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# Genetic BACH1 deficiency alters mitochondrial function and increases NLRP3 inflammasome activation in mouse macrophages

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## ABSTRACT

BTB-and-CNC homologue 1 (BACH1), a heme-regulated transcription factor, mediates innate immune responses via its functional role in macrophages. BACH1 has recently been shown to modulate mitochondrial metabolism in cancer cells. In the current study, we utilized a proteomics approach and demonstrate that genetic deletion of BACH1 in mouse macrophages is associated with decreased levels of various mitochondrial proteins, particularly mitochondrial complex I. Bioenergetic studies revealed alterations of mitochondrial energy metabolism in BACH1<sup>-/-</sup> macrophages with a shift towards increased glycolysis and decreased oxidative phosphorylation. Moreover, these cells exhibited enhanced mitochondrial membrane potential and generation of mitochondrial reactive oxygen species (mtROS) along with lower levels of mitophagy. Notably, a higher inducibility of NLRP3 inflammasome activation in response to ATP and nigericin following challenge with lipopolysaccharide (LPS) was observed in BACH1-deficient macrophages compared to wild-type cells. Mechanistically, pharmacological inhibition of mtROS markedly attenuated inflammasome activation. In addition, it is shown that inducible nitric oxide synthase and cyclooxygenase-2, both of which are markedly induced by LPS in macrophages, are directly implicated in BACH1-dependent regulation of NLRP3 inflammasome activation. Taken together, the current findings indicate that BACH1 is critical for immunomodulation of macrophages and may serve as a target for therapeutic approaches in inflammatory disorders.

## 1. Introduction

BTB-and-CNC homologue 1 (BACH1), is a nuclear transcription factor that belongs to the basic leucine zipper (bZip) factor family [1] with key regulatory functions in innate immunity including hematopoiesis of higher eukaryotes [2]. BACH1 is a heme-regulated protein that represses gene expression of heme-oxygenase 1 (HO-1) [1], a heme degrading enzyme, and that of SPI-C, a macrophage-specific Ets transcription factor [3]. The BACH1/HO-1 pathway controls cellular heme homeostasis and counter-acts heme-dependent oxidative stress. BACH1 is highly abundant in mononuclear cells and mediates the differentiation of monocytes into erythrophagocytes [4].

Macrophages are an integral part of the innate immune system and activation of these cells is critical for regulating inflammatory responses.

A substantial body of evidence supports the notion that macrophage functions are associated with alterations in energy metabolism [5], showing that M1-activated pro-inflammatory and pro-angiogenic macrophages are characterized by enhanced glycolytic metabolism [6]. Modulation of the antioxidant inducible enzyme HO-1 has also been shown to mediate polarization of macrophages towards an M2 anti-inflammatory phenotype [7]. Moreover, BACH1 has been linked to an improvement in the pathogenesis of various inflammation-related diseases such as atherosclerosis, colitis and myocardial ischemia-reperfusion injury [8–10]. Independent studies have recently demonstrated that BACH1 is involved in modulating mitochondrial metabolism. In particular, genetic BACH1-deficiency in triple negative breast cancer cells significantly increased the expression of mitochondrial respiratory enzymes leading to augmented oxygen consumption

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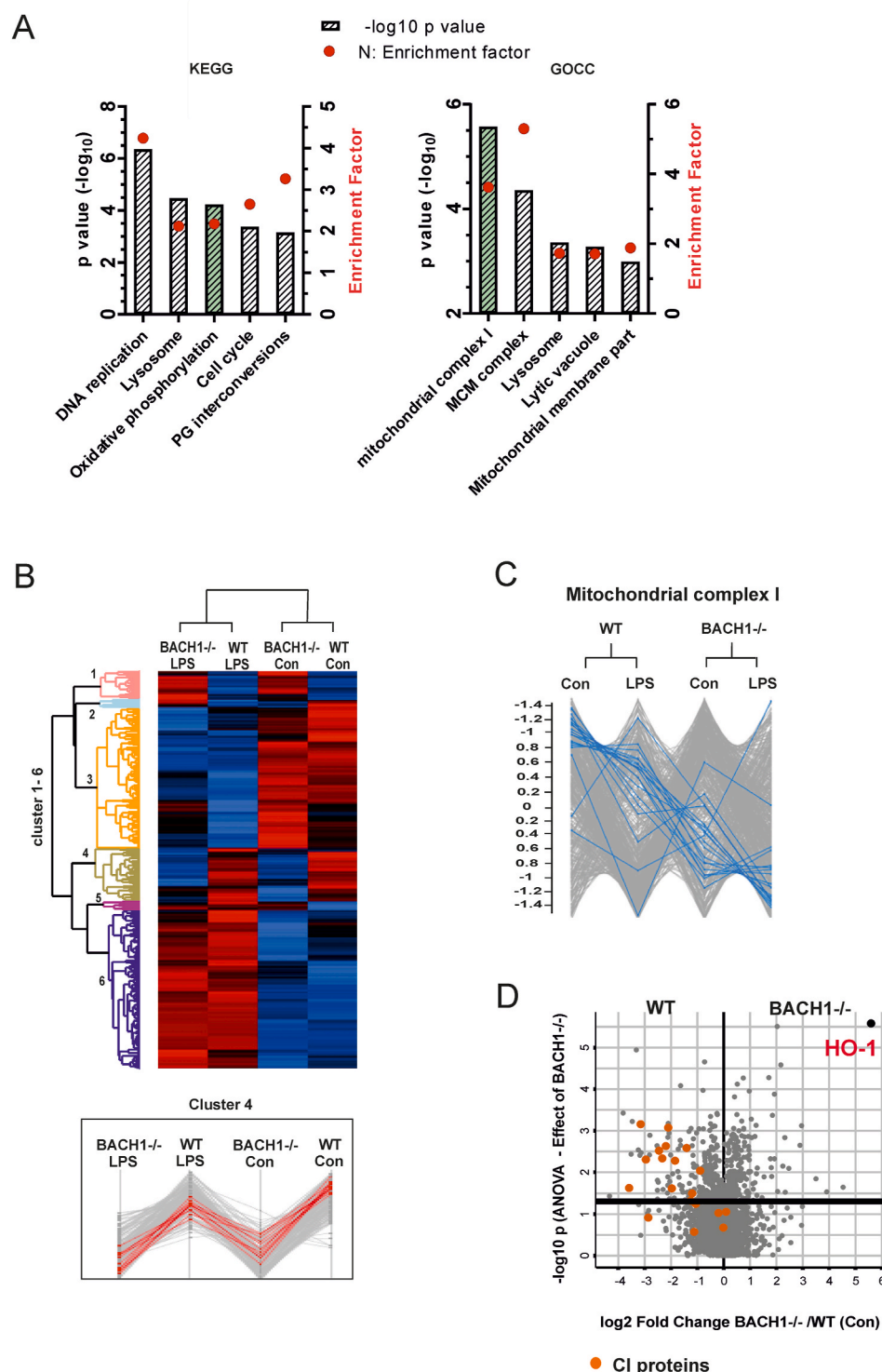
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rate (OCR) while decreasing extracellular acidification rate (ECAR), an index of glycolysis [11]. In lung cancer cells, BACH1 was shown to regulate glycolysis and exert a pro-metastatic role [12], suggesting that BACH1 modulates metabolism in a cell-dependent fashion. However, despite the recognized importance of metabolism in determining pro- and anti-inflammatory activities in macrophages and the involvement of BACH1 in inflammation, no study so far has addressed whether BACH1 affects macrophage metabolism.

The aim of the current study was to investigate whether and how BACH1 affects mitochondrial function and whether this has an impact

on the regulation of the NLRP3 inflammasome in murine bone marrow-derived macrophages (BMDMs). It is demonstrated that BACH1<sup>-/-</sup> BMDMs exhibit reduced mitochondrial complex 1 (CI) protein levels, resulting in altered mitochondrial bioenergetics and increased generation of mitochondrial reactive oxygen species (mtROS). We also report that BACH1<sup>-/-</sup> BMDMs challenged with lipopolysaccharide (LPS) are characterized by an increased activation of the NLRP3 inflammasome. The enhanced inflammasome inducibility is mechanistically dependent on down-regulation of inducible nitric oxide synthase (iNOS) and overexpression of cyclooxygenase-2 (COX-2) in BACH1<sup>-/-</sup> cells. Our



findings highlight a regulatory role of BACH1 for controlling mitochondrial metabolism and the inflammatory response in mouse macrophages.

## 2. Results

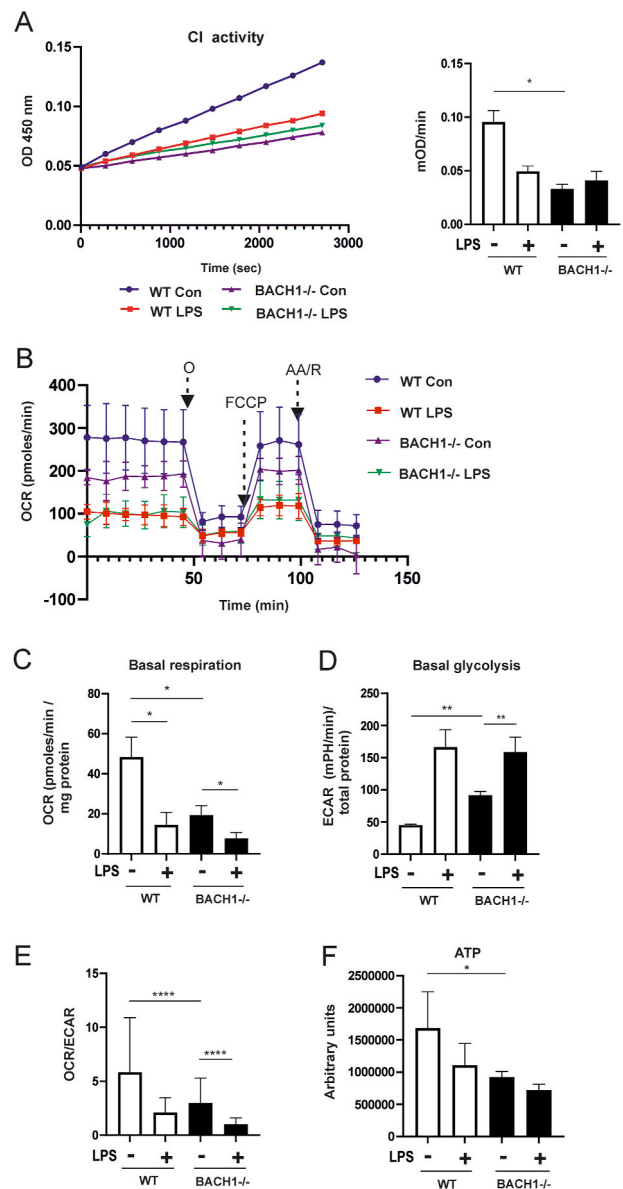
### 2.1. Proteomics analysis in BACH1<sup>-/-</sup> macrophages reveals low mitochondrial complex I levels

In an explorative proteomics approach to identify proteins that might be affected by deficiency of BACH1, BMDMs from BACH1<sup>-/-</sup> and wild-type (WT) mice were treated with the toll-like receptor (TLR)-4 ligand LPS with untreated cells serving as controls and subjected to high throughput mass spectrometry. Subsequent data processing, as described in the methods section (Fig. S1 “Data Processing”) was followed by the analysis of 2486 proteins identified in one of the four conditions. Differentially regulated proteins in BACH1<sup>-/-</sup> and WT BMDMs were analyzed by bioinformatics methods. A principle component analysis (Fig. S2A) showed distinct clustering of the biological replicates of each condition indicating a high degree of reproducibility. Statistical analyses resulted in the identification of 1105 proteins significantly affected by the genetic defect, LPS or combined effects. Among the 469 proteins affected by the BACH1 deficiency, proteins of the oxidative phosphorylation pathway and proteins of the CI were significantly enriched (Fig. 1A). A cluster analysis of all 1105 affected proteins showed that the majority of the CI proteins were enriched in a cluster of proteins with decreased expression in BMDMs from BACH1<sup>-/-</sup> mice independent from LPS treatment (Fig. 1B and C, S2B). The expression of mitochondrial complex II, but not III and IV, was also lower in BACH1<sup>-/-</sup> macrophages (Fig. S2C). HO-1, the inducible isozyme of heme degradation, was the most strongly up-regulated protein (Fig. 1D). This is consistent with earlier findings [4,13,14] showing that expression of this enzyme and that of the Ets1 transcription factor SPI-C are highly up-regulated in BACH1<sup>-/-</sup> BMDMs (Fig. S2D). Collectively, the data indicate that BACH1 deficiency results in reduced levels of mitochondrial proteins, in particular that of CI, in macrophages.

### 2.2. BACH1 deficiency impairs mitochondrial bioenergetics and function in macrophages

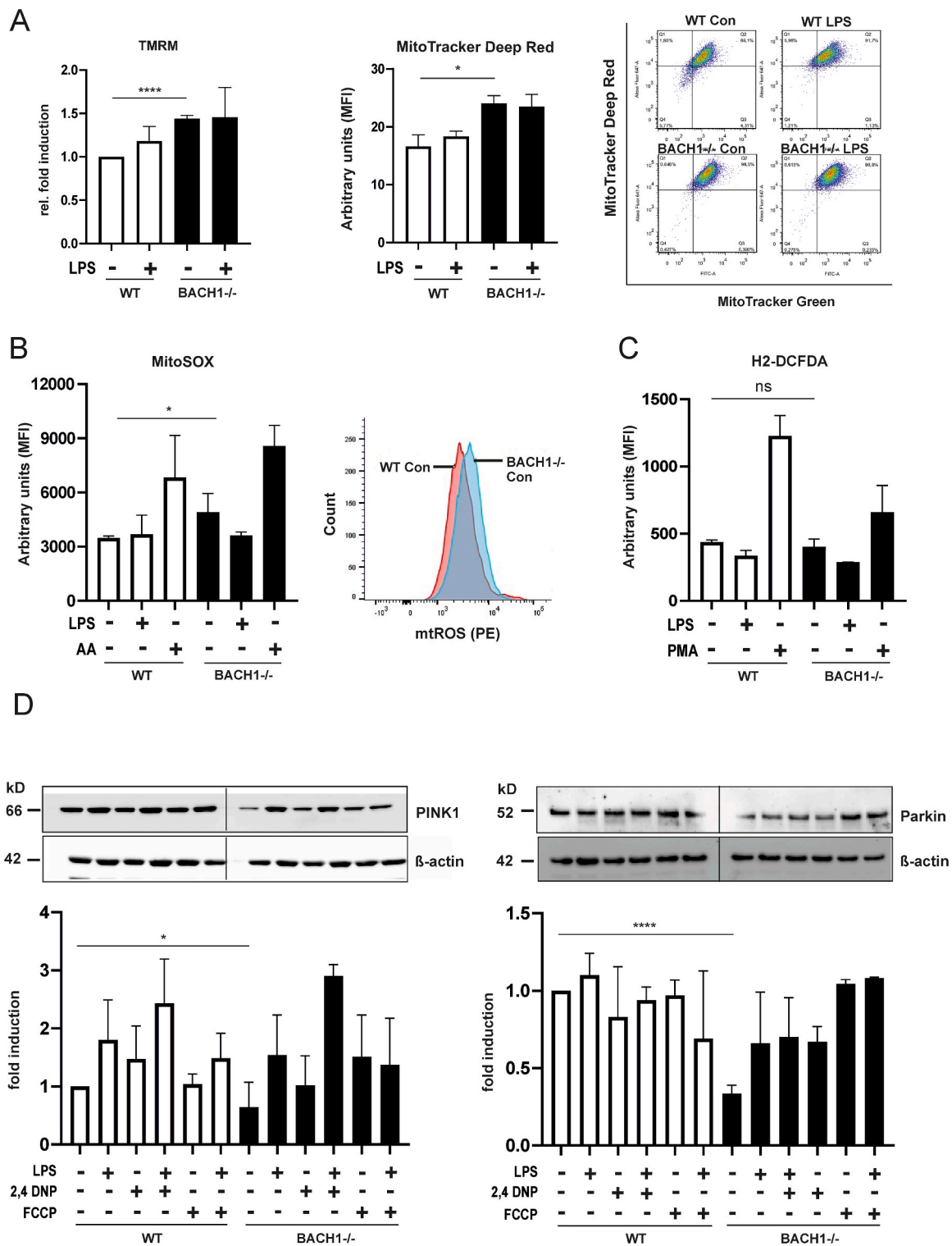
To evaluate whether the decreased expression of respiratory patterns is functionally relevant, the enzymatic activity of CI was determined in BACH1<sup>-/-</sup> and WT cells. CI activity was significantly reduced in untreated BACH1<sup>-/-</sup> BMDMs as compared to WT cells (Fig. 2A). After challenge with LPS, CI activity was 50% less than in WT macrophages, but did not change in BACH1<sup>-/-</sup> cells (Fig. 2A). Due to the crucial role of CI in mitochondrial metabolism, we next asked whether mitochondrial bioenergetics were also affected in these cells. To this end, Mito Stress profiles were assessed with a Seahorse XF analyzer. We first observed that BACH1<sup>-/-</sup> cells displayed a lower basal OCR and a higher ECAR, resulting in a significantly decreased OCR/ECAR ratio compared to WT macrophages (Fig. 2B, C, 2D, 2E). Stimulation of macrophages with LPS similarly decreased OCR and increased ECAR levels in BACH1<sup>-/-</sup> and WT BMDMs leading to reduced OCR/ECAR ratio compared to untreated cells (Fig. 2B, C, 2D, 2E). Consistent with this profile, we found that intracellular ATP was significantly lower in BACH1<sup>-/-</sup> compared to WT BMDMs and was further decreased in LPS-challenged cells (Fig. 2F). Altogether, these data indicate that genetic BACH1 deficiency in mouse macrophages is associated with impairment of key mitochondrial respiratory proteins thus altering mitochondrial metabolism and energy production.

We next determined the levels of mitochondrial membrane potential ( $\Delta\psi_m$ ) and mtROS [15,16] in untreated and LPS-stimulated macrophages. A higher basal  $\Delta\psi_m$  along with increased generation of mtROS was detected in BACH1<sup>-/-</sup> macrophages relative to WT BMDMs



**Fig. 2. Decreased CI enzyme activity and altered mitochondrial metabolism in BACH1<sup>-/-</sup> macrophages.** (A) WT and BACH1<sup>-/-</sup> cells were treated with or without LPS for 16 h and CI activity was measured (left). A representative bar graph of the measured enzyme activity plotted in mOD/min (right) is shown. (B) Macrophages were treated with LPS (1  $\mu$ g/ml) for 16 h and subjected to Mito-Stress assay using a Seahorse XF-analyzer. Oxygen consumption rate (OCR), (C) basal respiration, and (D) extracellular acidification rate (ECAR) were measured after the sequential addition of oligomycin (1 mg/ml), FCCP (0.7  $\mu$ M), and rotenone (1  $\mu$ M) plus antimycin A (1  $\mu$ M). (E) OCR/ECAR ratio of macrophages in the conditions described above (F) ATP levels were assessed in cells after LPS treatment. All values shown are means  $\pm$  SEM of three independent experiments. Statistical analysis was performed using t-test; \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001, \*\*\*\* $p$  < 0.0001. Con, control; WT, wild-type; CI, mitochondrial complex I; O, oligomycin; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; AA, antimycin A; R, rotenone.

(Fig. 3A and B). Interestingly, total cellular ROS were not affected by genetic BACH1 deficiency in BMDMs (Fig. 3C). In our experimental setting, LPS did not change mtROS production in BMDMs. To validate these findings, mtROS were also measured in RAW 264.7 macrophages, which are known to produce more ROS in response to LPS [17–19]. As shown in Fig. S3, LPS clearly caused a more pronounced increase in mtROS production in these cells.

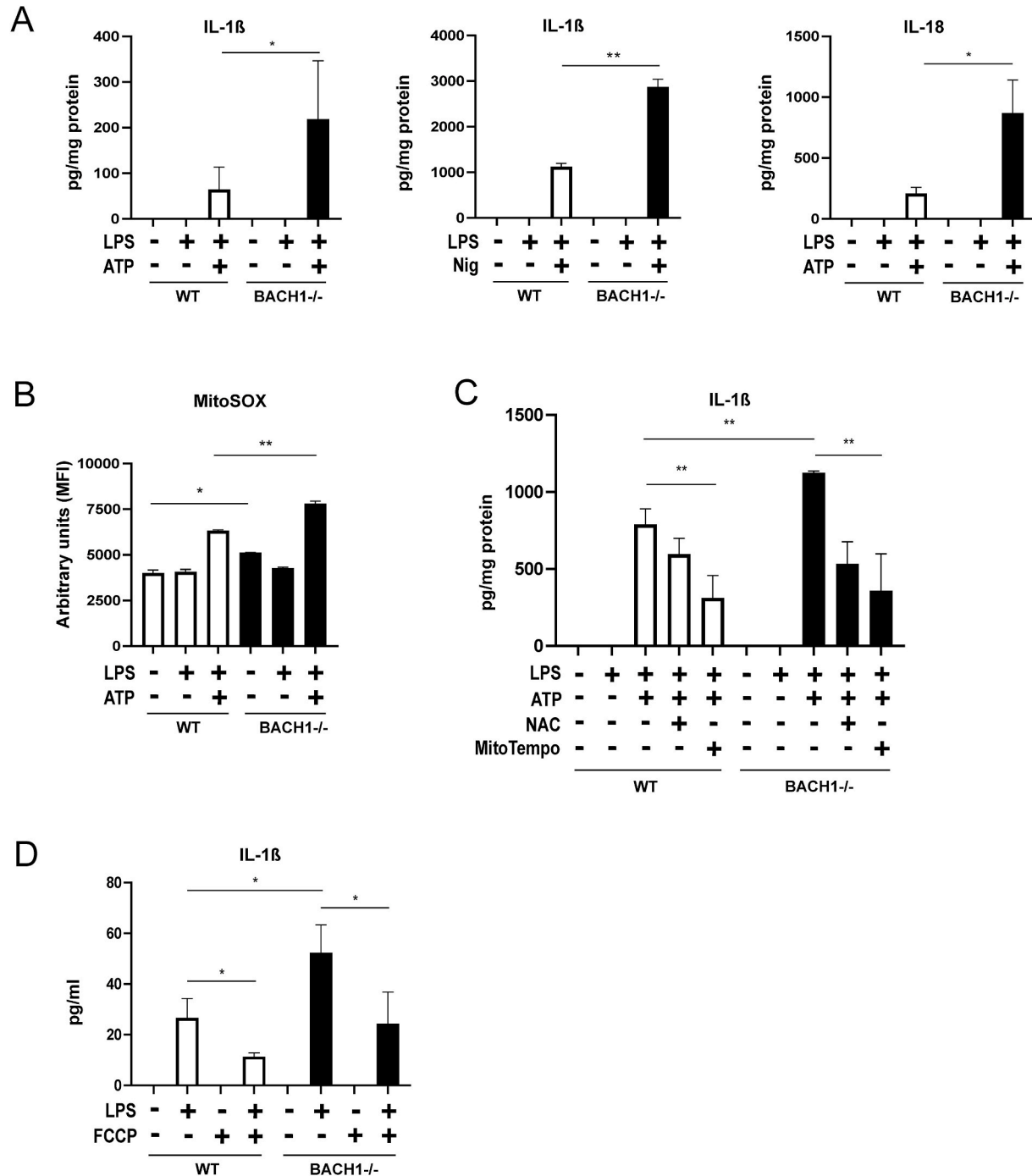


**Fig. 3. Increased  $\Delta\psi_m$  and mtROS but not mitophagy in BACH1<sup>-/-</sup> macrophages.** WT and BACH1<sup>-/-</sup> cells were treated with or without LPS for 16 h. Cells were stained with (A) MitoTracker Deep Red and (B) MitoSOX and analyzed by FACS, as indicated. Fluorescence intensity was quantified as a measure of  $\Delta\psi_m$  or mtROS production, respectively. As a positive control for mtROS production, LPS-primed macrophages were treated with antimycin A (AA, 5 mM, 30 min). (C) For determination of total ROS, LPS-primed cells were treated with H2-DCFDA for 20 min and analyzed by FACS. As a positive control, cells were treated with PMA for 30 min. (D) WT and BACH1<sup>-/-</sup> cells were stimulated with LPS alone or co-stimulated with 2,4 DNP (1  $\mu$ M) or FCCP (5  $\mu$ M) for 16 h. Western blot of total cell lysates probed with antibodies against PINK1, Parkin and  $\beta$ -actin (top) and a representative bar graph of densitometric protein quantification normalized to  $\beta$ -actin (bottom). All values shown are means  $\pm$  SEM of three independent experiments. Statistical analysis was performed using *t*-test; \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001. Con, control; WT, wild-type; rel, relative; AA, antimycin; MFI, mean fluorescence intensity; PMA, phorbol 12-myristate 13-acetate; DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



Since impaired mitochondrial function is often accompanied by defects in mitophagy (for a review see Ref. [20]) and has been shown to be associated with changes in mitochondrial bioenergetics markers such as  $\Delta\psi_m$  and mtROS [21], we investigated whether alterations of mitochondrial function in BACH1-deficient BMDMs are correlated with markers of mitophagy activity. To this end we determined the levels of PINK1 and Parkin, two essential proteins participating in the mitophagy

process. Under basal conditions, PINK1 and Parkin expression were lower in BACH1<sup>-/-</sup> relative to WT BMDMs (Fig. 3D), suggesting reduced activity of mitophagy in these cells. However, treatment with LPS caused an increase of PINK1 and Parkin levels, similar to that elicited by the mitochondrial uncouplers 2, 4-dinitrophenol (DNP) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), in both cell types. These data combined indicate that deletion of BACH1 in



**Fig. 4. Increased NLRP3 inflammasome activation in BACH1<sup>-/-</sup> BMDMs and role of ROS.** WT and BACH1<sup>-/-</sup> BMDMs were treated with LPS for 16 h for all the experiments. (A) LPS-primed BMDMs were treated with ATP (5 mM) or nigericin (20  $\mu$ M) for 15 min and production of IL-1 $\beta$  and IL-18 cytokine in cell culture supernatants were measured with ELISA. (B) LPS-primed BMDMs were treated with ATP for 15 min and production of mtROS was measured by MitoSOX. (C) BMDMs were pretreated with NAC (5 mM) and MitoTempo (50 mM) for 30 min before LPS stimulation followed by ATP treatment (5 mM, 15 min). Secretion of IL-1 $\beta$  in cell culture supernatants was measured with ELISA. (D) BMDMs were treated with LPS and FCCP for 16 h, as indicated. IL-1 $\beta$  secretion in cell culture supernatants was measured with ELISA. All values shown are means  $\pm$  SEM of at least three independent experiments. Statistical analysis was performed using *t*-test; \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001. WT, wild-type; Nig, nigericin; MFI, mean fluorescence intensity; NAC, N-acetylcysteine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

BMDMs also causes alterations in  $\Delta\psi_m$  and ROS levels, which may be dependent on abnormal mitophagy.

### 2.3. NLRP3 inflammasome activation is increased in BACH1<sup>-/-</sup> macrophages

Inflammasomes are multi-protein complexes of the innate immune system and mediate inflammatory responses [22]. Because the NLRP3 inflammasome is activated via a complex sequence of events in which mitochondria play a critical regulatory role [23,24] and BACH1 deficiency is clearly associated with mitochondrial abnormalities, we investigated the activation of the NLRP3 inflammasome in BACH1<sup>-/-</sup> macrophages and compared it to WT cells. Macrophages were primed with LPS (signal 1) and then treated with ATP or nigericin (signal 2), known activators of the NLRP3 inflammasome [25]. Fig. 4A shows that secretion of IL-1 $\beta$  and IL-18 were markedly higher in BACH1<sup>-/-</sup> BMDMs after treatment with ATP and nigericin.

LPS-primed BACH1<sup>-/-</sup> macrophages also exhibited higher levels of mtROS in response to ATP as compared to WT cells (Fig. 4B). Furthermore, when macrophages challenged with LPS and ATP were pre-treated with MitoTempo, a mitochondrial superoxide scavenger, or the antioxidant N-acetylcysteine (NAC), IL-1 $\beta$  production was markedly reduced in both BACH1<sup>-/-</sup> and WT BMDMs (Fig. 4C). As a control, cells were treated with FCCP, an uncoupler of oxidative phosphorylation (OxPhos) that decreases mtROS production [26,27]. LPS-dependent IL-1 $\beta$  secretion was significantly diminished by treatment with FCCP in both cell types (Fig. 4D). These findings indicate that the higher mtROS levels observed in BACH1<sup>-/-</sup> macrophages may be responsible for the exacerbated NLRP3 inflammasome activation compared to WT cells.

Because BACH1<sup>-/-</sup> macrophages have been previously associated with an M2-polarized phenotype [2,9], we also determined anti-inflammatory markers in our experimental setting. Higher levels of arginase and CD206, but lower IL-10, were found in BACH1<sup>-/-</sup> untreated and LPS-stimulated cells (Fig. S4A). Interestingly, LPS caused an increased secretion of the pro-inflammatory cytokines TNF- $\alpha$  and IL-6 in BACH1<sup>-/-</sup> BMDMs relative to WT cells (Fig. S4B). The data indicate that BACH1<sup>-/-</sup> deficiency modifies the inflammatory response of macrophages and amplifies the production of pro- and anti-inflammatory mediators.

### 2.4. Differential regulation of iNOS and COX-2 is associated with inflammasome activation in BACH1<sup>-/-</sup> macrophages

We next asked whether other classical inflammatory markers of activated macrophages were affected by BACH1<sup>-/-</sup> deficiency and measured the expression of inducible nitric oxide synthase (iNOS) and COX-2 proteins.

We observed that up-regulation of iNOS expression and secretion of nitrite, a product of NO, were markedly reduced in BACH1<sup>-/-</sup> BMDMs after challenge with LPS (Fig. 5A and B). Furthermore, the over-expression of COX-2 after LPS was further amplified in BACH1<sup>-/-</sup> macrophages treated with LPS compared to WT cells (Fig. 5C). We then evaluated the involvement of these enzymes in NLRP3 inflammasome activation in BACH1 deficiency. Since NO production was lower in BACH1<sup>-/-</sup> cells, we pre-treated cells with the NO donor DETA NON-Oate (DETA-NO) and then stimulated with LPS (signal 1) followed by treatment with ATP (signal 2). Interestingly, DETA-NO strongly reduced IL-1 $\beta$  levels in both BACH1<sup>-/-</sup> and WT cells (Fig. 5D). As a control, the iNOS inhibitor 1400W led to an increased secretion of IL-1 $\beta$  in macrophages (data not shown). Furthermore, macrophages were also pre-treated with celecoxib, an inhibitor of COX-2 activity, and we observed that levels of IL-1 $\beta$  were significantly suppressed (Fig. 5E). These data indicate that decreased levels of iNOS inducibility and amplification of COX-2 expression after LPS challenge play an important role in the exacerbation of the NLRP3 response observed in BACH1-

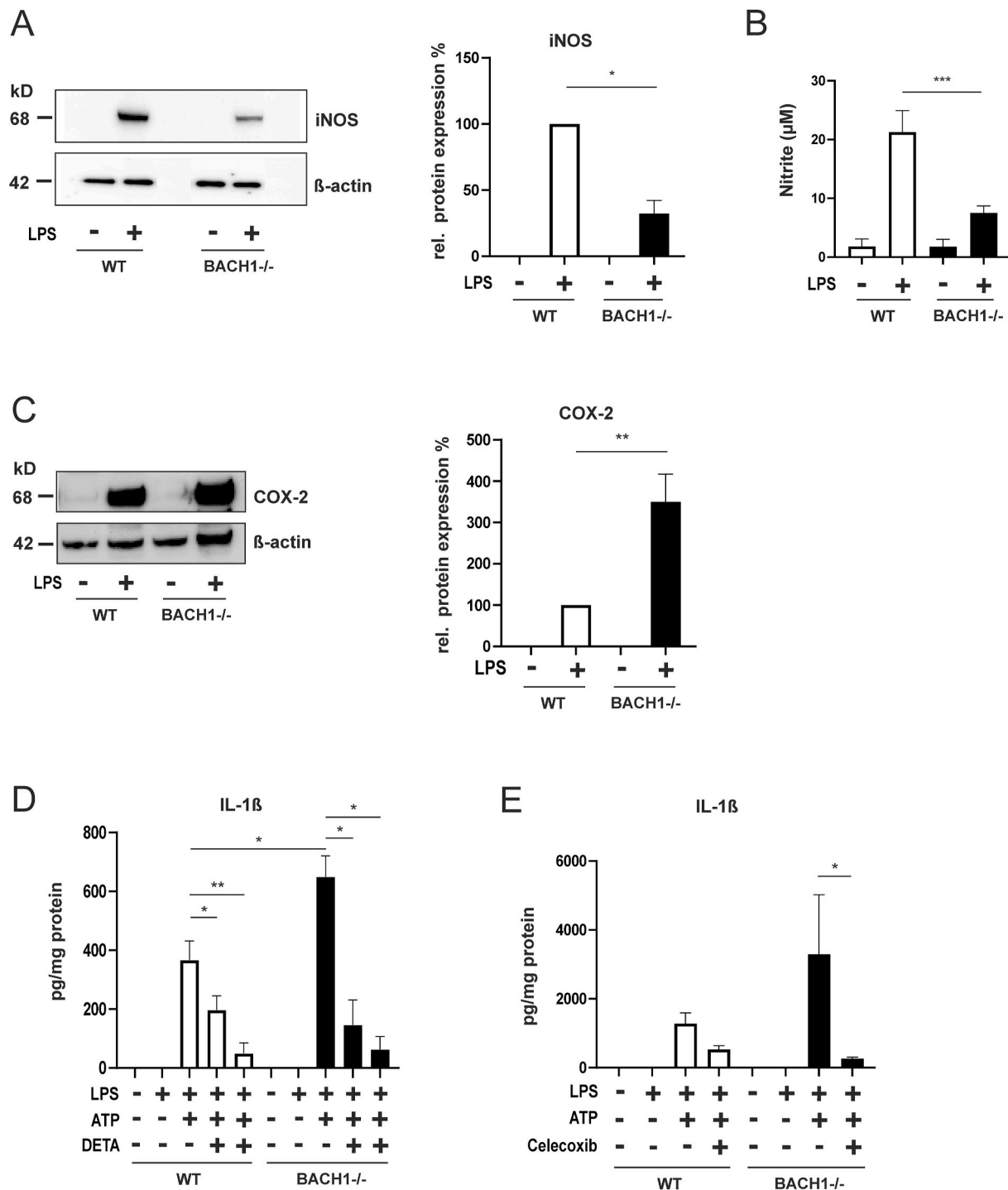
deficient macrophages.

## 3. Discussion

BACH1, a member of the bZIP family of transcription factors, is a heme sensor protein [1] and nuclear repressor of the heme-degrading enzyme HO-1 [13] that affects macrophage function in inflammatory conditions. Different metabolic profiles characterize macrophage responses to inflammatory stimuli and recent evidence in cancer cells indicates that BACH1 affects the regulation of OxPhos and glycolysis [11, 12], prompting us to examine whether BACH1 deficiency modifies murine macrophage metabolism and inflammatory responses. The major findings of the current study are that 1) BACH1<sup>-/-</sup> macrophages exhibit an altered expression pattern of mitochondrial proteins, with significantly reduced levels of CI, thus resulting in changes of cellular bioenergetics, ROS production and mitochondrial function, and, 2) lack of BACH1 is associated with an increased inducibility of NLRP3 inflammasome activation in mouse macrophages, which is partially dependent on altered iNOS and COX-2 expression during stimulation with LPS.

A most striking finding of our study is that BACH1-deficient macrophages exhibit lower CI protein levels, which was accompanied by decreased CI enzyme activity. These results suggest that BACH1 not only controls expression of HO-1 and SPI-C, but also that of CI. This is in line with recent data showing that BACH1 regulates the transcription of electron transport chain and glycolytic genes in breast and lung cancer cells, respectively [11,12]. Thus, it appears that the transcriptional activity of BACH1 has functional consequences on cellular bioenergetics. Indeed, we found that BACH1 deficiency is associated with a shift towards decreased OxPhos and increased glycolysis as well as lower ATP production in mouse macrophages. In addition, we observed increased  $\Delta\psi_m$  and mtROS levels, further indicating an altered mitochondrial function in BACH1<sup>-/-</sup> cells. Interestingly, these metabolic alterations typically occur during macrophage reprogramming in response to activation by LPS [18], leading us to propose that, in unstimulated conditions, BACH1-deficient macrophages already display a 'reprogrammed-like' metabolic profile. The functional role of  $\Delta\psi_m$  in these cells is complex because it is affected by numerous cellular events that may cause changes in mitochondrial activities [19]. In fact, an increase in  $\Delta\psi_m$  generally results in higher mtROS levels [28], as observed in our study. Importantly, the generation of mtROS plays an important role in the regulation of innate immune responses, the complexity of which is currently under intense investigation [29]. It should be noted that the rise of mtROS in BACH1<sup>-/-</sup> cells was not associated with higher levels of total cellular ROS or increased mitophagy. These findings suggest that the changes in  $\Delta\psi_m$  and mtROS in BACH1-deficient macrophages are not strong enough to promote mitophagy. It is important to emphasize that CI and complex III are well recognized sites of ROS leakage from mitochondria [28]. The fact that CI protein and activity are markedly lower in BACH1<sup>-/-</sup> cells implies that complex III could be the major source of ROS in this system. Thus, we suggest that the increased generation of mtROS in BACH1<sup>-/-</sup> macrophages probably contributes to enhanced signaling activities that may have repercussions for the inflammatory response.

In fact, NLRP3 inflammasome activation and levels of mtROS were more pronounced in BACH1-deficient macrophages as indicated by a further increase of IL-1 $\beta$  and IL-18 after stimulation with LPS and ATP compared to WT cells. This finding was unexpected because an M2 anti-inflammatory phenotype of macrophages has been reported in various experimental *in vivo* and *in vitro* models of genetic BACH1 deficiency [8, 9] and has been attributed, in part, to high expression of the anti-inflammatory enzyme HO-1 [7,30–32]. In addition, increased expression of HO-1 has been shown to attenuate inflammasome activation but these findings have been reported for sterile inflammation in the hypoxic lung. Since we confirmed that BACH1-deficient macrophages exhibited high levels of HO-1 and M2 markers in our



**Fig. 5.** Role of iNOS and COX-2 for regulation of NLRP3 inflammasome activation in BACH1<sup>-/-</sup> macrophages. WT and BACH1<sup>-/-</sup> BMDMs were stimulated with LPS for 16 h in all experiments. (A) Western blot of total cell lysates probed with antibodies against iNOS and  $\beta$ -actin (left) and bar graphs of the densitometric protein quantifications normalized to  $\beta$ -actin from three independent experiments are shown (right). Protein expression in LPS-treated WT cell was considered 100%. (B) Nitrite levels (an indicator of nitric oxide production) were measured using the Griess reagent. (C) Western blot of total cell lysates probed with antibodies against COX-2 and  $\beta$ -actin (left) and bar graphs of densitometric protein quantifications normalized to  $\beta$ -actin from three independent experiments are shown (right). Protein expression in LPS-treated WT BMDMs was considered as 100%. (D) Macrophages were pre-treated with DETA NONOate (25 and 50  $\mu$ M) for 30 min followed by LPS stimulation for 16 h. Cells were then exposed to ATP (5 mM) for 15 min and IL-1 $\beta$  secretion in culture medium was measured by ELISA. (E) BMDMs were pre-treated with celecoxib (0.1  $\mu$ M) for 30 min followed by LPS stimulation for 16 h. Cells were then treated with ATP (5 mM) for 15 min and IL-1 $\beta$  secretion in culture medium was measured by ELISA. All values shown are mean  $\pm$  SEM of three independent experiments. Statistical analysis was performed using *t*-test; \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.  $\beta$ -actin, beta-actin. WT, wild-type; rel, relative; DETA, DETA NONOate.



experimental setting [14,33,34], activation of the NLRP3 inflammasome and M2 polarization of BACH1<sup>-/-</sup> cells appear to be mutually independent [35]. Because HO-1 is critical for iron metabolism in macrophages [36], it is conceivable that BACH1 deficiency may also affect both the inflammatory and metabolic phenotypes of macrophages via iron-dependent pathways [37]. The altered mitochondrial metabolism in BACH1<sup>-/-</sup> cells characterized by decreased activity of CI and OCR/ECAR ratio likely contributes to the increased inducibility of the NLRP3 inflammasome because these organelles are critically involved in regulating this pathway [23,38]. Moreover, the reduced inflammasome activation in the presence of NAC and MitoTempo corroborate that mtROS are an important signal in inflammasome activation in WT and BACH1<sup>-/-</sup> macrophages. Notably, others have shown that the CI inhibitor rotenone led to an increase of mtROS production that caused an increased activation of the NLRP3 inflammasome in macrophages [23,39]. Interestingly, a link between mtROS and lysosomes in NLRP3 inflammasome activation has been reported [40] and our data on proteomics highlight that, in association with mitochondrial dysfunction, lysosomes were also affected by genetic BACH1 deficiency (Fig. 1A).

Our findings also suggest a mechanistic involvement of the enzymes iNOS and COX-2, two LPS-inducible proteins [41,42] that regulate inflammasome activation [43–45]. Regarding iNOS, it is known that this protein is a negative regulator of inflammasome activation [43,44,46,47] because iNOS-derived NO inhibits the formation of the ASC pyroptosome and S-nitrosylation of the NLRP3 inflammasome complex [43,44]. In agreement with findings of earlier reports [14,34], our results show that iNOS expression and NO production are markedly lower in BACH1<sup>-/-</sup> cells challenged with LPS compared to WT macrophages, suggesting that less NO is available to suppress inflammasome activation in the absence of BACH1. Indeed, when BACH1<sup>-/-</sup> cells were supplied with exogenous NO (DETA) to compensate for reduced endogenous NO levels, inflammasome activation was significantly decreased, corroborating a role of NO in this process. Moreover, genetic deficiency of iNOS has been shown to cause generation of high mtROS levels and subsequent mitochondrial damage [44], in line with the phenotype found in our BACH1<sup>-/-</sup> macrophages. Overexpression of COX-2 in BACH1<sup>-/-</sup> cells also contributes to enhanced inflammasome activation, because inhibition of COX-2 enzyme activity with celecoxib results in a strong decrease in IL-1 $\beta$  production (Fig. 5E). This is likely due to inhibition of pro-inflammatory prostaglandins released by this enzyme [48]. It is important to point out that iNOS and COX-2 are heme-containing proteins the synthesis of which is dependent on the availability of intracellular labile heme. Because the BACH1/HO-1 axis has been shown to regulate intracellular heme levels in LPS-stimulated macrophages [49], genetic BACH1 deficiency with consequent increase in HO-1 may indirectly impact iNOS and COX-2 expression, as previously reported [50,51].

In conclusion, our findings add to the emerging notion that BACH1 is an important regulator of cellular bioenergetics with functional consequences for immunomodulatory activities and reprogramming of macrophages. In particular, we report an atypical phenotype of LPS-stimulated BACH1-deficient macrophages characterized by the coexistence of both pro-inflammatory (enhanced inflammasome activation) and anti-inflammatory (high expression levels of HO-1 and M2 markers) profiles, which may represent an intermediate state within the spectrum of macrophage activation. It will be important to verify whether a similar profile is reproduced in animals lacking BACH1 and challenged with endotoxin in order to better understand the full implication of this heme-sensitive repressor in inflammatory responses. A better comprehension of the role of BACH1 in the regulation of cellular metabolism and immunomodulation may also lead to potential therapeutic approaches in inflammatory disorders [52], as recently shown for the targeted regulation of BACH1 by the anti-diabetic drug metformin in preclinical animal models of cancer [11,12].

## 4. Materials and methods

### 4.1. Reagents

Recombinant cytokine macrophage colony-stimulating factor (M-CSF) was purchased from PeproTech Inc., USA. Penicillin-streptomycin was purchased from C.C.Pro GmbH (Oberdorla, Germany). Lipopolysaccharide serotype 0111:B4 was from InvivoGen (San Diego, CA, USA). Accutase was obtained from Capricorn Scientific (Ebsdorfergrund, Germany). Mitochondrial complex inhibitors, 2',7'-dichlorodihydrofluorescein diacetate (H2-DCFDA), phorbol 12-myristate 13-acetate (PMA) and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless indicated otherwise.

### 4.2. Mice

BACH1<sup>-/-</sup> mice were generated at Tohoku University (Sendai, Japan) [13,53] and transferred to the Central Animal Laboratory, Hannover Medical School. They were backcrossed for more than 10 generations on a C57BL/6J background and compared with WT control mice. Adult male mice (10–14 weeks of age) were used. Mice were kept on a standard laboratory diet and were housed in cages under standardized environmental conditions (12 h light/dark cycle, 23  $\pm$  1  $^{\circ}$ C and 55  $\pm$  1% relative humidity). All animal experiments were approved by the Committee for Animal Welfare.

### 4.3. Isolation and cell culture of BMDMs

Mouse bone marrow cells were flushed from the tibia and femur of male C57BL/6J mice and cells were cultured for 7 days in DMEM high glucose media containing 10% fetal bovine serum (FBS) (Merck), 100 U/ml penicillin, 100 mg/ml streptomycin and 25 ng/ml recombinant mouse M-CSF. On day 7, cells were plated in a 12-well plate at 3  $\times$  10<sup>5</sup> cells per well. All treatments were performed in 1% serum containing 12.5 ng/ml M-CSF and LPS was used at a concentration of 1  $\mu$ g/ml.

### 4.4. Cell culture of RAW264.7 cells

RAW264.7 macrophages were obtained from American Type Culture Collection (Rockville, MD, USA) and were cultured as previously described [54].

### 4.5. Analysis of mRNA expression

Total RNA isolation was performed using an RNeasy mini kit (Qiagen GmbH, Hilden, Germany). cDNA synthesis was carried out using high Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Primers for quantification of mRNA levels of HO-1, SPI-C, arginase and GAPDH were from Applied Biosystems. Amplification was performed with TaqMan Gene Expression Master Mix on a StepOnePlus<sup>TM</sup> Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Thermal cycling was performed at 95  $^{\circ}$ C for 10 min followed by 40 cycles at 95  $^{\circ}$ C for 15 s and 60  $^{\circ}$ C for 1 min. GAPDH was used as a control for normalization of cDNA values. The  $\Delta\Delta$ CT method was used to semi-quantify mRNA levels.

### 4.6. Mass spectrometry analysis

Samples were prepared for mass spectrometry analyses and measured as described recently [55]. In brief, 1.5  $\times$  10<sup>8</sup> cells were lysed in RIPA buffer, proteins were precipitated with acetone and 50  $\mu$ g of protein was separated by SDS-PAGE for each sample. Upon staining with Coomassie, each lane on the SDS gel was cut into five fractions which were digested separately over night with Trypsin Gold (Promega) according to manufacturer's instructions. A Linear Trap Quadrupole (LTQ) Orbitrap-Velos mass spectrometer coupled to a reversed-phase liquid

chromatography system was used to analyse samples. A nanoflow ultrahigh pressure liquid chromatography system (rapid separation liquid chromatography; Thermo Fisher Scientific) with a trapping column (2 cm length, 75  $\mu$ m inner diameter, and 3  $\mu$ m C18 particle size) and a reversed-phase separating column (50 cm length, 75  $\mu$ m inner diameter, and 2  $\mu$ m C18 particle size) was used for reversed phase chromatography followed by ionisation and electrospray injection of peptides using a Nano Spray Flex Ion Source II. For overview scans, stored in profile mode, the Orbitrap analyzer with a resolution of 60 k at  $m/z$  400 and a mass range of  $m/z$  300 to 1600 was used. Fragmentation of ten most intensive peptides (double and triple charged, minimum intensity of 2000 counts) were performed in the LTQ by collision-induced dissociation. Resulting mass spectra were recorded in the LTQ and stored as centroid  $m/z$  value and intensity pairs. Dynamic exclusion was set to 70 s and a mass window of 10 ppm of  $m/z$ .

#### 4.7. Proteomics data analysis

MaxQuant version 1.6.11.0 [56] was used for the identification and label-free quantification (LFG) of proteins from mass spectrometry raw files (See Data Processing in supplemental section). Preconfigured settings were used including digest mode set to Trypsin (specified) allowing two missed cleavages, carbamidomethyl (C) set as fixed and oxidation (M) and acetylation (N-term) set as variable modifications and mass tolerance set to 20 ppm (precursor) and 0.5 Da (MS/MS). Uniprot database (reviewed + unreviewed, 188,357 entries, database downloaded on March 09, 2020) was used to match mass spectra. Perseus software 1.6.15 [57] was applied for data processing (see data processing in supplemental section). Briefly, proteins marked as contaminants, and proteins only identified by a modified peptide (only identified by side) as well as proteins matching with the reverse database were excluded. Analysis was further restricted to proteins identified by at least two peptides and detected in both replicates in at least one condition. Additionally, LFQ values were normalized by transformation into the base-2 logarithm, and missing values were replaced by low LFQ values drawn from the normal distribution of the dataset (imputation; width: 0.3, down shift: 1.8, separately for each column). Principal component analysis (PCA) was used to identify variation and reproducibility in samples. Medians were calculated for each condition as well as fold changes to compare protein abundance across conditions. Two-way ANOVA was used to identify proteins which expression was affected by the genotype (BACH1), the treatment (LPS) or combined affects (genotype and treatment). Protein abundance of affected proteins ( $p \leq 0.05$ , median LFQ values) were visualized as a heat map after z-normalization based on hierarchical clustering using complete linkage based on Euclidean distance method. For enrichment analyses, protein annotations from databases GOCC (Gene Ontology Cellular Compartment) and KEGG (Pathways) were added based on majority protein IDs. Fisher exact tests were performed to screen for enrichment of attributed cellular compartments or pathways among affected proteins. Results were visualized by Perseus Software and Graphpad Prism software version 8.

#### 4.8. Seahorse analysis

OCR and ECAR were measured using a Seahorse XF24 extracellular flux analyzer (Seahorse Bioscience, Inc, North Billerica, MA, USA). Mito stress assay was performed as previously described [58].

#### 4.9. CI enzyme activity assay

CI enzyme activity was measured using Complex I Enzyme activity assay kit (colorimetric) (Abcam, ab109721) according to the manufacturer's protocol. Enzyme activity was measured for 45 min at 450 nm using a plate reader.

#### 4.10. Protein isolation and Western blot analysis

At the end of the experiments, cells were washed with PBS and proteins were extracted using RIPA buffer containing protease inhibitor mixture (Chemcruz, Santacruz, USA) and stored at  $-20^{\circ}\text{C}$  until analyzed. Protein concentrations were measured with the BCA assay (Interchim, Montlucon, France) and samples were subjected to Western blot analysis. Whole cell-lysates were separated by SDS-PAGE, transferred to PVDF membranes (Bio-Rad) and incubated with primary antibodies against PINK1 (1:200, Santa Cruz), Parkin (1:100, Santa Cruz), iNOS (1:1000, Novus Biologicals), COX-2 (1:1000, Abcam) and  $\beta$ -actin (1:5000, Sigma- Aldrich). Secondary antibodies, polyclonal goat anti-rabbit IgG/HRP and rabbit anti-mouse IgG/HRP (Dako, Jena, Germany) were used at a dilution of 1:800. Chemiluminescent bands on autoradiograms were visualized with a ChemiDocTM Touch Imaging System (Bio-Rad, Basel, Switzerland) and quantified using the Image Lab software (Biorad, Germany).

#### 4.11. Flow cytometry

Flow cytometry analysis was performed on a FACS Canto II flow cytometer and analyzed with FACS Diva Software (BD Biosciences, San Jose, CA, USA).

#### 4.12. ROS production

To determine mtROS and total ROS production, cells were stained with MitoSOX red (2.5  $\mu$ M) (Thermo Fisher Scientific, Inc. Waltham, MA, USA) and H2-DCFDA (10  $\mu$ M), respectively, for 15 min at  $37^{\circ}\text{C}$  and analyzed by FACS.

#### 4.13. Mitochondrial function measurements

$\Delta\psi$ m was assessed by staining cells with 20 nM tetramethylrhodamine, methyl ester, perchlorate (TMRM) or 100 nM MitoTracker Deep Red (Thermo Fisher Scientific) for 15 min. For total mitochondrial mass, cells were incubated with 50 nM MitoTracker Green FM (Thermo Fisher scientific) for 15 min at  $37^{\circ}\text{C}$  and analyzed by FACS.

#### 4.14. Other biochemical assays

Measurements of ATP (Cayman Chemical, MI, USA), cytokines (Biolegend, San Diego, CA, USA), and nitrite (Promega, Madison, Wisconsin, USA) were performed using the respective assay kits according to the manufacturers' instruction.

#### 4.15. Statistical analysis

All statistical data analysis was performed using One-way ANOVA with Post Tukey's test or Student's *t*-test as indicated in the figure legends using GraphPad Prism Version 8 (GraphPad Prism Software Inc.)

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### Author contributions

PP, VV, RF, RM, KI and SI planned and designed the experiments of the study. PP conducted the experiments. KC and FFRB performed sample preparation for MS and bioinformatics analysis. PP, RF, RM and SI wrote the manuscript. All the authors reviewed and contributed to the final version of the manuscript.

## Declaration of interests

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

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