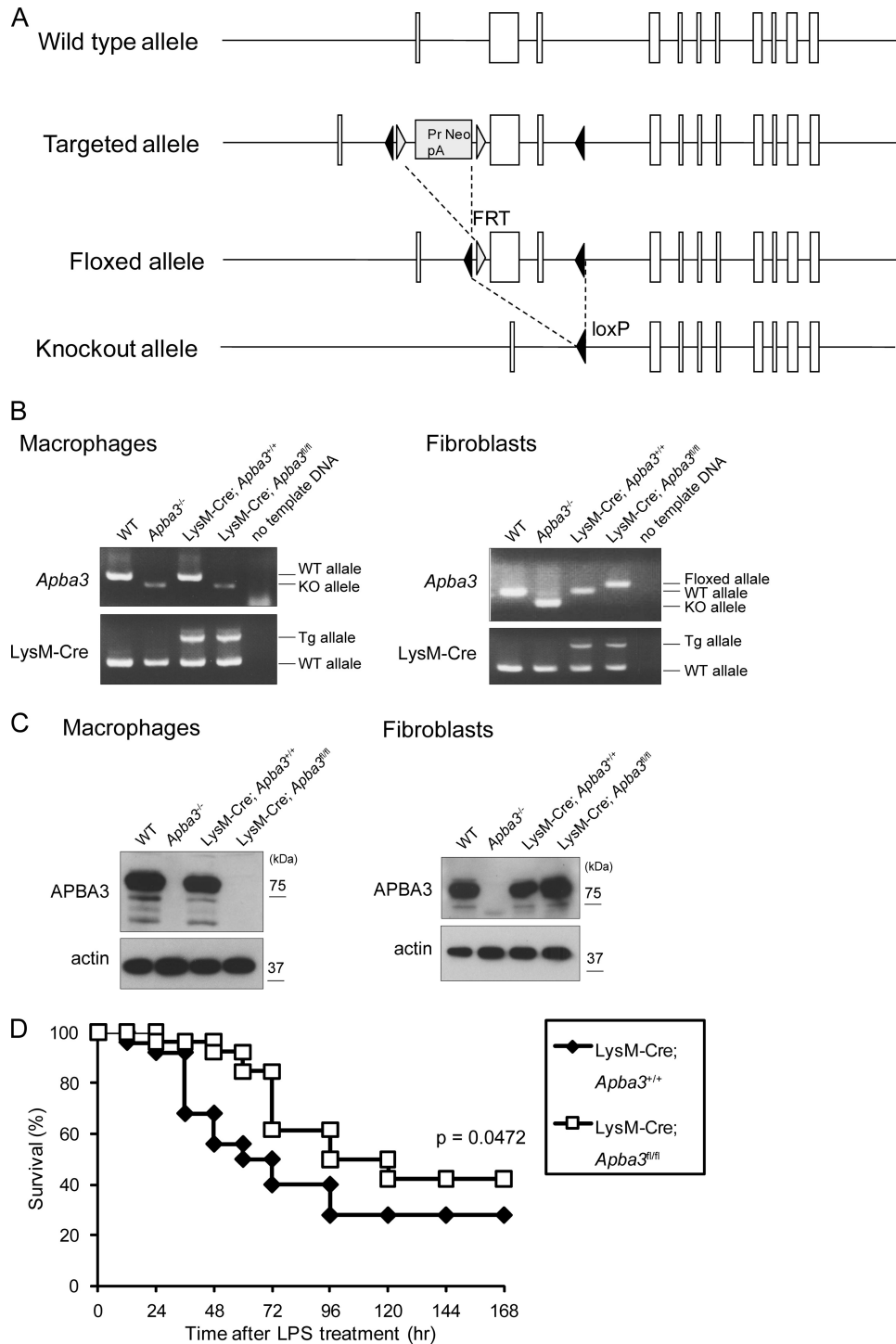
FIGURE 6. **APBA3 regulates motility and invasive activity in macrophages.** A and B, motility (A) and invasive activity (B) of WT and Apba3<sup>-/-</sup> macrophages. C, forced expression of APBA3-NT restores motility in Apba3<sup>-/-</sup> macrophages to a level similar to that observed in WT cells. D, peritoneal exudate macrophages in WT and Apba3<sup>-/-</sup> mice were counted 4 days after the intraperitoneal injection of thioglycorate medium. The data in A–D were analyzed using the Student's *t* test. \*, *p* < 0.05; \*\*, *p* < 0.01.

not the fibroblasts isolated from the tails of LysM-Cre, Apba3<sup>fl/fl</sup> mice (Fig. 7B). Immunoblot analysis also revealed the loss of APBA3 protein in macrophages but not in fibroblasts isolated from LysM-Cre, Apba3<sup>fl/fl</sup> mice (Fig. 7C). Then we subjected LysM-Cre, Apba3<sup>fl/fl</sup> mice and LysM-Cre, Apba3<sup>+/+</sup> mice (control) to LPS-induced septic shock. Twenty-eight percent of the control mice had survived 168 h after LPS injection, whereas 42.3% of the LysM-Cre, Apba3<sup>fl/fl</sup> mice survived (Fig. 7D). Apba3<sup>fl/fl</sup> mice showed a comparable rate of survival as the LysM-Cre mice (data not shown). These results indicate that APBA3 expressed in myeloid cells

including macrophages plays a significant role in the LPS-induced septic shock *in vivo*.

## DISCUSSION

In this study, we generated Apba3<sup>-/-</sup> mice and myeloid lineage-specific, conditional knock-out mice to examine the role of APBA3 in regulating FIH-1 in macrophages in a cell type-specific manner. As with the Apba3<sup>-/-</sup> mice previously generated by another group, we did not observe any gross abnormality in the mutant mice. However, they did exhibit a significant resistance to septic shock induced by LPS treatment. Serum



**FIGURE 7. Conditional knock-out of APBA3 in myeloid cells increases resistance to LPS-induced septic shock.** *A*, genomic structure of the murine *Apba3* locus and targeting allele before and after FLPe-mediated excision of the Neo cassette and after Cre-mediated recombination. *B*, PCR analysis of WT; *Apba3*<sup>-/-</sup>; LysM-Cre, *Apba3*<sup>+/+</sup>; and LysM-Cre, *Apba3*<sup>fl/fl</sup> macrophages (*left panel*) and fibroblasts (*right panel*). *C*, Western blot analysis of WT; *Apba3*<sup>-/-</sup>; LysM-Cre, *Apba3*<sup>+/+</sup>; and LysM-Cre, *Apba3*<sup>fl/fl</sup> macrophages (*left panel*) and fibroblasts (*right panel*). *D*, survival of LysM-Cre;*Apba3*<sup>+/+</sup> ( $n = 25$ ) and LysM-Cre, *Apba3*<sup>fl/fl</sup> mice ( $n = 26$ ) after LPS injection. The data in *D* were analyzed using the logrank test.

levels of TNF- $\alpha$  and IL-12 p70 were lower in the mutant mice than in the WT mice. 2-DG treatment increased resistance to septic shock in the WT but not the mutant mice. Therefore, cells secreting cytokines dependent upon ATP generated by APBA3-dependent glycolysis play crucial roles in the LPS-induced cytokine storm in WT mice. Macrophages derived from the mutant mice exhibited reduced ATP content, expression of

HIF-1 target genes, glycolytic activity, cytokine production, motility, and invasiveness compared with cells from WT mice. Finally, we confirmed that lethality of septic shock was also mitigated in myeloid lineage-specific conditional KO mice. Thus, we have demonstrated the physiological importance of APBA3 in the regulation of HIF-1 in macrophages. Because we have previously demonstrated that MT1-MMP regulates HIF-1



## APBA3 Promotes Macrophage Function

in the mouse (14, 17), the physiological importance of the proposed MT1-MMP/APBA3/FIH-1 axis in the regulation of HIFs has been shown using both MT1-MMP- and APBA3-deficient mouse models.

In addition to HIF-1, macrophages also express HIF-2, which shares common but not identical target genes. Both HIF-1 $\alpha$  and HIF-2 $\alpha$  subunits also share the same oxygen-dependent regulatory proteins. Prolyl hydroxylases hydroxylate two proline residues within the oxygen-dependent degradation domain of HIF-2 $\alpha$ , as is observed for HIF-1 $\alpha$ , although HIF-2 $\alpha$  is less susceptible to modification when oxygen is moderately abundant (28, 29). This differential sensitivity presumably derives from differences in amino acid sequence surrounding the proline residues. FIH-1 also hydroxylates an asparagine residue in the C-terminal activation domain of HIF-2 $\alpha$ , although HIF-2 $\alpha$  is also less sensitive to this modification than HIF-1 $\alpha$  (30). Because APBA3 inhibits FIH-1, deficiency of the former is expected to affect both HIF-1 and HIF-2 via FIH-1. Despite the similarity between HIF1 $\alpha$  and HIF-2 $\alpha$  in structure and regulation, their physiological functions differ during development and in adult somatic tissues. Glycolysis in macrophage is one such example. Although HIF-1 $\alpha$ -deficient macrophages exhibit a 20% reduction in ATP production (5), HIF-2 $\alpha$ -deficient macrophages do not exhibit any alteration in ATP levels (31). Thus, glycolytic activity in macrophages appears to be maintained primarily by the activity of HIF-1. Thus, the effect of APBA3 deficiency on ATP production in macrophages is largely mediated by its effect on HIF-1 $\alpha$ .

Neutrophils also belong to the myeloid lineage and employ glycolysis for the production of ATP. HIF-1 $\alpha$ -deficient neutrophils and macrophages are reported to exhibit decreased ATP production caused by decreased glycolysis (5). However, APBA3-deficient neutrophils did not exhibit any alteration in ATP content, whereas APBA3 deficient macrophages did (Fig. 3, A–C). The differential role of APBA3 between neutrophils and macrophages may reflect a difference in expression of MT1-MMP in these cells; macrophages express MT1-MMP, but neutrophils do not. Therefore, FIH-1 is not inactivated by APBA3 so as to activate HIF-1 in neutrophils. An important question that is then raised is how HIF-1 is activated in neutrophils? Answering this question may lead to discovery of a yet unknown mechanism that regulates HIF-1 $\alpha$ .

APBA3 and FIH-1 are expressed in a wide variety of cells, although expression of MT1-MMP is limited to cells of mesenchymal origin and invasive cancer cells that have undergone the epithelial-mesenchymal-transition (32, 33). We have demonstrated that activation of HIF-1 can be observed in different types of cells, provided that they express MT1-MMP. For example, MEFs express MT1-MMP and also HIF-1 target genes, whereas ATP levels are not affected because the cells make use of OXPHOS predominantly for ATP production (14). Because MT1-MMP is expressed in mesenchymal cells such as fibroblasts, endothelial cells, and pericytes, a lack of APBA3 presumably affects HIF-mediated gene expression in these cells. However, we did not observe any apparent defects in these cells in the mutant mice. Therefore, a phys-

iological role for the MT1-MMP/APBA3/FIH-1 axis in the regulation of HIF transcription factors in mesenchymal cells remains to be elucidated.

In conclusion, we have demonstrated the physiological importance of APBA3 in the regulation of HIF-1 and its downstream effectors in macrophages. Because APBA3-deficient mice showed no gross apparent abnormality, APBA3 might be a useful molecular target to develop therapeutics to attenuate macrophage activity contributing to septic shock, excessively inflammatory conditions, or cancer.

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