Cadmium-mediated lung injury is exacerbated by the persistence of classically activated macrophages

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Heavy metals released into the environment have a significant effect on respiratory health. Lung macrophages are important in mounting an inflammatory response to injury, but they are also involved in repair of injury. Macrophages develop mixed phenotypes in complex pathological conditions and polarize to a predominant phenotype depending on the duration and stage of injury and/or repair. Little is known about the reprogramming required for lung macrophages to switch between these divergent functions; therefore, understanding the mechanism(s) by which macrophages promote metabolic reprogramming to regulate lung injury is essential. Here, we show that lung macrophages polarize to a pro-inflammatory, classically activated phenotype after cadmium-mediated lung injury. Because metabolic adaptation provides energy for the diverse macrophage functions, these classically activated macrophages show metabolic reprogramming to glycolysis. RNA-Seq revealed up-regulation of glycolytic enzymes and transcription factors regulating glycolytic flux in lung macrophages from cadmium-exposed mice. Moreover, cadmium exposure promoted increased macrophage glycolytic function with enhanced extracellular acidification rate, glycolytic metabolites, and lactate excretion. These observations suggest that cadmium mediates the persistence of classically activated lung macrophages to exacerbate lung injury.

Results

Cadmium promoted the persistence of the pro-inflammatory macrophage phenotype

Lung macrophages have a critical role in mounting an immune response to injury (6–8). Cadmium has been shown to suppress the innate immune response of macrophages (6, 17). We have shown that cadmium inhibits lung macrophage host defense by inhibiting the Rho GTPase, Rac2 (6); however, it is not known if cadmium regulates the phenotypic switching of macrophages to influence lung repair. We found that cadmium promotes macrophage polarization to the pro-inflammatory, classically activated phenotype. TNFα and iNOS mRNA expression were increased 3–8-fold, respectively, in macrophages exposed to CdCl₂ (Fig. 1, A and B). The alternatively activated markers, arginase 1, TGF-β1, IL-10, and PDGF-B were significantly reduced compared with vehicle exposed (Fig. 1, C–F).

To determine whether cadmium mediated the persistence of a pro-inflammatory phenotype, macrophages were exposed to
Cadmium for 0, 1, 3, 6, 12, 24, or 48 h. Cadmium-exposed macrophages showed a time-dependent increase in TNFα and iNOS mRNA expression with maximal expression seen 24-48 h after exposure (Fig. 1, G and H). Arginase 1 and TGF-β1 expression were drastically reduced in macrophages exposed to cadmium for 3 h (Fig. 1, I and J), whereas IL-10 and PDGF-B were significantly reduced 1 h after cadmium exposure (Fig. 1, K and L). The reduction in anti-inflammatory gene expression from cadmium-exposed macrophages persisted over the duration of the time course.

Further examining the role of cadmium gene regulation, TNFα promoter activity showed nearly 4-fold increase in cadmium-exposed macrophages and was similar to LPS-exposed macrophages, which was used as a positive control (Fig. 1M). iNOS promoter activity increased nearly 10-fold in cadmium-exposed macrophages (Fig. 1N). In contrast, arginase 1 and TGF-β1 promoter activities were significantly reduced in cadmium-exposed macrophages (Fig. 1, O and P). Cadmium exposure did not alter macrophage apoptosis as no changes were detected in the mitochondrial or cytoplasmic localization of...
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Bcl-2, cytochrome c, or Bax expression (Fig. S1, A and B). Moreover, caspase-3 activity remained unchanged over the duration in cadmium-exposed macrophages (Fig. S1C). These data suggest that cadmium induces a rapid and persistent polarization of macrophages to a pro-inflammatory phenotype.

Cadmium-induced lung injury and the pro-inflammatory phenotype of lung macrophages

To determine the biological relevance of our in vitro observations, we exposed WT mice to cadmium at the mean concentration found in bronchioalveolar lavage (BAL) fluid from cigarette smoke-exposed mice (6). BAL was performed 7 days later. Cadmium-exposed mice showed over 2.5-fold increase in the number of BAL cells (Fig. 2A) and greater than 95% of the cells were macrophages (Fig. 2B). Cadmium exposure induced the polarization of lung macrophages to a pro-inflammatory phenotype. Lung macrophages isolated from cadmium-exposed WT mice showed over 5-fold increase in TNFα and iNOS mRNA expression compared with vehicle-exposed (Fig. 2, C and D). In contrast, arginase 1, TGF-β1, IL-10, and PDGF-B mRNA expression were significantly reduced in lung macrophages from cadmium-exposed mice (Fig. 2, E–H). Cadmium exposure did not alter the expression or localization of Bcl-2, cytochrome c, or Bax in lung macrophages (Fig. S2, A and B) and caspase-3 activity remained unchanged between vehicle and cadmium-exposed mice (Fig. S2C).

Because macrophages are phagocytic cells and to confirm that there is uptake and accumulation of cadmium in lung macrophages, transmission EM (TEM) analysis of isolated lung macrophages from cadmium-exposed mice revealed an accumulation of dark aggregates (~1 nm in diameter, blue arrows, for reference ribosomes are indicated with orange arrowheads) found in the cytoplasm of macrophages, whereas this was absent in vehicle-exposed (Fig. 2I). Lung histology from cadmium-exposed mice revealed increased lobar consolidation associated with hemorrhage, whereas vehicle-exposed mice showed normal lung architecture (Fig. 2, J and K). These results were confirmed by increased albumin concentration in the BAL fluid indicating the presence of lung injury in cadmium-exposed mice (Fig. 2L). Moreover, the ratio of wet to dry lung weight was significantly increased in the cadmium-exposed mice (Fig. 2M). These results suggest a correlation between the cadmium-mediated lung macrophage phenotype and lung injury.

Cadmium increased lung macrophage mitochondrial ROS

The redox-sensitive transcription factor, nuclear factor (NF)-κB, is a critical mediator of the macrophage inflammatory response and is required for pro-inflammatory gene expression (18–21). Using a promoter construct driven by NF-κB, we found that cadmium-exposed macrophages had significantly greater NF-κB–driven luciferase activity than vehicle exposed and the cadmium-induced increase in activity was significantly greater than LPS-exposed (Fig. 3A). Moreover, the transcription factors associated with classical activation, p-STAT1, and the p65 subunit of NF-κB, were localized to the nuclear fraction in macrophages exposed to cadmium, whereas p65 remained in the cytosol in vehicle-exposed (Fig. 3, B and C).

Mitochondrial ROS (mtROS) are suggested to act as signal-transducing molecules that trigger inflammation by driving pro-inflammatory cytokine production (22, 23). We determined that cadmium-exposed macrophages showed a significant increase in mtROS generation (Fig. 3, D and E). These results were validated in vivo. The increased mtROS production in lung macrophages from cadmium-exposed mice (Fig. 3F) correlated with the activation of redox-regulated transcription factors. Lung macrophages from cadmium-exposed mice showed increased nuclear localization of the p65 subunit of NF-κB and p-STAT1 (Fig. 3G), whereas the p65 subunit of NF-κB remained in the cytosol in macrophages from vehicle-exposed mice (Fig. 3H).

Validating that cadmium-mediated regulation of transcription factor expression was induced by mtROS generation, cadmium-exposed macrophages were treated with mitoTEMPO, a specific scavenger of mtROS. MitoTEMPO treatment significantly reduced cadmium-mediated mtROS generation to the level seen in vehicle control (Fig. 3I). Nuclear localization of the p65 subunit of NF-κB and p-STAT1 were reduced with mitoTEMPO treatment, whereas the p65 subunit of NF-κB was increased in the cytosolic fraction with mitoTEMPO treatment in macrophages exposed to vehicle or cadmium. (Fig. 3, J and K).

Abrogating cadmium-mediated mtROS generation in macrophages influenced macrophage phenotypic switching. Treatment with mitoTEMPO reduced TNFα and iNOS gene expression to the level seen with vehicle alone (Fig. 3, L and M). In contrast, mitoTEMPO treatment rescued anti-inflammatory gene expression in cadmium-exposed macrophages similar to vehicle-exposed levels (Fig. 3, N–Q). These data suggest that cadmium-induced mtROS regulates redox-regulated transcription factors.

Cadmium promoted glycolytic flux in lung macrophages

Because hypoxia-inducible factor 1α (HIF-1α) is a critical regulator of macrophage inflammation (24), we determined that cadmium-exposed macrophages had increased HIF-1α expression in the nuclear fraction (Fig. 4A). Inhibiting cadmium-mediated mtROS generation with mitoTEMPO treatment abolished HIF-1α expression. Studies suggest classically activated macrophages utilize glycolytic metabolism, which can be rapidly activated to fuel responses for injury (25–27). Additionally, HIF-1α influences the metabolic reprogramming of macrophages to glycolysis (28). A critical activator of glucose metabolism, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), was similarly increased in macrophages exposed to cadmium and the expression of PFKFB3 was redox-regulated (Fig. 4B).

To measure glycolytic flux, lactate excretion was measured in conditioned media from cadmium-exposed macrophages. Lactate was significantly increased in cell culture media from cadmium-exposed macrophages, whereas mitoTEMPO reduced lactate to control levels (Fig. 4C). Measuring glycolytic function via extracellular acidification rate (ECAR), cadmium-exposed macrophages showed increased ECAR compared with vehicle-exposed macrophages (Fig. 4D).
Studies indicate PPAR\(\gamma\) plays a critical role in mediating the alternative activation of macrophages (15, 29). We determined that PPAR\(\gamma\) expression was absent in CdCl\(_2\)-exposed macrophages (Fig. 4E). Treating macrophages with mitoTEMPO induced alternative activation with STAT6 activation and PPAR\(\gamma\) nuclear expression in cadmium-exposed macrophages. These data suggest that inhibiting mtROS generation in cadmium-exposed macrophages induces the phenotypic switching of macrophages and reduced glycolytic metabolism, a key feature of alternatively activated macrophages.

To further understand the bioenergetics in cadmium-exposed macrophages, RNA-Seq was performed in lung macrophages isolated from vehicle- and cadmium-exposed mice. Key glycolytic enzymes, hexokinase 2 (Hk2), phosphofructokinase (Pfkm), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate kinase (PKM), and lactate dehydrogenase A (LDHA) were greater in cadmium-exposed lung macrophages (Fig. 4F). The transcription factors, c-myc and Hif1\(\alpha\), considered master regulators of glycolytic flux, were increased in lung macrophages from cadmium-exposed mice.

Because cellular metabolism plays an important role during macrophage polarization, we validated that HIF-1\(\alpha\) was increased in lung macrophages from cadmium-exposed mice.
Cadmium promoted a shift from mitochondrial oxidation toward glycolytic metabolism. Confirming the RNA-Seq data, lung macrophages isolated from cadmium-exposed mice showed increased PFKFB3 expression (Fig. 4H). Furthermore, BAL fluid from cadmium-exposed mice had elevated lactate levels, a measure of glycolytic flux (Fig. 4I). The increased flux was confirmed by an increase in ECAR in the cadmium-exposed mice (Fig. 4J). These data suggest that cadmium regulates the metabolic reprogramming to glycolysis, a critical bioenergetic characteristic in classically activated macrophages.

**Figure 3. Cadmium increases lung macrophage mitochondrial ROS.** A, NF-κB activity was measured by luciferase assay in THP-1 cells exposed to vehicle, CdCl₂ (50 μM), IL-4 (20 ng/ml, negative control), or LPS (100 μg/ml, positive control) for 3 h (n = 6). Immunoblot analysis of exposed THP-1 cells in isolated (B) nuclear and (C) cytosolic extracts. mtROS generation in exposed THP-1 cells by (D) pHFA assay (n = 9) and (E) mitoSOX (antimycin A, 100 μM for 30 min, positive control) (n = 10). F, mtROS generation in BAL cells isolated from WT mice exposed to vehicle or CdCl₂ for 7 days (n = 6). Immunoblot analysis of BAL cells isolated from exposed mice in (G) nuclear and (H) cytosolic extracts. THP-1 cells were treated with vehicle or mitoTEMPO (10 μM, 16 h) and exposed to vehicle or CdCl₂ for 3 h. I, mtROS generation (n = 3). Immunoblot analysis in isolated (J) nuclear and (K) cytosolic fractions. L, TNFα; M, iNOS; N, arginase 1; O, TGF-β1; P, IL-10; and Q, PDGF-B mRNA expression (n = 3). ***p < 0.0001. Values are shown as mean ± S.D.

(Cadmium altered macrophage glycolytic intermediates)

Because cadmium treatment drastically reduced PPARγ expression and to understand the role of cadmium in promoting glycolysis, glycolytic intermediates and amino acid levels were measured by MS in macrophages expressing PPARγ and exposed to cadmium (Fig. 5A). Expression of PPARγ or treatment of cadmium did not alter glucose, glucose 6-phosphate, ribose 5-phosphate, or fructose 1,6-bisphosphate levels (Fig. 5, B–D). Although macrophages exposed to cadmium showed no change in glyceraldehyde 3-phosphate, PPARγ-expressing macrophages showed increased levels (Fig. 5E). Suggesting an
increase in glycolysis, cadmium-exposed macrophages had a significant increase in 3-phosphoglycerate (Fig. 5F). Whereas serine levels remained unchanged, cysteine and glycine synthesis were reduced in cadmium-exposed and PPAR\textsubscript{g}-expressing macrophages. Further implicating enhanced glycolysis, phosphoenolpyruvate, and alanine levels were increased in cadmium-exposed macrophages and PPAR\textsubscript{g} expression reduced levels to that seen in controls (Fig. 5G). These studies suggest that cadmium promotes glycolysis in lung macrophages and PPAR\textsubscript{g} may provide a therapeutic mechanism to abrogate cadmium-mediated lung injury.

**PPAR\textsubscript{g} induced the alternative phenotype in cadmium-exposed macrophages**

Because PPAR\textsubscript{g} is a critical regulator of the anti-inflammatory phenotype in macrophages, we questioned if overexpression of PPAR\textsubscript{g} could alter the cadmium-mediated phenotypic switching of macrophages. Macrophages expressing PPAR\textsubscript{g} showed significantly reduced mtROS generation after cadmium exposure (Fig. 6A). The down-regulation of cadmium-mediated mtROS completely abrogated gene expression of TNF\textalpha and iNOS mRNA in cadmium-exposed macrophages expressing PPAR\textsubscript{g} (Fig. 6, B and C). The reverse was seen with the anti-inflammatory markers. PPAR\textsubscript{g}-expressing macrophages had significantly greater arginase 1, TGF-\beta\textsubscript{1}, IL-10, and PDGF-B mRNA expression (Fig. 6, D–G). More importantly, cadmium exposure did not alter expression of these genes in PPAR\textsubscript{g}-expressing macrophages.

Because PPAR\textsubscript{g} regulated macrophage phenotypic switching in cadmium-exposed macrophages, we determined if PPAR\textsubscript{g} altered the activation of redox-regulated transcription factors. Macrophages expressing PPAR\textsubscript{g} showed an absence of the transcription factors associated with classical activation, p-STAT1, and the p65 subunit of NF-\kappaB in the nuclear fraction in macrophages exposed to cadmium, whereas p-STAT6 was highly up-regulated (Fig. 6H). Furthermore, PPAR\textsubscript{g}-expressing
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Macrophages decreased HIF-1α expression, showed reduced lactate levels, and failed to undergo metabolic reprogramming toward glycolysis (Fig. 6, I and J). Cadmium exposure showed no effect in the presence of PPARγ. Taken together, these studies suggest that PPARγ-mediated phenotypic switching is associated with metabolic reprogramming in macrophages exposed to cadmium.

Discussion

Altered amino acid metabolism is a hallmark in defining macrophage phenotype. Pro-inflammatory macrophages convert l-arginine to nitric oxide by increasing iNOS activity, whereas anti-inflammatory macrophages have increased arginase 1, which converts l-arginine to urea (25). Classically activated, pro-inflammatory macrophages utilize glycolytic metabolism, which can be rapidly activated to fuel responses for injury (24, 25, 28). Although relatively inefficient in ATP production, the conversion of pyruvate into lactate is essential to restore NAD+ and maintain flux through the glycolytic pathway. Glycolytic flux is controlled by several enzymes, including hexokinase, phosphofructokinase, and pyruvate kinase. Although the nonoxidative part of glycolysis did not show accumulation of intermediates, oxidative glycolytic intermediates as well as exit products (alanine) were increased, suggesting that enzyme flux capacity was reached for pyruvate kinase and potentially GAPDH. This view is strengthened by the lack of significant differences of exit pathways, such as serine metabolism or pentose phosphate pathway intermediates. Moreover, changes in the concentration of metabolites can regulate flux via allosteric regulation of enzymes (26). Here, we show...
that cadmium-mediated lung injury results in the persistence of classically activated lung macrophages and induces the metabolic reprogramming of these cells to glycolysis to exacerbate lung injury.

The exposure of cadmium is closely associated with the development of lung diseases. Cadmium is one of the metal compounds present in cigarette smoke and each pack of cigarettes contains 30 μg of cadmium, but nearly 3 μg of cadmium is present in a single cigarette (27). In total, cigarette smokers have at least twice as much cadmium in body content than non-smokers, and cadmium has no physiologic role in humans. Cadmium has an extremely long t1/2, up to 15-20 years (27). Thus, the physiologic effect of cadmium may be acute as well as being prolonged. Our data demonstrates the accumulation of cadmium in lung macrophages in an acute exposure; however, the accumulation in macrophages is not known in the prolonged setting.

Cadmium is not redox-active, but the generation of ROS is a critical mediator for cadmium-triggered tissue injury (30). Studies implicate cadmium-induced oxidative stress is mediated through cellular redox disruption by depletion of antioxidant enzymes (31). We show that cadmium-mediated mtROS generation regulates the polarization of macrophages, and studies indicate that mtROS are known to sustain inflammation by mediating pro-inflammatory cytokine secretion (32). Our data indicate that quenching mtROS induced the macrophage phenotype to become alternatively activated. Inhibiting cadmium-mediated mtROS reduced ECAR and may induce metabolic reprogramming to oxidative phosphorylation.

HIF-1α is a key transcription factor that regulates cell metabolism (24). Oxidative stress has been implicated in HIF-1α signaling, and mtROS has been shown to stabilize and activate HIF-1α (33, 34). There is some controversy on the role of cadmium and ROS in the regulation of HIF-1α. Cadmium-
mediated ROS increased HIF-1α levels to induce malignant transformation of bronchial epithelial cells (35), whereas another study suggested that the inhibition of HIF-1α by cadmium was not secondary to oxidative stress (36). Thus, the regulation of HIF-1α by cadmium may be cell- and/or stimulus-specific.

The link between HIF-1α and NF-κB is also controversial. HIF-1α has been suggested to regulate NF-κB (37), and activation of HIF-1α may result from the inhibition of NF-κB (31). Other studies suggest NF-κB is a direct modulator of HIF-1α transcription during inflammatory conditions, such as hypoxia (38). Our data extended these findings by showing that cadmium-mediated mtROS generation regulates NF-κB activation and increases HIF-1α expression to potentially regulate metabolic reprogramming to glycolysis.

Evidence suggests that lactate competes with glucose as a mitochondrial substrate in the lung, and type II alveolar epithelial cells have been shown to readily utilize lactate (39). The effect of enhanced glycolysis is cell type-specific as increased glycolysis has been shown to protect alveolar epithelial cells from lung injury in an LPS model (40). Further studies are warranted to better understand the cross-talk between macrophages and alveolar epithelial cells, as we suggest the increased lactate is macrophage-derived and may play a critical role in lung injury.

PPARγ is a key regulator of lipid metabolism and inflammation and is expressed in various cell types throughout the human body. Our data indicate PPARγ may be a novel target for regulating macrophage metabolic reprogramming and influencing lung repair after cadmium exposure. PPARγ can be activated by synthetic ligands. Rosiglitazone and pioglitazone belong to the thiazolidinedione class of PPARγ agonists and are potent insulin-sensitizing drugs. Rosiglitazone has been shown to reduce bleomycin-induced lung fibrosis via inhibition of TGF-β1–mediated differentiation of lung fibroblasts (41, 42). Rosiglitazone decreased pulmonary artery remodeling in a rat model of pulmonary hypertension (43). Moreover, pioglitazone prevented LPS-induced acute lung injury in mice (44). Although the use of thiazolidinediones is associated with many undesirable side effects, such as weight gain, edema, and heart disease in diabetic patients (45), new classes of PPARγ agonists may be able to effectively target specific cells and tissues preserving the efficacy of these drugs whereas reducing their side effects.

Taken together, we show that cadmium-mediated mtROS in macrophages regulates redox-regulated transcription factors to maintain the persistence of a pro-inflammatory phenotype. These observations also suggest that the cadmium-induced pro-inflammatory phenotype in macrophages may hinder the resolution of lung injury.

**Experimental procedures**

**Mice**

All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham and were performed in accordance with NIH guidelines. WT C57BL6 mice were purchased from JAX Laboratory (Bar Harbor, ME). 8- to 12-week-old male and female mice were intratracheally administered 100 ng/kg of CdCl2 or saline, as a vehicle control, after being anesthetized with 3% isoflurane using a precision Fortec vaporizer. Mice were fed ad libitum standard chow and kept at 12-h light/12-h dark cycles. Mice were euthanized 7 days after exposure and BAL was performed.

**Cell culture**

Human monocyte (THP-1) and mouse alveolar macrophage (MH-S) cell lines were obtained from American Type Culture Collection (Manassas, VA). Macrophages were maintained in RPMI 1640 media (Thermo Fisher Scientific) with 10% fetal bovine serum and penicillin/streptomycin supplements. All experiments were conducted in RPMI containing 0.5% serum. Cells were treated with vehicle or 50 μM CdCl2 for 3 h or the indicated time. Macrophages were treated with IL-4 (20 ng/ml, negative control) or LPS (100 μg/ml) as a positive control.

**Quantitative real-time PCR**

Total RNA was isolated, reverse transcribed, and quantitative real-time PCR was performed as described previously using previously published primer sets (46, 47). Data were calculated by the cycle threshold ($\Delta \Delta C_T$) method, normalized to β-actin or HPRT, and expressed in arbitrary units.

**Plasmids, transfections, and luciferase assays**

The PPARγ plasmid (8895) and iNOS promoter (19296) were purchased from Addgene (48, 49). TNFα promoter, arginase 1 promoter/ enhancer, TGF-β1 promoter, and NF-κB gene expression were evaluated using luciferase reporter plasmids as previously described (19, 46, 47). The correct reading frame and sequence was verified by the Heflin Center Genomics Core at the University of Alabama. Cells were transfected using X-treme GENE 9 Transfection Reagent (Promega) and normalized to control (firefly).

**TEM**

BAL cells were fixed in 2.5% paraformaldehyde and 2.5% glutaraldehyde in Sorensen’s phosphate buffer as previously described (46). Cells were processed and sectioned with a diamond knife (Diatome, Electron Microscopy Sciences) at 70-80 nm and sections were placed on copper mesh grids. Sections were stained with uranyl acetate and lead citrate for contrast and viewed on a Tecnai Twin 120kv TEM (FEI).

**ECAR**

ECAR was determined using a Seahorse XF24 bioanalyzer (Seahorse Bioscience). In brief, 7.5 × 10^6 macrophages per well were subjected to ECAR measurement in the XF24 extracellular flux analyzer with sequential additions of the following conditions: glucose (10 mM), oligomycin (0.5 μM), and 2-deoxyglucose (50 μM).
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**Albumin**

Albumin levels were determined in BAL fluid using the Mouse Albumin ELISA Kit (Immunology Consultants Laboratory) according to the manufacturer’s protocol. Samples were diluted 1/500,000.

**Lactate**

Lactate was measured in conditioned media and BAL fluid using the Lactate Colorimetric/Fluorometric Assay Kit (BioVision) according to the manufacturer’s protocol.

**Next-generation RNA-Seq**

mRNA-Seq was performed on the Illumina NextSeq500 as described by the manufacturer (Illumina Inc., San Diego, CA). Briefly, RNA quality was assessed using the Agilent 2100 Bioanalyzer. RNA with an RNA Integrity Number of ≥7.0 was used for sequencing library preparation. RNA passing quality control was converted to a sequencing ready library using the NEBNext Ultra II Directional RNA library kit as per the manufacturer’s instructions (New England Biolabs, Ipswich, MA). The cDNA libraries were quantitated using quantitative PCR in a Roche LightCycler 480 with the Kapa Biosystems kit for Illumina libraries were quantitated using quantitative PCR in a Roche LightCycler 480 with the Kapa Biosystems kit for Illumina

**Data assessment for RNA-Seq**

STAR (version 2.5.3a) was used to align the raw RNA-Seq fastq reads to the reference genome from Gencode. Following alignment, HTSeq-count was used to count the number of reads mapping to each gene. Normalization and differential expression were applied to the count files using DESeq2. Data isolation of mitochondria and cytoplasm fractions

Mitochondria were isolated by lysing the cells in mitochondria buffer containing 10 mM Tris, pH 7.8, 0.2 mM EDTA, 320 mM sucrose, and protease inhibitors. Lysates were homogenized using a Kontes Pellet Pestle Motor and centrifuged at 2000 × g for 8 min at 4°C. The supernatant was removed and incubated at 4°C, and the pellet was lyzed, homogenized, and centrifuged again. The two supernatants were pooled and centrifuged at 12,000 × g for 15 min at 4°C. The pellet was washed in the mitochondrial buffer twice and then resuspended in mitochondria buffer without sucrose.

**Data assessment for metabolomics**

Raw data files are preprocessed directly after data acquisition and stored as Chroma TOF-specific *.peg files, as generic *.txt result files and additionally as generic ANDI MS *.cdf files as previously described (50). ChromaTOF versus 2.32 is used for data preprocessing without smoothing, 3 s peak width, baseline subtraction just above the noise level, and automatic mass spectral deconvolution and peak detection at signal/noise levels of 5:1 throughout the chromatogram. Apex masses are reported for use in the BinBase algorithm. Result *.txt files are exported to a data server with absolute spectra intensities and further processed by a filtering algorithm implemented in the metabolomics BinBase database.

**Isolation of mitochondria and cytoplasm fractions**

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**Determination of mitochondrial ROS generation**

Mitochondrial H$_2$O$_2$ production was determined fluorometrically by p-hydroxyphenylacetic acid (pHPA) assay. Freshly isolated mitochondria were incubated in phenol red-free Hanks’ balanced salt solution supplemented with 6.5 mM glucose, 1 mM HEPES, 6 mM sodium bicarbonate, 1.6 mM pHPA, and 0.95 µg/ml of HRP Fluorescence of pHPA-dimer was measured using a spectrophuometer at excitation of 320 nm and emission of 400 nm (46). Treatment of cells with antimycin A (100 µM for 30 min) was used as a positive control. MitoSOX, a mitochondrial superoxide indicator (Thermo Fisher Scientific), was used to detect mitochondrial superoxide anion according to the manufacturer’s protocol. Equal numbers of cells were subjected to
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fluorescent reading (excitation, 510 nm; emission, 580 nm) as previously described (51).

Immunoblot analysis

Primary antibodies used were: Bax (2772S), Bcl-2 (3498), cytochrome c (4272), HIF-1α (79233), Lamin A/C (2032), PFKP3 (13123), phospho-STAT1 (7649), phospho-STAT6 (9361), STAT1 (9172), VDAC (4866) (Cell Signaling); β-actin (A5441) (Sigma); NF-κB p65 (sc-372) (Santa Cruz Biotechnology); and PPARγ (A0270) (ABclonal).

Caspase-3 activity analysis

Caspase-3 activity was measured using EnzChek Caspase-3 Assay Kit Number 2 (Molecular Probes) according to the manufacturer’s protocol. Cells were lysed in 1× lysis buffer, subjected to a freeze-thaw cycle, centrifuged to remove cellular debris, and loaded into individual microplate wells. The 2× reaction buffer with substrate was immediately added to the samples, and fluorescence was measured (excitation/emission 496/520 nm). A supplied inhibitor was used as a negative control in all experiments (11).

Statistics

Statistical comparisons were performed using a Student’s t test when only two groups of data are presented, or one-way analysis of variance with a Tukey’s post hoc test or two-way analysis of variance followed by Bonferroni post-test when multiple data groups are present. All statistical analysis was expressed as mean ± S.D. and p < 0.05 was considered to be significant. GraphPad Prism statistical software was used for all analysis.

Data availability

All data are contained within the manuscript. Data have been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus database (GEO) with GEO accession number GSE155166.

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Abbreviations—The abbreviations used are: PPARγ, peroxisome proliferator-activated receptor γ; BAL, bronchoalveolar lavage; TNF, tumor necrosis factor; PDGF, platelet-derived growth factor; TEM, transmission EM; IL, interleukin; mtROS, mitochondrial ROS; HIF-1α, hypoxia-inducible factor 1α; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; ECAR, extracellular acidification rate; HPRRT, hypoxanthine-guanine phosphoribosyltransferase; LPS, lipopolysaccharide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ROS, reactive oxygen species; pHPA, p-hydroxyphenylacetic acid.

References

1. Lampe, B. J., Park, S. K., Robins, T., Mukherjee, B., Litonjua, A. A., Amarsirwardena, C., Weisskopf, M., Sparrow, D., and Hu, H. (2008) Association between 24-hour urinary cadmium and pulmonary function among community-exposed men: the VA Normative Aging Study. Environ. Health Perspect. 116, 1226–1230 CrossRef Medline
2. Mannino, D. M., Holguin, F., Greves, H. M., Savage-Brown, A., Stock, A. L., and Jones, R. L. (2004) Urinary cadmium levels predict lower lung function in current and former smokers: data from the Third National Health and Nutrition Examination Survey. Thorax 59, 194–198 CrossRef Medline
3. Farooq, O., Ashizawa, A., Wright, S., Tucker, P., Jenkins, K., Ingerman, L., and Rudiss, C. (2012) Agency for Toxic Substances and Disease Registry (ATSDR) toxicological profiles. in Toxicological profile for cadmium, Agency for Toxic Substances and Disease Registry (US), Atlanta, GA
4. UNEP (2008) Interim review of scientific information on cadmium. United Nations Environment Program, Geneva
5. Raja, R., Nayak, A. K., Shukla, A. K., Rao, K. S., Gautam, P., Lal, B., Tripathi, R., Shahid, M., Panda, B. B., Kumar, A., Bhattacharyya, P., Bardhan, G., Gupta, S., and Patra, D. K. (2015) Impairment of soil health due to fly ash-fugitive dust deposition from coal-fired thermal power plants. Environ. Monit. Assess. 187, 679 CrossRef Medline
6. Larson-Casey, J. L., Gu, L.,Jackson, P. L., Briles, D. E., Hale, J. Y., Blalock, S. L., Paine, R., 3rd., and Standiford, T. J. (1997) Alveolar macrophages are required to reduce the severity of cigarette smoke-induced pneumonia. Am. J. Respir. Crit. Care Med. 156, 1288–1301 CrossRef Medline
7. Broug-Holub, E., Toews, G. B., and Iwaarden, J. F., Strieter, R. M., Kunkel, J. E., Wells, J. M., Deshane, J. S., Wang, Y., Davis, D., Antony, V. B., Massicano, A. V. F., Lapi, S. E., and Carter, A. B. (2018) Macrophage Rac2 is required for protective pulmonary defenses in murine Klebsiella pneumoniae: elimination of alveolar macrophages increases neutrophil recruitment but decreases bacterial clearance and survival. Infect. Immun. 85, 1139–1146 CrossRef Medline
8. Knapp, S., Leemans, J. C., Florquin, S., Branger, J., Maris, N. A., Pater, J., van Rooijen, N., and van der Poll, T. (2003) Alveolar macrophages have a protective antiinflammatory role during murine pneumococcal pneumonia. Am. J. Respir. Crit. Care Med. 167, 171–179 CrossRef Medline
9. Martinez, F. O., and Gordon, S. (2014) The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000Prime Rep. 6, 13 CrossRef Medline
10. He, C., Ryan, A. J., Murthy, S., and Carter, A. B. (2013) Accelerated development of pulmonary fibrosis via Cu/Zn-superoxide dismutase-induced alternative activation of macrophages. J. Biol. Chem. 288, 20745–20757 CrossRef Medline
11. Larson-Casey, J. L., Murthy, S., Ryan, A. J., and Carter, A. B. (2014) Modulation of the mevalonate pathway by Akt regulates macrophage survival
and development of pulmonary fibrosis. J. Biol. Chem. 289, 36204–36219

12. Murthy, S., Adamakova-Dodd, A., Perry, S. S., Tephly, L. A., Keller, R. M., Metwali, N., Meyerholz, D. K., Wang, Y., Glogauer, M., Thorne, P. S., and Carter, A. B. (2009) Modulation of reactive oxygen species by Rac1 or catalase prevents asbestos-induced pulmonary fibrosis. Am. J. Physiol. Lung Cell Mol. Physiol. 297, L846–L855 CrossRef

13. Schneider, C., Nobs, S. P., Kurrer, M., Rahrehaus, H., Thiele, C., and Kopf, M. (2014) Induction of the nuclear receptor PPAR-γ by the cytokine GM-CSF is critical for the differentiation of fetal monocytes into alveolar macrophages. Nat. Immuno. 15, 1026–1037 CrossRef Medicine

14. Jiang, C., Ting, A. T., and Seed, B. (1998) PPAR-γ agonists inhibit production of monocyte inflammatory cytokines. Nature 391, 82–86 CrossRef Medicine

15. Odegaard, J. I., Ricardo-Gonzalez, R. R., Goforth, M. H., Morel, C. R., Subramanian, V., Mukundan, L., Red Eagle, A., Vats, D., Brombacher, F., Ferrante, A. W., and Chawla, A. (2007) Macrophage-specific PPARγ controls alternative activation and improves insulin resistance. Nature 447, 1116–1120 CrossRef Medicine

16. Satarug, S., Garrett, S. H., Sens, M. A., and Sens, D. A. (2010) Cadmium, environmental exposure, and health outcomes. Environ. Health Perspect. 118, 182–190 CrossRef Medicine

17. Cox, J. N., Rahman, M. A., Bau, S., Liu, M., Wheeler, S. E., and Knoell, D. L. (2016) Cadmium attenuates the macrophage response to LPS through inhibition of the NF-κB pathway. Am. J. Physiol. Lung Cell Mol. Physiol. 311, L754–L765 CrossRef Medicine

18. Carter, A. B., Knudtson, K. L., Monick, M. M., and Hunninghake, G. W. (1998) The p38 mitogen-activated protein kinase is required for NF-κB-dependent gene expression: the role of TATA-binding protein (TBP). J. Biol. Chem. 274, 30858–30863 CrossRef Medicine

19. Carter, A. B., and Hunninghake, G. W. (2000) A constitutive active MEK of macrophages reveals distinct, opposing roles during liver injury and time metabolome profiling of the metabolic switch between starvation and growth. Cell 127, 533–543 CrossRef Medicine

20. Tojo, K., Tamada, N., Nagamine, Y., Yazawa, T., Ota, S., and Goto, T. (2013) DFS inhibition primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. Cell Metab. 6, 137–143 CrossRef Medicine

21. Wang, Y., Fang, I., Leonard, S. S., and Rao, K. M. (2004) Cadmium inhibits the electron transfer chain and induces reactive oxygen species. Free Radic. Biol. Med. 36, 1434–1443 CrossRef Medicine

22. Sato, K., and Hunninghake, G. W. (2000) A constitutive active MEK of macrophages reveals distinct, opposing roles during liver injury and time metabolome profiling of the metabolic switch between starvation and growth. Cell 127, 533–543 CrossRef Medicine

23. Chandel, N. S., Maltepe, E., Goldwasser, E., Mathieu, C. E., Simon, M. C., and Schumacker, P. T. (1998) Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc. Natl. Acad. Sci. USA. 95, 11715–11720 CrossRef Medicine

24. Chandel, N. S., McClintock, D. S., Feliciano, C. E., Wood, T. M., Mellendc, J. A., Rodriguez, A. M., and Schumacker, P. T. (2000) Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1α during hypoxia: a mechanism of O2 sensing. J. Biol. Chem. 275, 25130–25138 CrossRef Medicine

25. Jia, Y., Liu, L. Z., Jiang, Y., Zhu, Y., Guo, N. L., Barnett, J., Rojanasakul, Y., Agani, F., and Jiang, B. H. (2012) Cadmium increases HIV-1 and VEGF expression through ROS, ERK, and AKT signaling pathways and induces malignant transformation of human bronchial epithelial cells. Toxicol. Sci. 125, 10–19 CrossRef Medicine

26. Chun, Y. S., Choi, E., Kim, G. T., Choi, H., Kim, C. H., Lee, M. J., Kim, M. S., and Park, J. W. (2000) Cadmium blocks hypoxia-inducible factor (HIF-1)-mediated response to hypoxia by stimulating the proteasome-dependent degradation of HIF-1α. Proc. Natl. Acad. Sci. U.S.A. 97, 11715–11720 CrossRef Medicine

27. Rius, J., Guma, M., Schachtrup, C., Akassoglou, K., Zinkernagel, A. S., Nizet, V., Johnson, R. S., Haddad, G. G., and Karin, M. (2008) NF-κB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1α. Nature 453, 807–811 CrossRef Medicine

28. Lottes, R. G., Newton, D. A., Spyropoulos, D. D., and Baatz, J. E. (2015) Lactate as substrate for mitochondrial respiration in alveolar epithelial type II cells. Am. J. Physiol. Lung Cell. Mol. Physiol. 308, L953–961 CrossRef Medicine

29. Bouhlou, M. A., Derudas, B., Rigamonti, E., Dävser, R., Brozek, J., Haulon, S., Zawadzki, C., Jude, B., Torpier, G., Marx, N., Staels, B., and Chiniatti-Gbaguidi, G. (2007) PPARγ activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. Cell Metab. 6, 137–143 CrossRef Medicine

30. Wang, Y., Fang, I., Leonard, S. S., and Rao, K. M. (2004) Cadmium inhibits the electron transfer chain and induces reactive oxygen species. Free Radic. Biol. Med. 36, 1434–1443 CrossRef Medicine

31. Carbia-Nagashima, A., Gerez, J., Perez-Castro, C., Paez-Pereda, M., Silberstein, S., Stalla, G. K., Holsboer, F., and Arzt, E. (2007) RSUME, a small RWD-containing protein, enhances SUMO conjugation and stabilizes HIF-1α during hypoxia. Cell 131, 309–323 CrossRef Medicine

32. Bulau, A. C., Simon, A., Maddipati, R., Peltier, M., Park, H., Kim, K. Y., Sack, M. N., Kastner, D. L., and Siegel, R. M. (2011) Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNF1-associated periodic syndrome (TRAPS). J. Exp. Med. 208, 519–533 CrossRef Medicine

33. Chandel, N. S., Maltepe, E., Goldwasser, E., Mathieu, C. E., Simon, M. C., and Schumacker, P. T. (1998) Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc. Natl. Acad. Sci. USA. 95, 11715–11720 CrossRef Medicine

34. Tojo, K., Tamada, N., Nagamine, Y., Yazawa, T., Ota, S., and Goto, T. (2013) DFS inhibition primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. Cell Metab. 6, 137–143 CrossRef Medicine

35. Joseph, T., Crossno, J., Garat, C. V., Reusch, J. E. B., Morris, K. G., Dempsey, E. C., McMurry, I. F., Stenmark, K. R., and Klemm, D. J. (2007) Rosiglitazone attenuates hypoxia-induced pulmonary artery remodeling. Am. J. Physiol. Lung Cell. Mol. Physiol. 292, L885–L897 CrossRef Medicine
44. Grommes, J., Mörgelin, M., and Soehnlein, O. (2012) Pioglitazone attenuates endotoxin-induced acute lung injury by reducing neutrophil recruitment. Eur. Respir. J. 40, 416–423 CrossRef Medline

45. Nesto, R. W., Bell, D., Bonow, R. O., Fonseca, V., Grundy, S. M., Horton, E. S., Le Winter, M., Porte, D., Semenkovich, C. F., Smith, S., Young, L. H., and Kahn, R. (2004) Thiazolidinedione use, fluid retention, and congestive heart failure: a consensus statement from the American Heart Association and American Diabetes Association. Diabetes Care 27, 256–263 CrossRef

46. Larson-Casey, J. L., Deshane, J. S., Ryan, A. J., Thannickal, V. J., and Carter, A. B. (2016) Macrophage Akt1 kinase-mediated mitophagy modulates apoptosis resistance and pulmonary fibrosis. Immunity 44, 582–596 CrossRef

47. Larson-Casey, J. L., Vaid, M., Gu, L., He, C., Cai, G. Q., Ding, Q., Davis, D., Berryhill, T. F., Wilson, L. S., Barnes, S., Neighbors, J. D., Hohl, R. J., Zimmerman, K. A., Yoder, B. K., Longhini, A. L. F., et al. (2019) Increased flux through the mevalonate pathway mediates fibrotic repair without injury. J. Clin. Invest. 129, 4962–4978 CrossRef Medline

48. Hauser, S., Adelmann, G., Sarraf, P., Wright, H. M., Mueller, E., and Spiegelman, B. M. (2000) Degradation of the peroxisome proliferator-activated receptor γ is linked to ligand-dependent activation. J. Biol. Chem. 275, 18527–18533 CrossRef Medline

49. Lowenstein, C. J., Alley, E. W., Raval, P., Snowman, A. M., Snyder, S. H., Russell, S. W., and Murphy, W. J. (1993) Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon γ and lipopolysaccharide. Proc. Natl. Acad. Sci. U.S.A. 90, 9730–9734 CrossRef Medline

50. Fiehn, O., Wohlgemuth, G., Scholz, M., Kind, T., Lee, D. Y., Lu, Y., Moon, S., and Nikolau, B. (2008) Quality control for plant metabolomics: reporting MSI-compliant studies. Plant J. 53, 691–704 CrossRef Medline

51. Gu, L., Larson-Casey, J. L., and Carter, A. B. (2017) Macrophages utilize the mitochondrial calcium uniporter for profibrotic polarization. FASEB J. 31, 3072–3083 CrossRef Medline

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