University of Crete

Department of Medical School

Laboratory of Clinical Chemistry

Master thesis:

The role of PHF8 in metabolism during macrophage activation

Postgraduate Program: Molecular Biology Biomedicine

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Contents

Abstract

Macrophages as a population display great heterogeneity and serve various functions in immune response including elimination of pathogens, resolution of inflammation and tissue homeostasis. In response to different stimuli they exhibit great plasticity reflected at the transcriptional, epigenetic and metabolic level. Metabolism has a central role during macrophage activation. Different polarizing signals cause distinct metabolic changes, which govern macrophage activation and support the great macrophage plasticity. PHF8 is a Jumonji C domain containing histone lysine demethylase. Preliminary data have shown differences in inflammatory response in Raw264.7 cells overexpressing (O/E) PHF8 and Raw264.7 cells knock out (K.O.) for PHF8 with cells overexpressing PHF8 having lower levels of proinflammatory markers production and K.O. cells having enhanced proinflammatory phenotype after LPS stimulation. We performed RNA sequencing in these cells to gain further insight into the mechanism of PHF8 regulation of immune response. The results revealed differences in metabolic genes such as genes involved in lipid and protein biosynthesis, whose regulation of expression by PHF8 may underlie its inhibitory role in classic macrophage activation. In support to this notion mTORC1, a central complex which incorporates various signals and regulates metabolic pathways was positively regulated by PHF8 at the naïve state, but had impaired activation after LPS treatment in cells overexpressing PHF8. mTORC1 activates Hif1a, which in turn control glycolytic gene expression, a hallmark of M1 macrophages. Consistent with mTORC1 activation, major glycolytic genes such as hk3 and pfkp have increased expression at the naïve state but no further induction in PHF8 overexpressing cells, which could explain the reduced responsiveness of PHF8 O/E cells. Additionally in the RNA sequencing results we observed increased expression of E.R. stress induced genes, which we also validated by western blot. The negative role of PHF8 in classic macrophage activation was also observed in mouse BMDMs combined with enhanced TNF production at the endotoxin tolerant state in PHF8 knock down BMDMs. mTORC1 activation was also increased at the endotoxin tolerant state in PHF8 knock down BMDMs. Last but not least we showed that PHF8 expression is induced post α-KG treatment, a metabolite known to induce tolerance, indicating that PHF8 is a possible mediator of this phenotype. In conclusion, we showed in the present study that the histone demethylase PHF8 is a negative regulator of macrophage activation and an important regulator of macrophage metabolism revealing its potential role in shaping macrophage phenotype in the context of endotoxin tolerance.

Περίληψη

Τα μακροφάγα αποτελούν έναν εξαιρετικά ετερογενή πληθυσμό, τα οποία επιτελούν ποικίλους ρόλους στην ανοσολογική απόκριση όπως η αντιμετώπιση των παθογόνων, η επίλυση της φλεγμονής και η διατήρηση της ομοιόστασης του ιστού. Παρουσία διαφορετικών σημάτων αποκτούν διαφορετικούς φαινοτύπους που χαρακτηρίζονται από διακριτή μεταγραφική, επιγενετική και μεταβολική ρύθμιση. Ο μεταβολισμός έχει κεντρικό ρόλο στην ενεργοποίηση των μακροφάγων. Διαφορετικά σήματα προκαλούν αλλαγές στον μεταβολισμό, οι οποίες επηρεάζουν την ενεργοποίηση των μακροφάγων και υποστηρίζουν την λειτουργική τους ετερογένεια. Η PHF8 ανήκει στην οικογένεια των απομεθυλασών λυσίνης που περιέχουν τομέα jumonji C. Αρχικά δεδομένα του εργαστηρίου είχαν δείξει διαφορές στην έκφραση προφλεγμονωδών γονιδίων σε κυτταρικές σειρές Raw264.7 που υπερεκφράζουν την PHF8 και κυτταρικές σειρές Raw264.7 όπου η PHF8 έχει σιγηθεί. Οι κυτταρικές σειρές που υπερεκφράζουν την PHF8 εμφανίζουν χαμηλότερη έκφραση προφλεγμονωδών μαρτύρων , ενώ το αντίθετο παρατηρείται στις knock out κυτταρικές σειρές μετά από επαγωγή με LPS. Προκειμένου να διερευνήσουμε περαιτέρω τον μηχανισμό με τον οποίο η PHF8 ρυθμίζει την ανοσολογική απόκριση πραγματοποιήσαμε πείραμα αλληλούχισης RNA. Τα αποτελέσματα αποκάλυψαν διαφορές σε μεταβολικά γονίδια όπως γονίδια που εμπλέκονται στη βιοσύνθεση λιπιδίων και πρωτεϊνών, η ρύθμιση των οποίων από την PHF8 πιθανόν να αποτελεί ένα μηχανισμό μέσω του οποίου η PHF8 ρυθμίζει την ενεργοποίηση των μακροφάγων. Παράλληλα εντοπίσαμε διαφορές στην ενεργοποίηση του mTORC1, σύμπλοκο στο οποίο επιδρούν ποικίλα ερεθίσματα και ρυθμίζει μεταβολικά μονοπάτια. Κύτταρα που υπερέκφραζαν την PHF8 παρουσίαζαν αυξημένη ενεργοποίηση του συμπλόκου mTORC1 στα βασικά επίπεδα, ενώ αυτό δεν εμφάνιζε περαιτέρω ενεργοποίηση μετά από επαγωγή με LPS σε αντίθεση με τα κύτταρα αναφοράς. Το mTORC1 ενεργοποιεί τον μεταγραφικό παράγοντα Hif1a, ο οποίος με τη σειρά του ρυθμίζει την έκφραση γλυκολυτικών γονιδίων, χαρακτηριστικό των M1 μακροφάγων. Σε συμφωνία με τα αποτελέσματα σχετικά με την ενεργοποίηση του mTORC1 γλυκολυτικά γονίδια όπως η εξοκινάση 3 (hk3) και η φωσφοφρουκτοκινάση (pfkp) εμφανίζουν αυξημένη έκφραση στα βασικά επίπεδα αλλά δεν εμφανίζουν επιπλέον αύξηση μετά από χειρισμό με LPS στα κύτταρα που υπερεκφράζουν την PHF8, φαινόμενο που θα μπορούσε να εξηγεί την μειωμένη έκφραση κυτταροκινών στα κύτταρα που υπερεκφράζουν την PHF8. Επιπροσθέτως τα αποτελέσματα της αλληλούχισης RNA έδειξαν αυξημένη έκφραση γονιδίων που σχετίζονται με το stress ενδοπλασματικού δικτύου, αποτέλεσμα το οποίο επιβεβαιώσαμε και με ανάλυση κατά Western. Ο ανασταλτικός ρόλος της PHF8 στην κλασσική ενεργοποίηση των μακροφάγων όπως και αυξημένη παραγωγή TNF μετά από πρωτόκολλο ανοχής στην ενδοτοξίνη παρατηρήθηκε επίσης και σε BMDMs ποντικών μετά από σίγηση της PHF8. Τέλος δείξαμε ότι η PHF8 επάγεται μετά από χειρισμό με α-κετογλουταρικό, έναν μεταβολίτη που έχει δειχθεί ότι επάγει ανοχή. Συνεπώς η PHF8 θα μπορούσε να είναι ένας επιγενετικός ρυθμιστής που διαμεσολαβεί αυτόν τον φαινότυπο. Συμπερασματικά στην παρούσα εργασία δείξαμε ότι η απομεθυλάση ιστονών PHF8 είναι βασικός ρυθμιστής της ενεργοποίησης των μακροφάγων όπως και του μεταβολισμού, ενώ πιθανόν συντελεί στην ανάπτυξη της ανοχής στην ενδοτοξίνη.

B. Introduction

B.1 Innate immune response/macrophages

Innate immune cells are considered to be the first line of defense against invading pathogens. The large group of innate immune cells consists of macrophages, dendritic cells, NK cells, neutrophils, basophils, eosinophils and mast cells[1]. Macrophages are distributed throughout all body tissues and organs. They are produced both by self-maintaining tissue resident populations and by differentiation of blood circulating monocytes derived from bone marrow[2]. Macrophage primary functions include cytokine and chemokine production, phagocytosis and antigen presentation. In response to pathogens or noxious stimuli they initiate inflammation and recruit additional immune cells, while they also remove cell debris and dead cells, thus contributing to tissue homeostasis[3]. Macrophages express pattern recognition receptors (PRRs), which comprise of surface and endosomal receptors (TLRs), cytosolic receptors (RLRs), NOD like receptors (NLRs) and C-type lectin receptors (CLRs)[4],[5]. PRRs recognize pathogen's PAMPs (pathogen associated molecular patterns), DAMPs (Damage associated molecular pattern) produced after danger signal or cytokines and activate downstream signaling cascades leading to activation of key inflammatory orchestrators such as NF-Kb, AP-1 and IRFs[6].

B.1.1 M1 and M2 macrophages

Macrophages are an extremely heterogeneous population. In response to different stimuli they exhibit great plasticity characterized by distinct phenotypes and functions[7]. Macrophage activation states are broadly categorized into M1 or classic polarization and M2 or alternative polarization[8]. Signals inducing M1 macrophage polarization include LPS, IFN-γ or LPS+IFN-γ while IL4, IL10, IL13, TGF-β, glycocorticoids induce M2 polarization[9],[10]. More specifically M2 macrophages are divided into subcategories defined by different marker production and function. M2a macrophages produce after IL4 and IL13 stimulation, M2b after immunoglobulin complexes combined with TLR agonists and M2c after IL10, TGF-β or glycocorticoids[11]. M1 macrophages possess proinflammatory phenotype and enhanced bacterial-killing capacity, while M2 macrophages display anti inflammatory properties, they upregulate pro-fibrotic and tissue repair genes and mainly contribute to the resolution of inflammation, wound healing and parasite infections[9],[10]. Perturbations in M1/M2 polarization contribute to the pathology of several diseases including inflammatory disorders, obesity, type two diabetes, atherosclerosis and cancer[3].

Macrophage polarization is a tightly regulated process. Coordination of various inflammatory molecules, signaling cascades and transcription factors underlies the distinct macrophage functional phenotypes[12]. M1 macrophages are characterized by increased production of proinflammatory cytokines and chemokines including TNF, IL1, IL6, IL12, type I interferons, CXCL1-3, CXCL5 and CXCL8-10[13]. They produce high levels of NO and possess elevated levels of reactive oxygen intermediates (ROI)[14]. On the other hand M2 macrophages have enhanced expression and activity of arginase 1 and display upregulation of Dectin-1, DC-SIGN, mannose receptor, scavenger receptor A, scavenger receptor B-1, CD163, CCR2, CXCR1, and CXCR2[15],[10]. Additional M2 markers include Ym1 and Fizz[16].

M2 macrophages mainly generate anti-inflammatory cytokines such as IL10. There is a crosstalk between macrophage polarization pathways[17].

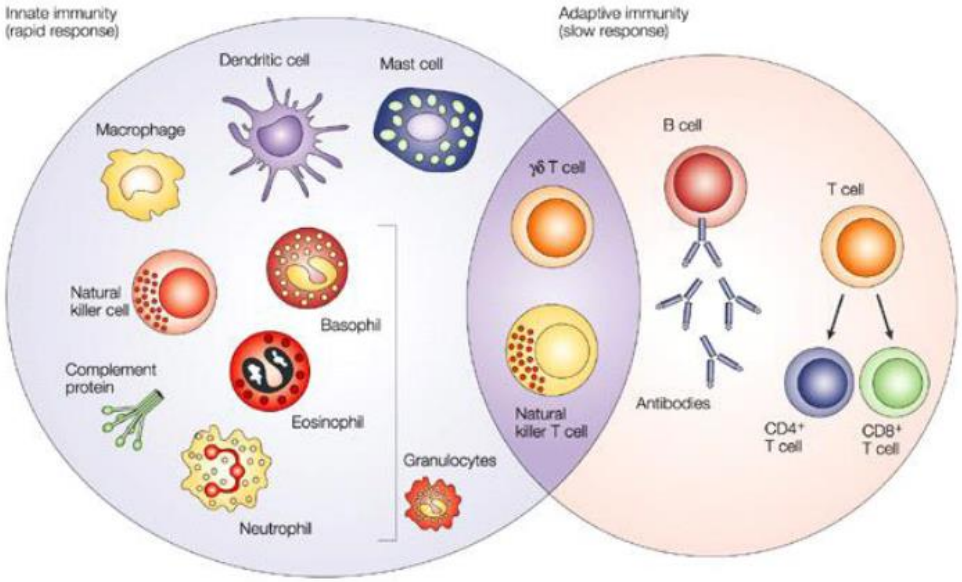

Figure 1: Immune system cells[18]

B.1.2 Transcriptional regulation of macrophage plasticity

The enhanced macrophage plasticity relies on the timely regulation of gene expression resulting from interaction of signaling molecules, transcription and epigenetic factors. Polarizing signals promote specific transcriptional programs determined by LDTFs (Lineage Determining Transcription Factors) such as PU.1, C/EBPs (CCAAT/enhancer binding proteins), AP-1 (activator protein 1), or RUNX1 (Runt-related transcription factor 1) as well as SDTFs (Signal Dependent Transcription Factors) including NF-κB, AP-1, Stat1 and Stat6. IRF/STAT is a key signaling pathway regulated differentially by M1 and M2 polarization signals[19]. Activation of IRF/Stat pathway via TLR ligants or IFNs coordinates M1 polarization via stat1. Two adaptor proteins: Myd88 and TRIF of TLR4 mediate the signaling cascade after LPS binding to TLR4. Activation of downstream kinases including IRAK4, TRAF6, IKKβ lead to NF-kB activation[20]. NF-κB is a transcription factor and a central mediator of the inflammatory program regulating genes such as TNF, IL6, IL1β, COX2 and IL12p40[21]. Proinflammatory signaling leads to I-κB proteasomal degradation after phosphorylation by IKK. This releases NF-κB p65/p50 heterodimer from the NF-κB/I-κB inhibitory complex and allows NF-κB heterodimer nuclear translocation and proinflammatory NF-κB dependent genes transcription[12],[22]. TRIF adaptor mediates IFNα and IFNβ production through IRF3 activation. IFNα and IFNβ binding to IFNR leads to Stat1 activation[23]. Stat transcription factors are regulated by members of the SOCS family (Suppressor of Cytokine Signaling), which are activated both by cytokine signaling pathways and directly by TLRs and modulate responsiveness to cytokines and TLR signaling, thus limiting inflammatory signaling[24]. Extensive TLR signaling also activates negative regulators such as IRAK-M, ST-2, SOCS1, a

short version of MyD88 (MyD88sh) and SHIPs as observed in cases of polymicrobial sepsis[25],[26],[27]. Correspondingly IRF/stat pathway engagement by M2 polarizing signals controls Stat3 activation by IL10 or Stat6 by IL4 and IL13 orchestrating M2 polarization associated with immune tolerance and tissue repairing[28]. Downstream of Stat6, activation of KLF4 and PPARγ promotes the M2 genes transcription[29],[30],. C-Myc and IRF4 transcription factors also participate in this process[31]. Activated Stat6 by IL4 induces direct repression of enhancers of proinflammatory genes by limiting binding of LDTFs (Lineage Determining Transcription Factors) such as PU.1, JUNB, C/EBPα and acetyltransferases such as p300, thus reducing chromatin accessibility and decreased LPS induced inflammasome activation, IL-1β production and pyroptosis genes expression[32]. IL10 binding to IL10R promotes M2 polarization by activating c-Maf, stat3 and by promoting NF-κB p50 homodimer[28, 33].

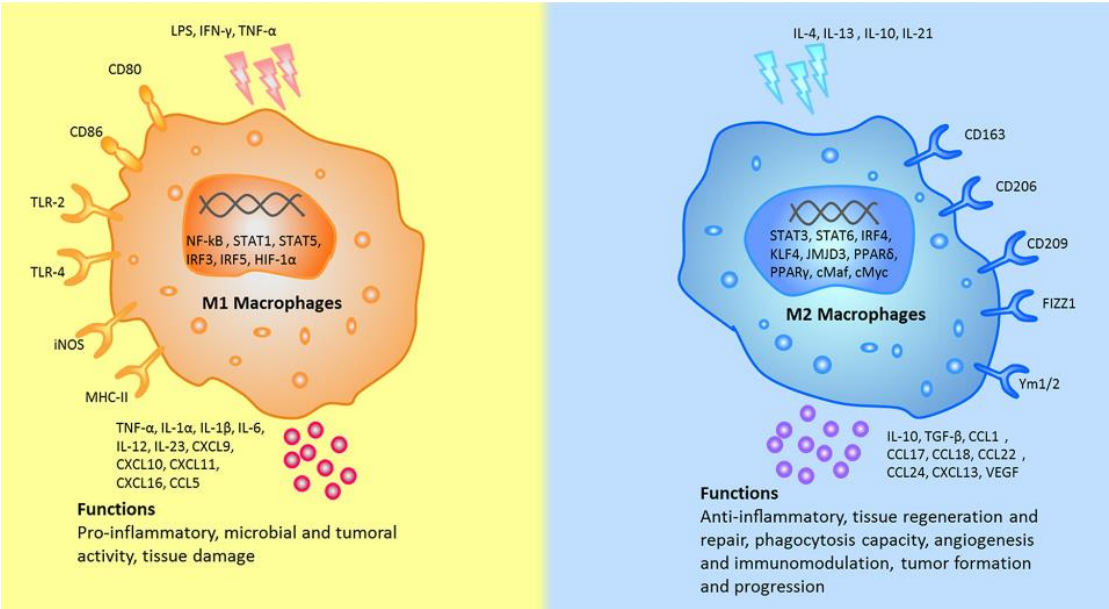

Figure 2: M1 and M2 macrophage polarization[34]

B.2 Epigenetics

B.2.1 Epigenetic regulation of macrophage activation

Epigenetics refers to the regulation of gene expression without changing the DNA sequence. Epigenetic modifications alter chromatin conformation, thus affecting DNA accessibility and transcriptional machinery assembly. Although traditionally considered as stable and hereditary epigenetic modifications are dynamically regulated by different signals providing a way to switch rapidly between transcriptional programs and subsequently determine macrophage activation phenotype[35]. Activation of inflammatory signaling cascades regulate epigenetic modifications at the promoters of inflammatory mediators, cytokines and chemokines, thus affecting transcriptional activation and immune function[36],[37]. Epigenetic regulation includes DNA modifications, histone post-translational modifications,

chromatin modifications and non coding RNAs[38]. Histone modifications include acetylation, methylation, phosphorylation, SUMOylation, ubiquitinilation of lysine, arginine, serine and other histone residues[39]. They occur at the gene promoters in proximity to the transcription start site and at the enhancers[40]. The dynamic nature of histone modifications and removal underlies the transcriptional changes that accompany inflammatory response as well as transition to inflammatory resolving phenotype. Broad epigenetic alterations characterize different macrophage phenotypes, including M1, M2 polarization as well as training and endotoxin tolerance[41].

Epigenetic regulation of macrophages is coordinated by tissue specific transcription factors as well as lineage specific transcription factors established during myeloid development. Lineage Determining Transcription Factors (LDTFs) can open chromatin conformation allowing the recruitment of further regulatory elements[42]. A hierarchical model for regulation of macrophage functions has been proposed, where a small number of LDTFs compete with nucleosomes for DNA binding in a cell specific manner. PU.1 is considered a pioneer factor and a master regulator of chromatin accessibility[43]. PU.1 binding further recruits other transcription factors including both other LDTFs, for example C/EBP family members and AP1 as well as SDTFs (Signal Dependent Transcription Factors) such as STATs, IRFs and NF-κB, thus priming DNA accessibility[44]. Moreover PU.1 recruitment marks cell-specific enhancers and contributes to basal DNA priming by controlling $H_3K_4me_1$ enhancer modification and $H_3K_4me_3$ at the promoters, setting a genome landscape prepared for appropriate response after stimulation[43]. Differentiated macrophages prior to stimulation have relatively “open” chromatin conformation on proinflammatory loci defined by master pioneer transcription factors such as PU.1 and C/EBP family members enhancer binding[43]. In the absence of activating signals enhancers are also occupied by repressive complexes and repressive histone marks such as $H_3K_{27}me_3$, H_3 K $_9me_2$, $H_4K_{20}me_3$, thus maintaining a poised state[45]. After TLR stimulation SDTFs activation including MAPKs, NF-κB and IRFs control eviction of corepressors and removal of inhibitory histone marks, therefore promoting proinflammatory genes expression[46]. Additionally activating histone marks such as $H_3K_4me_3$ and $H_3K_{27}Ac$ increase at the promoters of inflammatory genes[47].

B.2.2 Epigenetic modifications

B.2.2.1 Histone acetylation/Histone deacetylation

Histone acetylation status affects chromatin architecture and accessibility of transcriptional machinery. In general histone acetylation loosens chromatin structure and increases transcription while deacetylation associates with compact chromatin structure and diminished accessibility of the transcriptional machinery[48]. Inflammatory signals promote increase in histones acetylation levels[49]. Promoters of immediately induced genes upon proinflammatory stimulation bear high levels of H_3K_9Ac[50]. Also proinflammatory stimulation increases acetylation of several histone marks such as H_4K_5, H_4K_8, H_4K_{12} at the promoters of primary response genes[51]. Histone acetyltransferase p300 has been shown to increase H_3K_{27} and H_3K_{18} acetylation at the promoters of inflammatory genes[52]. Correspondingly deacetylation by HDACs is a mechanism of proinflammatory gene silencing at the phase of resolution of inflammation and endotoxin tolerance[53]. In the absence of TLR stimulation promoters of proinflammatory genes are occupied by HDACs, transcription

repressors such as BCL6 and demethylases inducing closed chromatin and gene silencing[54]. However depending on the HDAC enzyme and the promoters regulated, HDACs can have both positive and negative effect on TLR and interferon signaling. In general class 1 HDACs have a negative role on inflammatory genes regulation either by histone deacetylation or other mechanisms[55]. For example HDAC1 represses promoters of TLR-induced genes such as COX2, IFN-β and IL12p40 (interleukin 12 subunit p40)[55],[56]. TET2-recruites HDAC1 and HDAC2 to repress IL6 production during late macrophage activation[57]. HDACs also negatively control NF-kB transcriptional activity. HDAC3 indirectly inhibits NF-kB signaling by deacetylating p65 subunit and thus promoting interaction with the NF-kB inhibitory subunit IkB[56]. Also NF-kB repressor subunit p50 recruits HDAC1 at the promoters of proinflammatory genes including ccl2, cxcl10, GM-csf, mp13 in order to mediate their repression[53]. However HDACs also promote M1 polarization. HDAC3 is required for M1 polarization as evidenced by HDAC3 macrophage depletion models in which macrophages exhibit hyperresponsiveness to IL4[58]. Interestingly general HDAC inhibition has been shown to reduce proinflammatory response in terms of cytokine, chemokine and other inflammatory mediators, has protective role against sepsis[59, 60].

B.2.2.2 Histone methylation

Histone methylation can have both activating and repressive role on transcription determined by the residue methylated and the number of methyl groups added. Histone Lysine methyltransferases (KMTs) catalyze the transfer of one, two or three methyl groups from S' adenosyl methionine (SAM) to the ε amino group of the lysine residue. Apart from DOT1L (H_3K_{79} methylation) all KMTs identifies contain a SET (Su(var)3-9, enhancer-of-zeste, trithorax) catalytic domain, essential for their enzymatic activity[61]. There are nine families of lysine metyltransferases (EZ, SET1, SET2, SMYD, SUV39, SUV4-20, RIZ SET8/PR-SET7 and SET7/9) categorized based on the homology of their SET domain[61]. KMTs exhibit strict specificity for their enzymatic activity and they impact gene transcription by affecting recruitment of readers and other transcription regulators. Different histone marks associate with transcriptional activation of transcriptional repression. $H_3K_4me_3$ catalyzed primarily by the COMPASS complex (complex of proteins associated with Set1), which consists of six family members in humans, characterizes active chromatin state and its presences increases at the promoters of proinflammatory genes after classic activation polarizing signals. Silencing of different components of the COMPASS complex such as ASH2, WDR5, SET1 reduces proinflammatory mediators production, including IL1β, IL6, MCP1, TNF upon LPS stimulation[62],[63]. Mechanistically it has been shown that MRTF-A transcription factor is recruited by p65 subunit of the NF-κB and subsequently recruits COMPASS complex components such as ASH2 to promote trimethylation of H3K4 and this way facilitate NF-kB mediated transcription[64]. Ablation of another COMPASS complex component, MLL1 (or KMT2A) also results in decreased proinflammatory genes production after LPS, IFN-γ or LPS+IFN-γ. Surprisingly MLL1 deficient BMDMs exhibited enhanced phagocytosis and bacterial killing suggesting distinct regulation for inflammatory response and bactericidal activity[65]. Histone methyltransferases coordinate effective immune response both by positively regulating the transcription of inflammatory genes and by silencing negative signaling regulators. For example, KMT2B methyltransferase increases the activating histone mark $H_3K_4me_3$ at the promoter of PIGP gene in primary macrophages, which mediates the

anchoring of CD14 at the plasma membrane to promote TLR signaling[66]. EZH1, the catalytic subunit of PRC2 (Polycomb Repressor Complex) catalyzes the trimethylation of H_3K_{27} and subsequent transcriptional silencing of Tollip, a negative regulator of TLR4 signaling[67]. $H_3K_9me_2$ and $H_3K_9me_3$ written by EHMT1 and EHMT2 are repressive histone marks, found at the promoters and enhancers of proinflammatory mediators in the absence of activating signal[68]. Stimulus induced removal of repressive marks mediated by demethylases induces transcriptional activation. Also inhibition of the H_3K_9 demethylation from IFN-β promoters through a noncanonical NF-kB pathway, is a mechanism employed by viruses to reduce antiviral immunity[69]. Another repressive histone mark observed at the promoters of TLR4 responsive promoters in the absence of stimulation is $H_4K_{20}me_3$ written by SMYD5[70].

B.2.2.3 Histone demethylation

Histone demethylases remove methyl groups from lysine or arginine residues. Since the discovery of the fist KDM in 2004, it was believed that histone methylation is irreversible. Lysine demethylases (KDMs) are divided into two families. The FAD dependent amine oxidases and the Jumonji C containing catalytic domain, which use α-ketoglutarate, $Fe2^+$ and molecular oxygen as cofactors[71]. The FAD dependent family of KDM1 consists of KDM1A/LSD1 and KDM1B/LSD2. They catalyze the removal of mono and dimethylated H3K4 residues but cannot remove trimethylated H_3K_4 [72]. The jmjC domain family of KDMs, KDM2-7 can remove all three methyl groups[71]. A great number of studies have described the involvement of different KDMs in inflammation and immune response. KDM6B orJMJD3 was found to be strongly induced after 2 hours of LPS and LPS +IFNγ. JMJD3 mediates the demethylation of H_3K_{27}me2/3 at the promoters of inflammatory mediators and immune modulatory genes thus inducing their transcription[73]. JMJD3 is directly targeted to the promoters by the p65 subunit of NF-kB, the master regulator of inflammatory response[74]. JMJD3 also regulates M2 genes. After IL-4 stimulation JMJD3 demethylates the repressive mark H_3K_{27}me2/3 of M2 program genes in a Stat6 dependent manner[75]. Interestingly enough jmjd3$^{-/-}$ macrophages display normal M1, but impaired M2 polarization[76]. KDM6B promotes IL6 expression by removing the repressive $H_3K_{27}me_3$ from IL6 promoter, while it also promotes IFN-β expression by an indirect mechanism. It interacts with the writer MLL4, which catalyzes $H_3K_4me_2$ at the enhancer of IFN-B. PHF2 is another lysine demethylase found upregulated at the early stage of LPS stimulation. PHF2, recruited by p65 subunit of NF-kB catalyzes the removal of the repressive histone modification $H_4K_{20}me_3$ from the promoters of TLR4 induced genes such as Cxcl10 and TNF[70]. Also a non-classical mechanism independent from its demethylase activity has been described for KDM2B. In activated macrophages KDM2B interacts with Brg1 (Brahma related gene 1), which is the core ATPase subunit of the SWI/SNF chromatin remodeling complex at the promoter of IL6 gene, thus promoting its transcription[77]. H_3K_9 demethylases control gene activation by removing the repressive marks $H_3K_9me_3$ and $H_3K_9me_2$ from promoters and enhancers of PRR induced genes and associate with NF-kB dependent inflammatory genes transcription[68],[78]. Upon PRR stimulation LSD2/KDM1b interacts with c-Rel subunit of NF-kB, which preassembles at low levels at the promoters of its target genes, thus promoting $H_3K_9me_2$ demethylation and gene transcription[78].

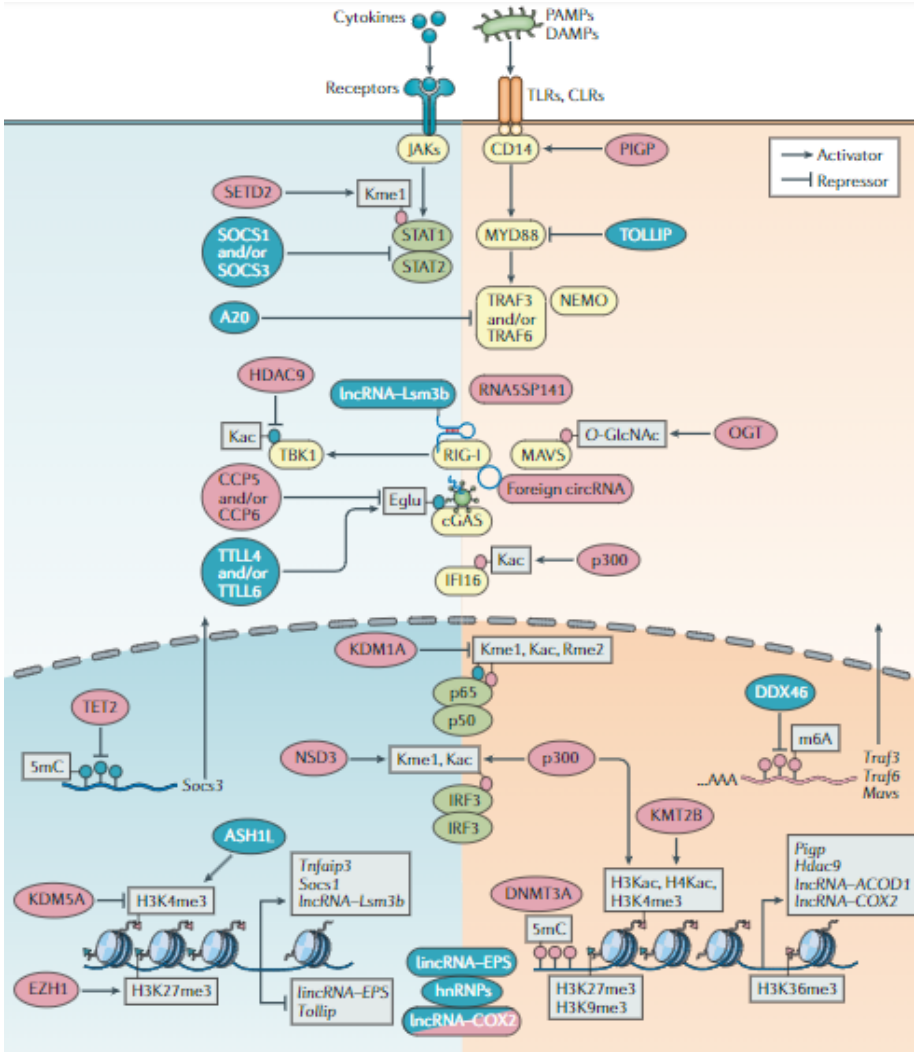

Figure 3: Epigenetic regulation of macrophage activation[37]

B.2.3 Histone demethylase PHF8

PHF8 or KDM7B is a histone lysine demethylase which belongs to the family of 2-oxoglutarate and ferrous ion dependent hydrolases. PHF8 (Plant Homeodomain Finger protein 8) contains two functional domains: an aminoterminal PHD finger which recognizes methylated lysine residues and jumonji C carboxyterminal domain which catalyzes histone demethylation[79]. It has a higher selectivity for mono and dimethylated residues and has been shown to have catalytic activity towards histone $H_3K_9me2/me1$, $H_4K_{20}me_1$, and/or $H_3K_{27}me_2$ residues[80],[81],[82],[83]. Loss of function mutations in PHF8 cause Siderius X-linked intellectual disability characterized by developmental delay, intellectual disability, craniofacial dismorphisms and cleft lip and/or cleft palate depending on the loss-of-function variant[84]. PHF8 has been extensively studied in the context of cancer. It has been described as an oncogene and its role in tumour development and metastasis has been highlighted in several types of cancer[85],[86],[87]. PHF8 is also involved in neuronal differentiation, cell cycle progression, rRNA synthesis, cell adhesion, cytoskeletal organization and somatic cell reprogramming[83],[88],[81],[89],[90]. PHF8 depletion has

been found to cause cognitive and memory impairments by upregulation of mTOR signaling in the hippocampus of PHF8$^{-/-}$ mice[91]. There is very little literature regarding the role of PHF8 in immune response. A study suggests PHF8 participates in T cell activation and proliferation in LPS-induced acute inflammation[92]. Also (Asensio-Juan,E., et al.) show that PHF8 interacts with HDAC1 and SIN1A at the promoters of IFNγ induced genes in the absence of IFNγ stimulus and represses their transcription by demethylating $H_4K_{20}me_1$ [93] Evidence from our lab has shown that, among other epigenetic and transcriptional regulators, PHF8 silencing in macrophages results in enhancement of iNOS expression and a pro-inflammatory phenotype (figure 4).

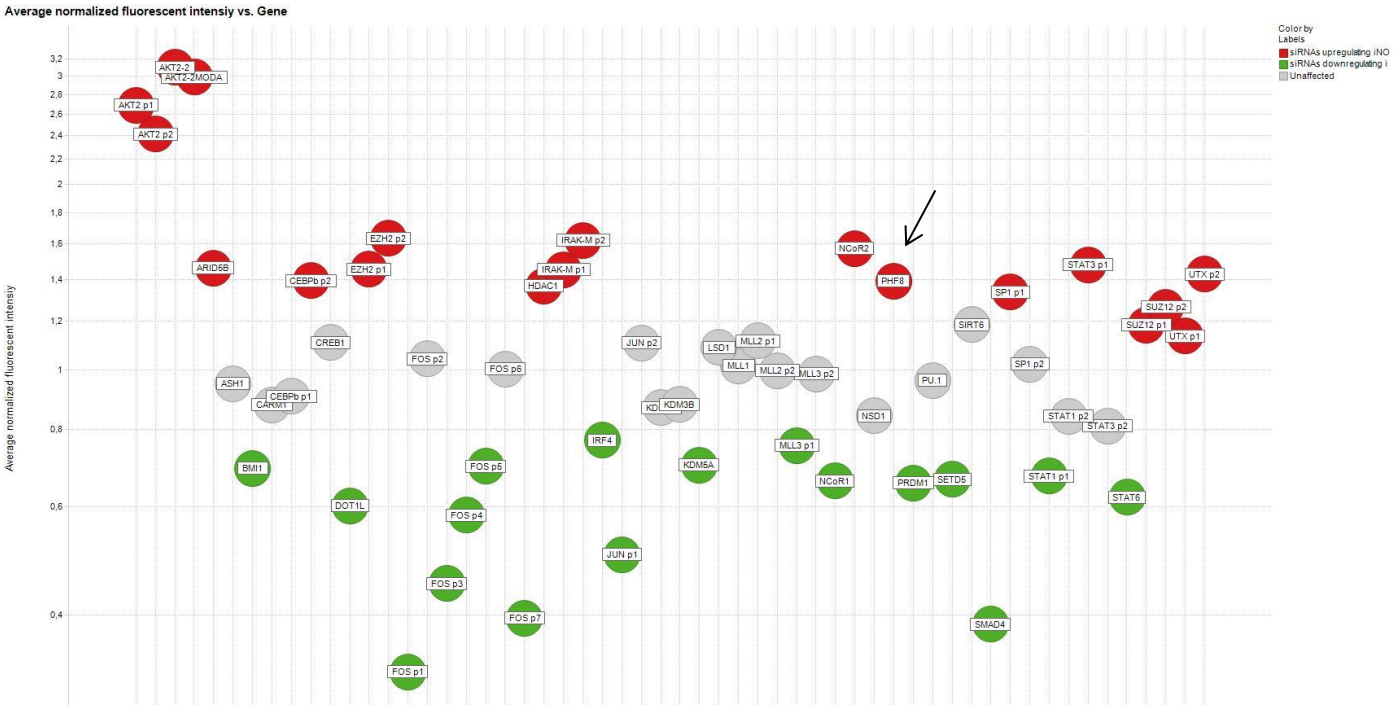

Figure 4: Knock down of PHF8 increases iNOS production measured as average fluorescence intensity. PHF8 is indicated in the arrow

B.3 Immunometabolism

B.3.1 Metabolic pathways in macrophages

During the last decades there is a growing body of evidence regarding the role of metabolism in immune response, as it is an energy demanding process, generating the field of immunometabolism. Cellular metabolism refers to the way that metabolic molecules are channeled into different pathways for energy generation and metabolic substrates production, a process that is transcriptionally and epigenetically regulated. Some key pathways include glycolysis, oxidative phosphorylation, amino acid metabolism, pentose phosphate pathway, fatty acid oxidation and fatty acid synthesis. Macrophages respond to environmental cues and acquire an array of phenotypes. During this process they also undergo metabolic rewiring and acquire distinct metabolic profiles which characterize

different activation states. Activated, M1 macrophages metabolically switch to glycolysis and pentose phosphate pathway, they upregulate fatty acid synthesis and display a broken TCA cycle. On the other hand M2 anti-inflammatory macrophages rely on OXPHOS and fatty acid oxidation for their metabolic needs while they have an intact TCA cycle. Also amino acid metabolism is differentially regulated in M1 and M2 macrophages[94],[95],[96].

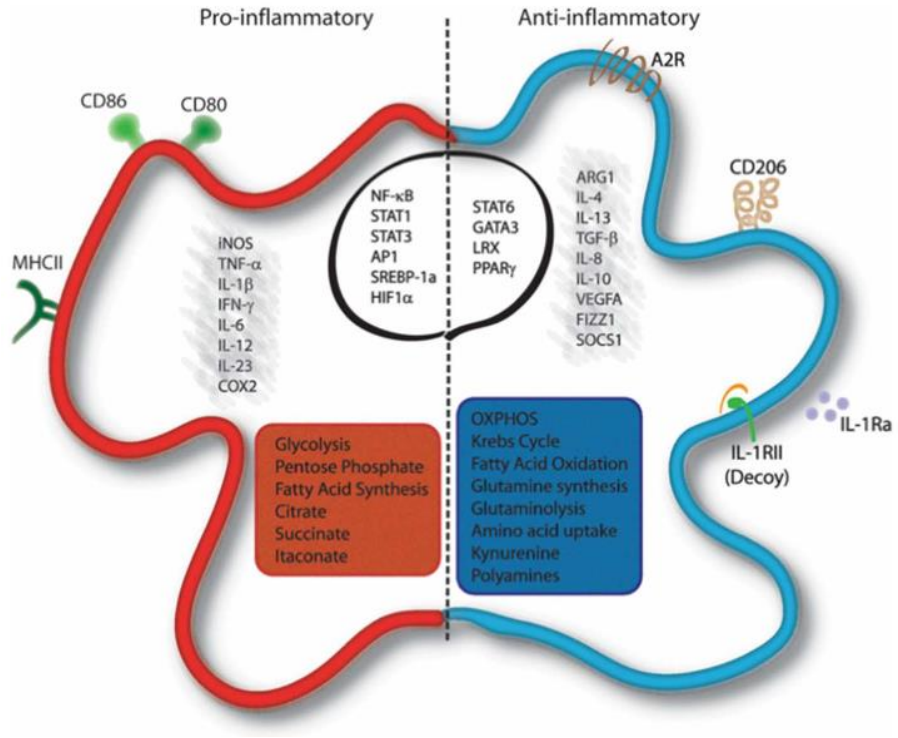

Figure 5: immunometabolic pathways at different macrophage activation states[94]

B.3.1.1 Glycolysis

Glycolysis is a common pathway often used by rapidly proliferating cells to oxidize glucose. Although it is an inefficient way of energy production, yielding only 2 ATP molecules per glucose molecule compared to OXPHOS which produces 36 ATP molecules per glucose unit, glycolysis is a rapid way of ATP production as it takes place at the cytoplasm and depends on the induction of glycolytic enzymes while OXPHOS requires mitochondrial mass production. More importantly glycolysis produces key metabolic intermediates which support biosynthetic processes. Specifically glycolysis provides substrates used in PPP for nucleotide biosynthesis, pyruvate acid which is converted into acetyl coA and fuels the TCA cycle, intermediates for amino acid and fatty acid biosynthesis and glycosylation reactions. LPS-activated macrophages as well as DCs, NK cells, effector T cells, activated B cells turn to glycolysis which allows the rapid ATP production and provides the building blocks, needed to support proinflammatory immune function[97]. Increase in glycolysis also increases flux through pentose phosphate pathway which provides NADPH, used by NADPH oxidase for respiratory burst and microbial killing[98],[99]. The switch to glycolysis is controlled by Hif1a which causes the transcription of all glycolytic genes as well as glucose transporters[100]. Inhibition of glycolysis dampens inflammatory response as well as training of

monocytes[101],[101],[102],[102]. Metabolic turn to glycolysis in actively proliferating cells has been described in tumour cells since 1927 and was termed Warburg effect[103]. However glycolysis is also important for anti-inflammatory macrophages. Glycolysis inhibition impairs M2 polarization upon IL4 stimulation, though it is suggested to be a rather indirect effect as glycolysis fuels the TCA cycle and reserves OXPHOS intact[104],[105].

B.3.1.2 TCA cycle and fatty acid metabolism

The TCA cycle in M1 macrophages appears fragmented. They display lower expression of isocitrate dehydrogenase (IDH) and succinate dehydrogenase leading to the accumulation of citrate and succinate[106]. Citrate is harnessed for itaconate production, an antimicrobial metabolite while succinate stabilizes Hif1a leading to the expression of proinflammatory genes[107],[108]. M1 and M2 macrophages also exhibit differences in fatty acid metabolic pathways. Fatty acid oxidation is upregulated in M2 macrophages and as well as observed in non-inflammatory and long-lived immune cells such as T_{reg} and memory T cells[109]. Proinflammatory macrophages switch to fatty acid synthesis supported transcriptionally by sterol regulatory element binding proteins (SREBPs). Fatty acid synthesis leads to inflammatory intermediates production such as prostanglandins and mediate inflammasome activation[110]. M1 macrophages are in high demand of fatty acids and phospholipids since they undergo expansion in their ER network and Golgi to support increased cytokine secretion and phagocytosis[111]. mTORC1 supports lipogenic activity by activating srebp1 and srebp2, master regulators of lipid synthesis[112]. Therefore, defective lipid biosynthesis leads to lower TNF and IL6 levels and impaired phagocytosis[113]. On the contrary M2 macrophages are characterized by high levels of OXPHOS and display enhanced fatty acid uptake[114]. It has been shown that fatty acid oxidation chemical inhibition impairs IL4 induced M2 polarization, while expression of a constantly active carnitine palmitoyl-transferase 1, the enzyme that targets long chain fatty acids in the mitochondria for oxidation, results in inhibition of palmitic acid induced M1 polarization[115]. A proposing mechanism by which fatty acid oxidation regulates M2 polarization is by activation of PPAR-γ and the proliferator-activated receptor-coactivator 1β, which control the expression of anti-inflammatory program genes[116]. Yet the exact mechanism is not fully understood.

B.3.1.3 Amino Acid metabolism

Amino acid metabolism also plays an important role and modulate immunity via multiple mechanisms. Amino acids support anabolic processes, they provide intermediates for post translational (PTMs) and epigenetic modifications and they also contribute to redox balance[117]. Differential metabolic use of amino acids supports distinct immune functions and characterizes different activation states. Depending on the metabolic pathway engaged, arginine can lead to either M1 or M2 phenotype. Proinflammatory signals cause upregulation of iNOS (NOS2). iNOS converts arginine to NO, which reacts with oxygen and ROS and produced several antimicrobial species. Anti-inflammatory macrophages induce arginase 1 activity which catabolizes arginine to ornithine, polyamines and urea, hallmarks of M2 phenotype[118]. In M1 macrophages glutaminolysis of glutamine causes anaplerosis of α-KG into the TCA cycle and supports succinate production needed for M1 polarization[119]. Glutamine is necessary for M2 polarization[120]. It fuels the hexosamine pathway, which provides with UDP-GlcNAc substrate used for protein and lipid glycosylation a phenotype observed in M2 polarized macrophages[121]. Glutamine deprivation leads to reduction in

M2 markers production after IL4 stimulation, an effect mediated by glutamine conversion to α-KG[120]. Also branched chain amino acids (BCAAs) are metabolized into CoA derivatives : acetyl CoA and succinyl-CoA and feed the TCA cycle[122]. Acetyl CoA is used for histone acetylation and epigenetically modulate gene transcription[123]. Acetylation of non-histone proteins also regulates their activation. NF-kB and NLRP3 are activated after acetylation as well as most glycolytic and TCA cycle enzymes[124],[125]. Accordingly in the presence of LPS stimulus BCAA transporters are upregulated and stimulate glucose uptake and immune response[126]. Serine also promotes glycolytic switch by activating PKM2, the enzyme catalyzing the conversion of PEP to pyruvate and supports the generation of 1 carbon units used in nucleotide synthesis and methionine recycling by feeding the folate and methionine cycle[127]. S-containing amino acids: methionine and cysteine feed the methionine cycle which lead to SAM production, the methyl groups donor for DNA, RNA and protein methylation, thus affecting gene expression and protein function[128]. Glutathione, a small molecule assembled from glycine, glutamate and cysteine serves for ROS detoxification and redox balance[129].

B.3.2 Metabolites in immune response

Pathogens and environmental cues cause metabolic rewiring in innate immune cells. These metabolic changes functionally support the distinct macrophage activation states[130]. Changes in cellular metabolic features impact on the transcriptional and epigenetic regulation, which in turn affects metabolic pathways and shapes immune response. Metabolites exert immunomodulatory roles[131]. They are used as cofactors for epigenetic enzymes and also as substrates for epigenetic modifications including methylation and acetylation reactions. They also serve as enzymatic cofactors and modulate the activity of transcription factors[123]. Histone acetylation increases chromatin accessibility and permits transcription[39]. During the induction of the immune response increased histone acetylation is related to the induction of proinflammatory genes[132]. Therefore availability of acetyl-CoA, the acetyl group donor for histone acetylation, impacts proinflammatory gene transcription while reduced levels of acetyl-CoA cause diminished cytokine production and endotoxin tolerance[133]. Apart from histones, post translational acetylation of other proteins also regulates their function[134]. NF-kB, the master regulator of proinflammatory genes transcription genes gets activated after acetylation at lysine 30 of RelA/p65 subunit[124]. Removal of the acetyl group by Sirt1 causes inactivation of NF-Kb[135]. S-Adenosyl methionine (SAM) is the methyl group donor which is used in DNA or histone methylation reactions and also in phospholipid synthesis[136]. In C. elegans SAM deprivation can attenuate immune response during *Pseudomonas aeruginosa* infection as a consequence of lower $H_3K_4me_3$ levels[137]. Also reduction in SAM levels boosts antiviral IFN-β immunity because of reduced H3K27me3 modification on antiviral gene promoters[138]. Buildup of succinate after LPS stimulation stabilizes Hif1a by inhibiting regulatory Hif hydrolases and leads to the induction of proinflammatory genes including IL-1β[139]. It also causes succinylation of several enzymes including pyruvate kinase 2(PKM2), pyruvate dehydrogenase, succinyl dehydrogenase(SDH), acyl coA synthase1, thus affecting their function[139]. Citrate is also used as a substrate for itaconate production, a metabolite with bactericidal properties and anti-inflammatory role[140]. Dimethyl itaconate pretreatment causes reduced NO, ROS, IL1β, IL18 and IL6 production after LPS stimulation while the

opposite is observed in IRG1$^{-/-}$ macrophages[141]. α-KG is another metabolite produced from the TCA cycle by isocitrate dehydrogenase[142]. It serves as cofactor for epigenetic enzymes and its role in epigenetic reprograming is well established. a-KG is a cofactor for JMJD and TET families of demethylases[74],[143]. Glutaminolysis derived α-KG has been shown to promote M2 polarization of macrophages through an JMJD3 dependent mechanism and reinforces M2 polarization of macrophages, an effect that is reinforced by high α-KG to succinate ratio while the opposite drives M1 polarization[120]. Mechanistically α-KG driven activation of JMJD3 causes demethylation of the repressive $H_3K_{27}me_3$ in the promoters of IL4 induced M2 genes including Arginase1, Ym1, Retnla, Mrc1[120]. It displays chemical antagonism with succinate, fumarate and 2-hydroxyglutarate and α-KG administration has been reported to counteract fumarate induced training[144],[145]. Additionally adipose derived α-KG exosomes transfer to macrophages and increase the M2 to M1 ratio by promoting TET mediated DNA demethylation and attenuating activation of Stat3/NF-kB[146]. Accumulated citrate produced by the break in TCA cycle upon M1 activation is exported to the cytoplasm and converted to acetyl CoA and oxaloacetate[147],[148]. Malonyl CoA produced by acetyl CoA restrains fatty acid oxidation by inhibiting CPT1a and is used in fatty acid and cholesterol synthesis[149]. Another metabolite with important role in immune regulation is NAD^+.NAD^+ is used as enzymatic and epigenetic cofactor including the sirtuin family of deacetylases. Many pathways reduce NAD^+ to NADH including glycolysis and TCA cycle while NADH fuels the electron transfer chain (ETC) in oxphos, finally favouring NAD^+ accumulation. Thus, the ratio of NAD^+ to NADH reflects cellular energy and metabolic state[150]. LPS stimulation and switch to glycolysis leads to NAD^+ depletion[151]. Increase of NAD^+ activates sirtuin deacetylases, which have been shown to promote anti-inflammatory responses. For instance Sirt1 controls oxidative metabolism and anti-inflammatory functions. It directly deacetylates and inactivates NF-kB, AP1 and Hif1a while it also removes activating histone acetylation modifications from proinflammatory genes[135],[152].

B.4 Trained immunity and endotoxin tolerance

Although immune memory has been traditionally considered as an adaptive immune system characteristic, recent findings have challenged this notion[153]. Memory-like features are present in innate immune cells too, with trained immunity and endotoxin tolerance being examples of this phenomenon. Trained cells are characterized by increased responsiveness to the secondary stimulation. Stimuli capable of inducing training include β-glucan, BCG and ox-LDL[154],[155]. On the other hand endotoxin tolerance describes the reduced responsiveness to LPS (endotoxin) following a pretreatment with LPS[156]. Metabolic changes as well as epigenetic reprograming has been shown to underlie trained and endotoxin tolerant phenotypes[154]. Training with β-glucan increases the activating histone mark H3K4me3 at the promoters of proinflammatory cytokine genes such as TNF, IL6, IL18, providing a form of epigenetic memory that facilitates transcription factors binding and regulates response to secondary stimulation[157]. Also important gain occurs in $H_3K_{27}Ac$ both at the promoters and enhancers of proinflammatory genes as well as $H_3K_4me_1$, a definitive mark of active and primed enhancers[158],[132]. On the other hand, promoters of proinflammatory genes in endotoxin tolerant cells lack the activating $H_3K_4me_3$ histone mark[41]. They are also characterized by high levels of the repressive histone mark

$H_3K_9me2/3$. Methyltransferase G9a induces $H_3K_9me_2$ at the promoter of TNF and IL1β, which leads to the recruitment of the reader HP1 and increase in DNA methylation by the DNA methyltransferase Dnmt3a/b, thus establishing closed chromatin state in inflammation related genes[159],[160].

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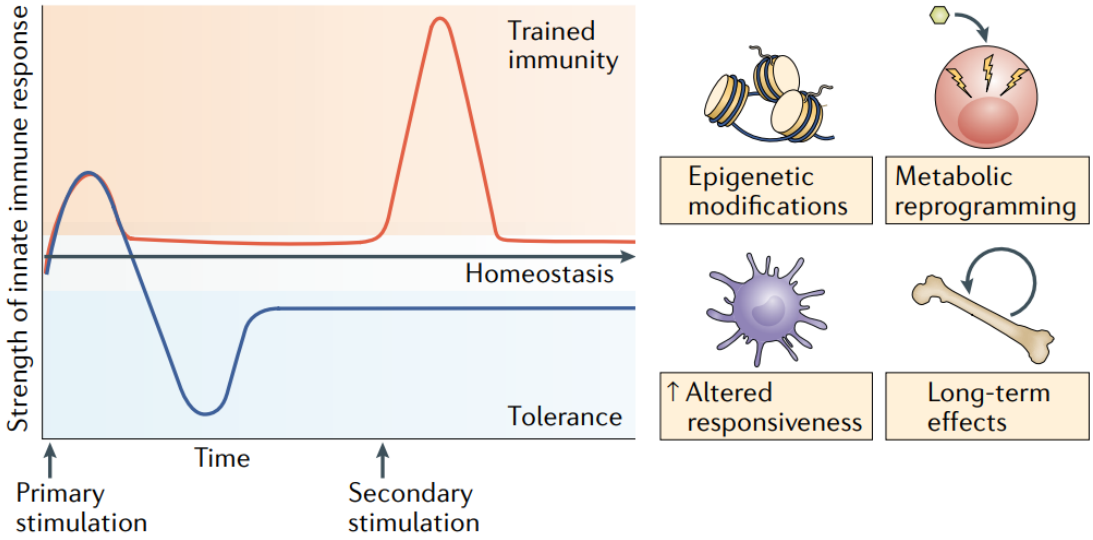


Figure 6: Trained immunity and endotoxin tolerance states[154]

B.4.1 Metabolic and epigenetic regulation of trained immunity

Persistent metabolic changes underscore trained immunity. Glucose, glutamine and cholesterol metabolism pathways have central role in the induction of innate immune memory[145]. Metabolic switch form oxidative phosphorylation to glycolysis in a mTOR/Hif1a dependent way has been shown to be crucial for induction of trained immunity[161]. The TCA cycle metabolite: Fumarate accumulated by glutaminolysis has been shown to mediate β-glucan induced trained immunity by inhibiting KDM5, a histone lysine demethylase responsible for $H_3K_4me_3$ demethylation from proinflammatory genes promoters. Interestingly enough fumarate treatment alone can partially recapitulate the trained immune phenotype[145]. Cholesterol synthesis pathway is another important mechanism for induction of trained immunity. The cholesterol synthesis pathway intermediate mevalonate promotes innate immune memory in an IFG1-R and mTOR dependent manner, an effect also reflected at the epigenetic level by increased $H_3K_4me_3$ at the promoters of IL6 and TNF[162]. Moreover the histone lysine methyltransferase (KMT) SET7 has been identified as epigenetic regulator of β-glucan induced trained immunity. Mechanistically SET7 writes the histone modification $H_3K_4me_1$ at the enhancer of the TCA cycle metabolic genes malate dehydrogenase 2 (MDH2) and succinate dehydrogenase (SDH), thus controlling epigenetic priming and persistent transcriptional activation of these key metabolic genes. Samuel T. Keating et al. at this paper also suggest OXPHOS dependency for

lower dose β-glucan induced training[163]. In summary metabolic changes controlled by epigenetic reprogramming, which is induced by metabolic alterations[163]

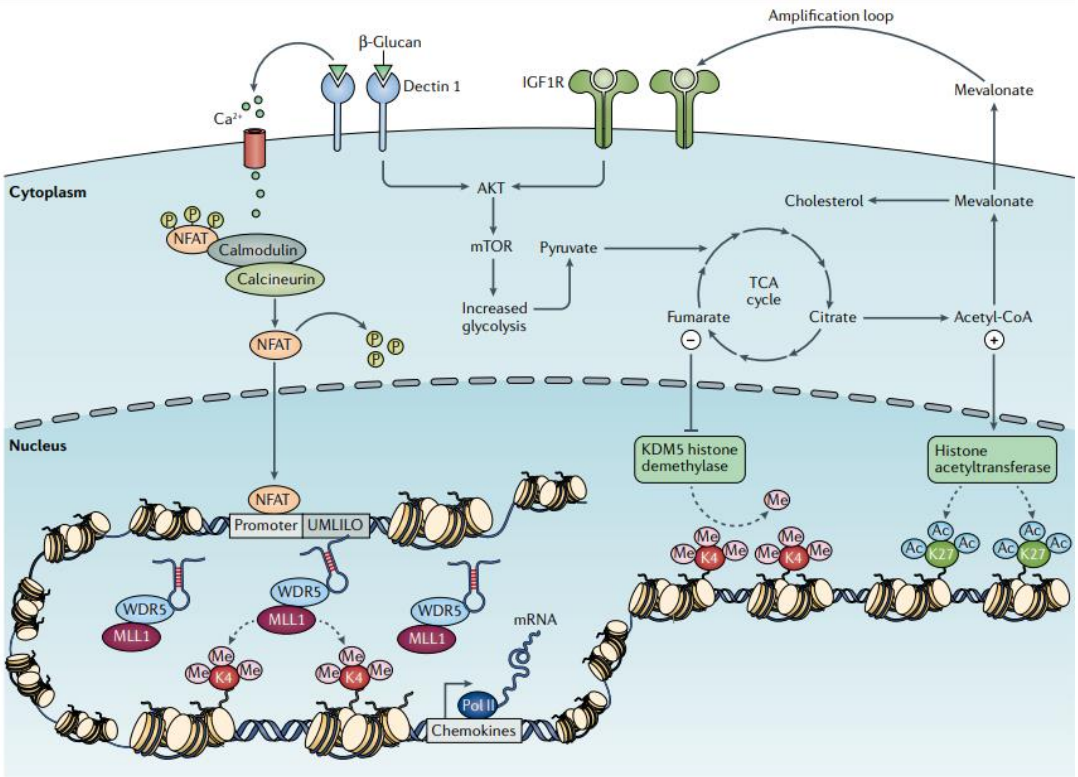


Figure 7: Interplay between epigenetics and metabolism in trained immunity[154]

B.5 Akt/mTOR pathway

Emerging studies indicate an extensive crosstalk between immunity and metabolism. mTOR and Akt are key regulators of this interplay[164]. mTOR is a serine threonine kinase present is two compexes in mammals: mTORC1and mTORC2 defined by the subunits raptor and rictor respectively. mTORC1 is a master metabolic regulator which incorporates signals regarding nutrient availability, oxygen, energy, growth factors and couples them to anabolic processes such as protein, lipid and nucleotide synthesis[112]. Polarizing signals cause metabolic switches which underlie macrophage functional capacity to respond appropriately to diverse stimuli[165]. These signals target mTORC1, which in turn controls macrophage metabolic features and activation. The PI3K/AKT/mTOR pathway integrates signals from various receptors including insulin receptors, growth factor receptors, PRRs, cytokine receptors, adipokine and hormone receptors. Signal binding to PRRs, for example TLR4, cytokine, chemokine and Fc receptors activate the PI3K/Akt pathway[164]. Activated PI3K type 1 converts PIP2 to PIP3 and leads to the recruitment of Akt and mTORC2 on the plasma membrane.mTORC2 activates Akt by phosphorylation, which in turn inactivates the tuberous sclerosis complex(TSC1/2) resulting in mTORC1 activation. mTORC1 by feedback inhibition inactivates mTORC2 and Akt[164]. BMDMs deficient in TSC, the negative regulator of mTORC1 display elevated basal levels of mTORC1 and mount hyperresponsive phenotype after LPS stimulation as well as stimulation with TLR2, TLR3 ligands and LPS with IFN-γ, while

induction of M2 markers is impaired[19]. Mechanistically attenuated Akt due to feedback inhibition by mTORC1 impairs M2 polarization[19]. mTORC1 targets include the translation initiation factor 4E binding protein (4EBP1), the ribosomal S6 Kinase, which in turn phosphorylates and activates the ribosomal protein s6 thus promoting protein synthesis[166]. The Akt family of protein kinases consists of 3 isoforms in mammals Akt1, Akt2 and Akt3[167]. Genetic models of specific isoform depletion reveals different roles in macrophage activation. Akt1 deletion leads to more pronounced M1 activation defined by increased IL-1β production, higher NO synthase activity and improved bacterial clearance[168]. Mechanistically in the absence of Akt1, Mir155 a master regulator of inflammatory phenotype is upregulated while miRNA let7e, which represses TLR4 is downregulated. Also C/EBPβ, a key transcription factor for M2 polarization is downregulated. On the contrary, Akt2 depleted macrophages exhibit enhanced M2 polarization and higher levels of the anti-inflammatory cytokine IL10 upon LPS stimulation. They are characterized by elevated levels of the M2 markers Arg1, Ym1 and Fizz1[168],[15]. Activated macrophages also undergo alterations in their metabolism. Akt and mTORC1 play a key role in metabolic reprogramming that supports immune function. Akt controls rapid glycolytic switch upon M1 activation at the posttranslational level by multiple mechanisms.mTORC1regulates HIF1a, the master regulator of glycolysis, which subsequently sustains long term glycolytic flux via transcriptional induction of glycolytic enzymes[139]. Also upregulation of glycolysis controlled by the mTOR/HIF1a pathway is a metabolic requirement for trained immunity[161]. Chemical inhibition of mTOR or genetic ablation HIF1a abrogates the enhanced cytokine production induced by β-glucan training[161]. Moreover mTORC1 promotes protein and lipid biosynthesis, needed for anabolic support of macrophage activation. It activates s6k and 4ebp1, thus enhancing translation[166]. It also activates srebp1 and srebp2 which orchestrate lipogenesis. Increased lipogenic activity during M1 polarization supports ER and Golgi expansion, needed for enhanced cytokine secretion and phagocytosis[111]. Additionally lipid biosynthetic pathways support the production of proinflammatory and anti-inflammatory mediators such as prostanglandins and lipoxins respectively[110].

B.6 E.R. stress

Endoplasmatic Reticulum (E.R.) is a large membranous organelle used for synthesis, folding, posttranslational modifications (for example N-glycosylation) and transport of proteins and lipids[169]. When the protein folding capacity of E.R. is exceeded misfolded and unfolded proteins accumulate resulting in E.R. stress. This activates the Unfolded Protein Response (UPR), responsible to alleviate E.R. stress and to restore proteostasis. Failure of UPR leads to apoptosis[170]. In order to relieve the cell from the accumulated misfolded load, the UPR response regulates the general attenuation of protein synthesis and upregulation of molecular chaperones in order to improve folding capacity. It also induces E.R. associated protein degradation (ERAD) to catabolize misfolded proteins through proteasomes and autophagy to support protein and cellular organelle degradation[171],[172]. The central regulators of the three distinct signaling UPR axes of include IRE1α (inositol requiring enzyme 1 α), PERK (protein kinase RNA like ER kinase) and ATF6 (activating transcription factor 6)[170]. They are E.R. transmembrane proteins bound with the E.R. immunoglobulin

heavy chain chaperone GRP78 (BiP or Hspa5) in the absence of E.R. stress. Accumulated misfolded proteins have higher affinity for BiP, thus promoting BiP dissociation and activation of UPR response regulators[173]. E.R. stress has been shown to be both the cause and the consequence of inflammation. Disturbed proteostatic network and E.R. stress lead to chronic inflammation, while inflammatory disorders and autoimmunities exhibit increased E.R. stress[174]. Kevin Shenderov et al. show that E.R. stress mediates the production of IL-1β in a caspase-8 and TRIF dependent pathway upon TLR4 stimulation[175]. The UPR effectors can directly activate the NF-kB and JNK-AP1 pathway by different mechanisms while E.R. stress also induces cytokine and chemokine production such as IL1, IL18, TNF, IL6 and MCP1[176],[177]. Additionally independent of UPR mediators E.R. stress promote inflammation by NLRP3 inflammasome activation in a ROS dependent manner while increase in cytoplasmic calcium ion levels and ROS leaked from E.R. also induce inflammatory response[178],[179]. Moreover extensive E.R. stress causes imbalance in M1-M2 polarization[180]. E.R. stress also acts in a non-cell autonomous manner as it promotes multivesicular bodies formation and exosome release, thus transmitting E.R. stress to the tissue microenvironment[181]. Tumor microenvironment (TME) is characterized by high levels of E.R. stress. Persistent E.R. stress transmitted to TME myeloid populations promotes tolerogenic immune activity[182]. Infiltrating macrophages are skewed towards M2 polarization, while infiltration of MDSCs (Myeloid Derived Suppressor Cells) increases, thus dampening antitumor immunity and promoting tumor progression and metastasis[182]. E.R. stress also characterizes several chronic inflammation and metabolic diseases such as obesity, type two diabetes, atherosclerosis, NAFLD and neurodegenerative disorders[183]. Although it is not clear whether E.R. stress is the cause or the consequence of these diseases, several studies connect E.R. stress with the inflammatory pathology, known to exacerbate chronic diseases[180]. In obesity metabolic E.R. stress in white adipose tissue drives ATMs (Adipose Tissue Macrophages) M1 polarization in an IRE1α dependent way, which contributes to chronic inflammation of adipose tissue and decreases energy expenditure of WAT. Deletion of myeloid lineage IRE1α rescues M1-M2 imbalance, insulin resistance and obesity induced by HFD[184]. Correspondingly atherosclerosis progression relies on macrophages. E.R. stress has been shown to be necessary and sufficient to induce M2 macrophage polarization, which contributes to increased foam cell formation after oxLDL exposure, characterized by enhanced cholesterol uptake, thus contributing to plaque progression[185]. Moreover E.R. stress induced chronic inflammation mediates fibrotic remodeling in IBD, while induction of E.R. stress in intestinal goblet cells causes development of spontaneous inflammation in colon[186].

C. Purpose of the study

Preliminary data of the lab have shown that histone demethylase PHF8 has a pivotal role in macrophage activation and potentially in the regulation of macrophage metabolism. As the induction of the immune response is closely correlated to metabolism the aim of this work was to find the mechanism in which PHF8 may be implicated in cell metabolism. To do so, we investigated the role of PHF8 in metabolism influenced pathways like mTORC signaling, ER-stress and endotoxin tolerance during LPS induced inflammation

D. Materials and methods

D.1 Mice

C57BL/6 w.t. and Akt2$^{-/-}$ mice were kept in a pathogen free animal facility in University of Crete, School of Medicine in a temperature-controlled room and 12h light/dark cycle, with free access to standard laboratory chow and water. Adult mice of 8-10 weeks old were used in all experiments. In the Akt2$^{-/-}$ compared to C56BL/6 experiment only female mice were used, while in the rest of the experiments male C57BL/6 mice were used. All animal procedures were in accordance with institutional guidelines and were approved by the University of Crete's Animal Care and Use Committee and the Veterinary Department of the Heraklion Prefecture (license number 150760/20-07-2017).

D.2 Aseptic peritonitis

Female C57BL/6 and Akt2$^{-/-}$ mice were injected intraperitoneally with 1.5 ml thioglycolate/animal using a 27 gauge syringe in order to induce aseptic peritonitis and macrophage recruitment in the peritoneal cavity. After 4 days the animals were euthanized.

D.3 Peritoneal macrophages and BMDMs collection

Femoral bones from male C57BL/6 mice were removed and transferred to sterile petri dishes. Bone marrow was flushed out of femurs using complete medium and a 27-gauge syringe. Peritoneal macrophages flushed out of the peritoneal cavity by injecting DMEM using a 21 gauge syringe. Cells were transferred to cell culture plates and incubated overnight.

D.4 Cell culture

Raw264.7 cells and primary peritoneal macrophages were cultured in DMEM medium supplemented with 10% FBS, 1% pen/strep.Raw264.7 PHF8 overexpressing cells were cultured in DMEM medium supplemented with 10% FBS, 1% pen/strep with the addition of 2μg/ml blasticidin and 400μg/ml hygromycin. Concerning Raw264.7 K.O.PHF8 cells, they were also cultured in complete medium supplemented with 2μg/ml puromycin. Bone Marrow Derived Macrophages (BMDMs) were prepared by plating bone marrow in medium containing 70% DMEM with 10%FBS, 1%pen/strep and 30% LCCM for 10 days.

D.5 Endotoxin Tolerance protocol

BMDMs were treated with LPS (100ng/ml) for 24 hours, then washed twice with 1x PBS, rested for 2 hours and then restimulated with LPS (100 ng/ml) for 2 hours.

D.6 Cell culture treatments

Cells were treated with 100ng/ml LPS (L2630, Sigma), 2.5μg/ml tunicamycin, α-KG (K1128, Sigma) diluted in 1x PBS in 0.1, 1 and 2 mM concentrations.

D.7 Mitotracker Green Assay

25*10^3 Raw264.7 cells per well were cultured and treated overnight. Then cell culture medium was removed and cells were washed using 1x PBS. 100μl of warm staining solution [1x PBS, 1% FBS, 35nM MitoTracker FM Green (M7514, Thermofisher)] was added to each well and the plate was incubated at 37°C for 30 min in cell culture incubator. After staining

was completed, staining medium was replaced with 1x PBS and fluorescence was measured using fluorescent microplate reader.

D.8 Elisa

Cytokine concentration of TNF and IL-6 in the supernatant was determined by ELISA using ELISA kits (R&D systems) according to the manufacturer's instructions.

D.9 Western Blot Analysis

Protein lysates from macrophages were resuspended in radioimmunoprecipitation assay buffer containing phosphatase and protease inhibitors (complete; Roche, Basel, Switzerland). Protein concentration of samples was determined using bicinchoninic acid kit. 15 ug of protein was electrophoresed on 15% or 8% denaturing polyacrylamide gel prior to wet transfer to 0.45-mm nitrocellulose membrane (Macherey–Nagel, Germany). Briefly, after blocking with 5% BSA in PBS-T (pH 7.4) for an hour at room temperature, the membranes were incubated with mouse polyclonal anti-mouse actin-β Ab (#4970, Cell Signaling), rabbit polyclonal anti-mouse LC3 Ab(#4108, Cell Signaling), rabbit polyclonal anti-mouse p-S6 Ab (S235/236, #2211, Cell Signaling)Ab, rabbit monoclonal anti-mouse p-4EBP1 Ab(T37/46, #2855, Cell Signaling), rabbit polyclonal anti-mouse IRE1a Ab(#3294, Cell Signaling),rabbit polyclonal anti-mouse PHF8 (#93801, Cell Signaling), mouse polyclonal anti-mouse Hsp90 (StressGen Biotechnologies), rabbit polyclonal anti-mouse GRP78 (#3177, Cell Signaling), rabbit polyclonal anti-mouse GRP94(#2104, Cell Signaling) at 4°C, overnight and then incubated with peroxidase-conjugated anti-mouse (#7076, Cell Signaling) or anti-rabbit (#50667388, Enzo life sciences) secondary Ab for 1 hour at room temperature followed by reaction with Chemiluminescent HRP Substrate (LumiSensor; GenScript). Image analysis was made using the Image J software.

D.10 RNA extraction and real time PCR

Total RNA was extracted from cells using the TRIzol reagent(Life technologies, Carlsbad, CA) and quantified at 260 and 280nm using spectrometry.500 ng of total RNA was used for cDNA synthesis(TAKARA, Shiga, Japan).The SYBR Green method was followed in the PCR reaction. Initiation was performed at 95°C for 3 min, denaturation at 95°C for 10 sec, annealing and extension at 60°C for 30 sec for 40 cycles. Melting curve was constructed continuously from 60°C to 95°C with 1% gain. Ribosomal protein S9 served as the housekeeping gene. Quantitative analysis of the fold change was performed using the Pfaffl method.

D.11 RNAseq

Libraries construction was made by the NEBNext® Ultra™ II RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, USA). Sequencing was held at the platform NextSeq 2000 (Illumina Inc., San Diego, CA, USA) with the use of NextSeq 2000 P3 Reagents (300 Cycles). Raw paired-end reads were quality filtered using TrimGalore. Reads with low quality (q-score<30) and length below 20 bases as well as adapter sequences and non-identified nucleotides (Ns) were removed from the data. For the differential expression analysis, filtered reads were aligned against the *Mus musculus* reference transcriptome using Salmon (v.0.13.1). The reference transcriptome that was used was the *Mus musculus* GRCm39 transcriptome (Date modified: 2022-12-13) from the Ensembl database. The resulting abundance tables were imported into R (v.4.2.1) and differential expression

analysis was performed at the gene level with the DESeq2 (v.1.36.0) R package. Differentially expressed genes (DEGs) were considered those with adjusted p-value lower than 0.05 and absolute log2(fold-change) greater than 1. In the case of Raw264.7 PHF8 K.O. cells the number of differentially expressed genes with log2(fold-change)>1 was very low, so we considered genes with log2(fold-change)<1.After sequencing, 375,842,528 raw paired-end reads were produced in total (15,660,105 on average per sample), of which, after quality filtering, 372,444,846 (15,518,535 on average per sample) reads were retained. Analysis produced 178129 transcripts and 58401 genes. Overall after removal of low variability transcripts, 20867 transcripts and 8533 genes were retained at the data set. Each condition included three technical replicate apart from naïve state of Raw264.7 PHF8 K.O., which included two technical replicates, as one showed quite differentiated expression profile from the other two replicates of the same group based as shown in the respective PCoA based on the FPKM values. Samples were checked for RNA integrity by agarose gel electrophoresis. The analysis was made by the laboratory of food science and nutrition at the Institute of applied biosciences-center for research and technology Hellas.

D.12 Transfection

60-70% confluent BMDMs were transfected with 5 pmol of siPHF8 or siScramble using RNAiMAX transfection reagent (13778100, ThermoFisher) and incubated for 24-72 hours before collection.

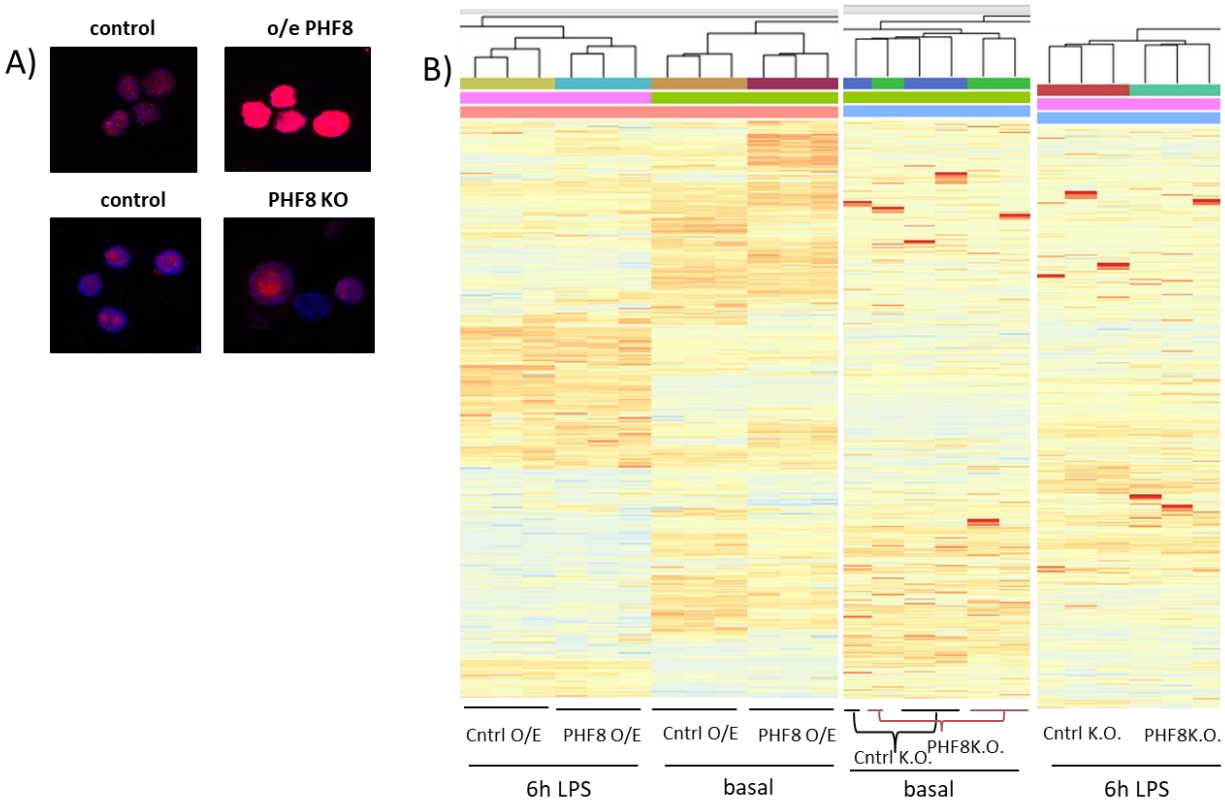
D.13 Statistical analysis

All numeric data were evaluated for normality using the Shapiro-Wilk test. The numerical data that passed the normality test and the PCR results were analyzed using Student's t-test. P-values<0.05 were considered statistically significant. The software GraphPad Prism 8.0.2 was used for the statistical analysis.

E. Results

E.1 RNA sequencing in Raw264.7 cells overexpressing PHF8

In order to study how PHF8 regulates macrophage activation we performed RNA sequencing analysis in Raw264.7 PHF8 overexpressing and PHF8 knock out cell lines at the naïve state and after 6 hours of LPS stimulation. Differentially expressed genes were considered those with adjusted p-value lower than 0.05 and absolute log2(fold change)>1. In case of Raw264.7 K.O cells, the number of genes meeting the above criteria was very low, so genes with absolute log2(fold change) lower than 1 were also considered. In Raw264.7 PHF8 overexpressing cells we detected 259 differentially expressed genes consisting of 184 upregulated and 75 downregulated genes. After LPS stimulation in cells overexpressing PHF8 87 genes were found differentially expressed and of those 37 were upregulated and 50 were downregulated. Correspondingly in Raw264.7 PHF8 K.O. cells 46 genes were found differentially expressed, 25 were upregulated and 21 were downregulated at the naïve state, while after 6 hours of LPS stimulation 117 genes were differentially expressed, 57 upregulated and 60 downregulated. We analyzed three technical replicates in each condition, except from Raw264.7 PHF8 K.O. cells at the naïve state, which included 2 K.O. replicates. According to the PCo analysis plots based on gene expression overexpression or K.O. samples were clustered together and separately from their corresponding control samples in each condition, thus evidencing distinct gene expression profiles in these cell lines. In general PHF8 overexpressing samples yielded a greater number of differentially expressed genes compared to K.O. cells (figure 8, C, D, E). This could be explained by the fact that Raw264.7 K.O. cells were a mixed population consisted of complete, partial knock out or even wild type PHF8 expressing cells(figure 8, A). We performed Gene Ontology analysis in our data set using the DAVID bioinformatics tool, in order to identify the biological processes our genes were involved. In general we observed enrichment in immune response related genes as well as metabolic genes. These results will be addressed later in detail.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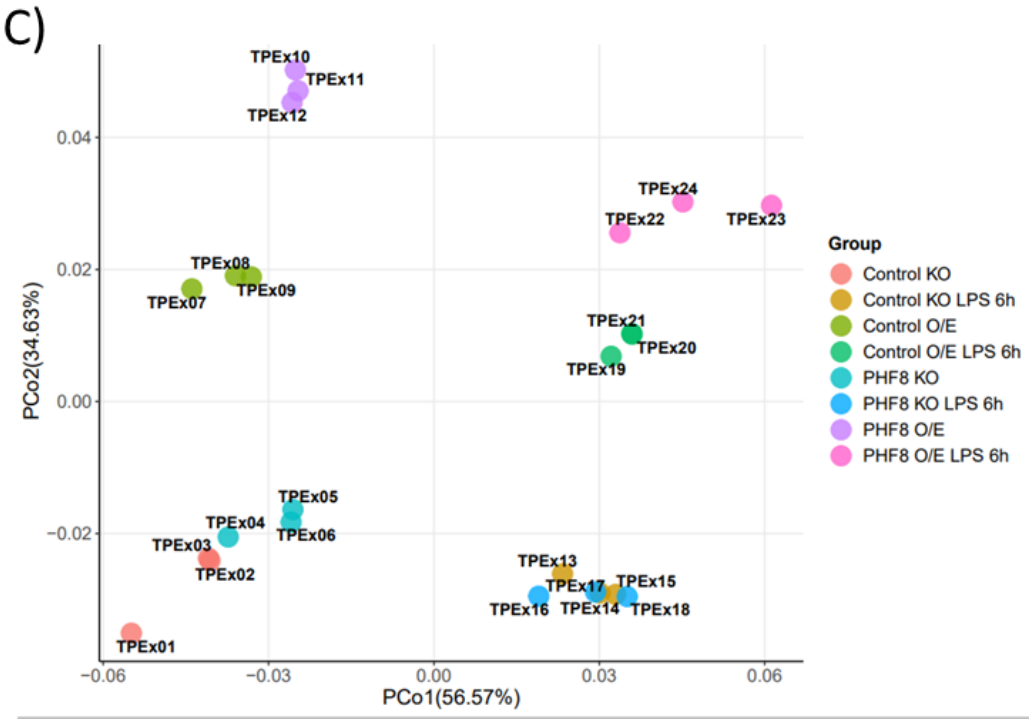
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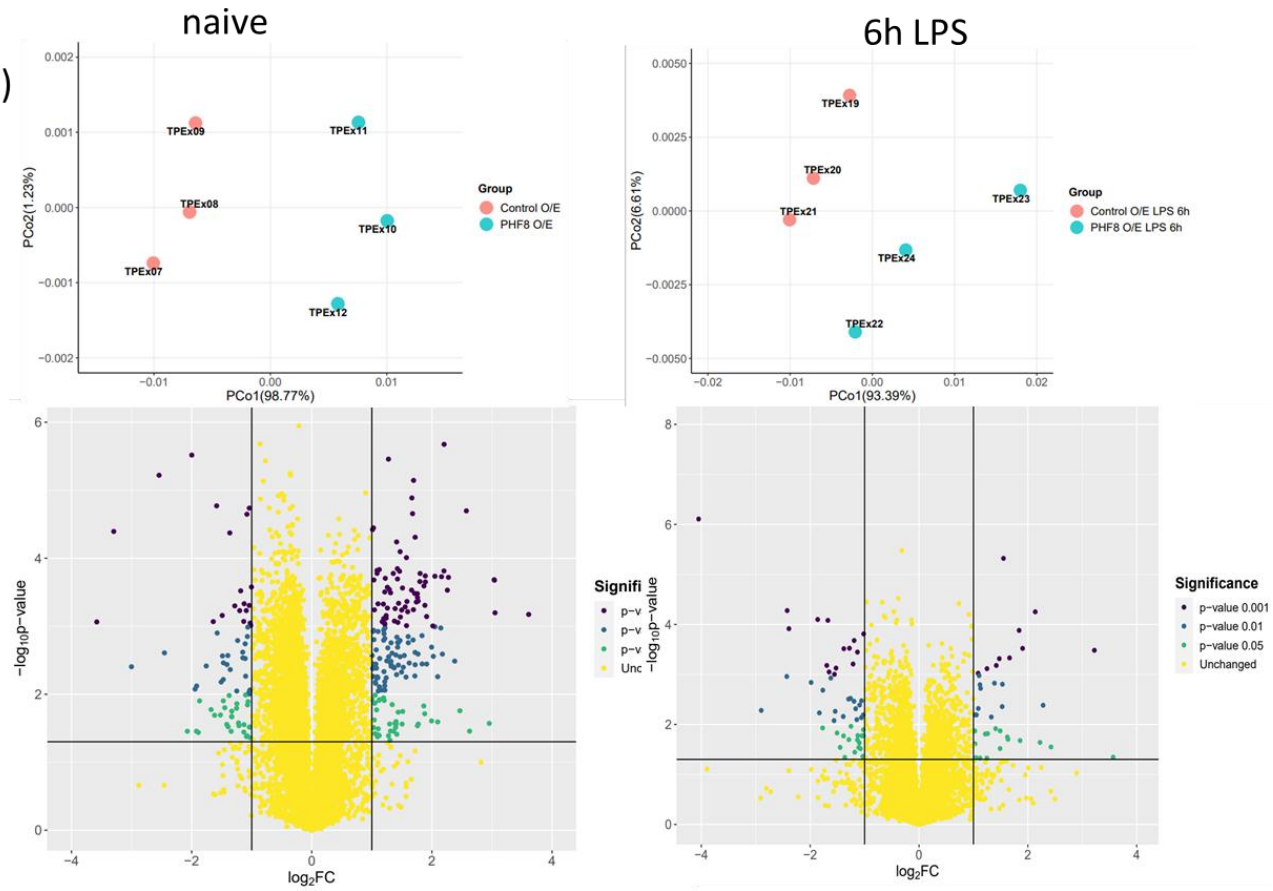
TPEx12
TPEx24
TPEx22
TPEx23
TPEx08
TPEx09
TPEx07
TPEx21
TPEx20
TPEx19
TPEx05
TPEx04
TPEx06
TPEx03
TPEx02
TPEx13
TPEx17
TPEx15
TPEx16
TPEx14
TPEx18
TPEx01
0.04
0.02
0.00
−0.02
PCo2(34.63%)
−0.06
−0.03
0.00
0.03
0.06
PCo1(56.57%)
Group
Control KO
Control KO LPS 6h
Control O/E
Control O/E LPS 6h
PHF8 KO
PHF8 KO LPS 6h
PHF8 O/E
PHF8 O/E LPS 6h

D)


naive
6h LPS
TPEx09
TPEx11
TPEx08
TPEx10
TPEx07
TPEx12
PCo2(1.23%)
PCo1(98.77%)
Group
Control O/E
PHF8 O/E
TPEx19
TPEx20
TPEx21
TPEx23
TPEx24
TPEx22
PCo2(6.61%)
PCo1(93.39%)
Group
Control O/E LPS 6h
PHF8 O/E LPS 6h
−log10p−value
log2FC
Significance
p−value 0.001
p−value 0.01
p−value 0.05
Unchanged

E)

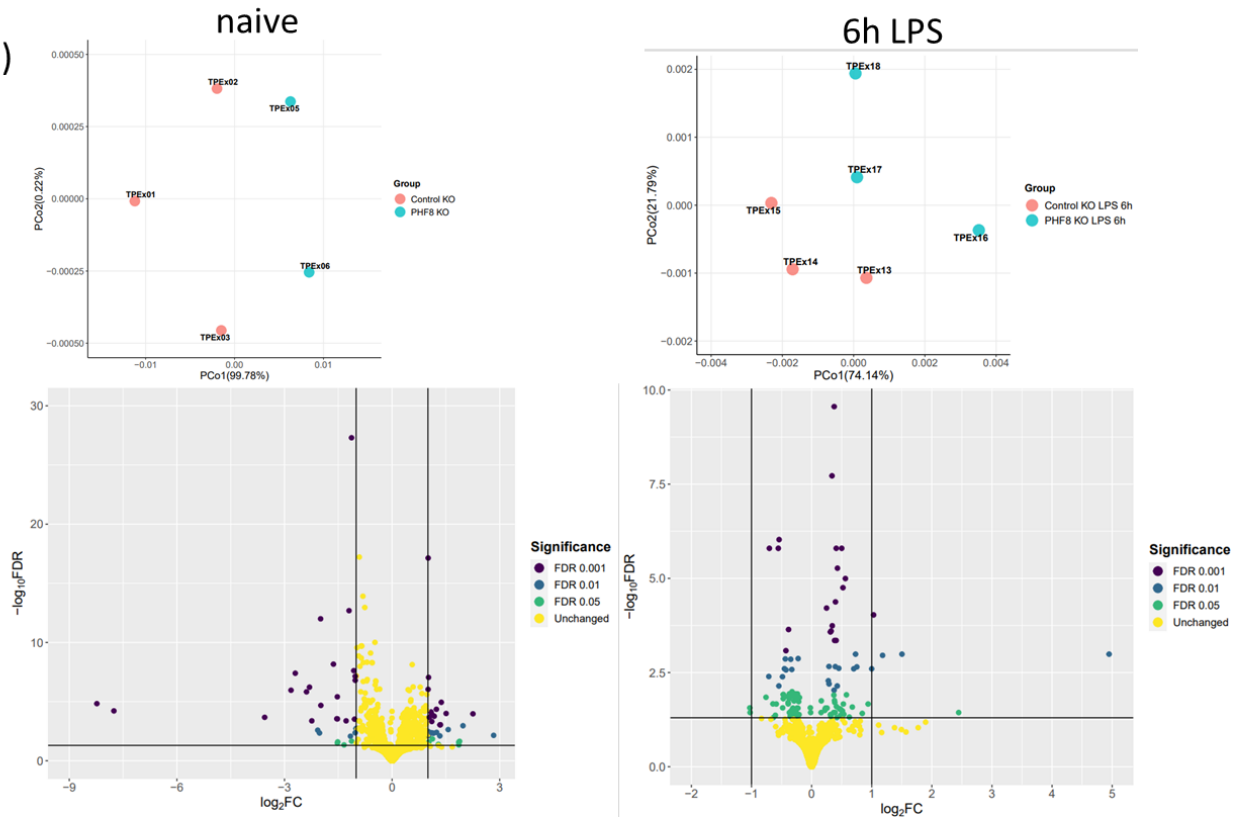
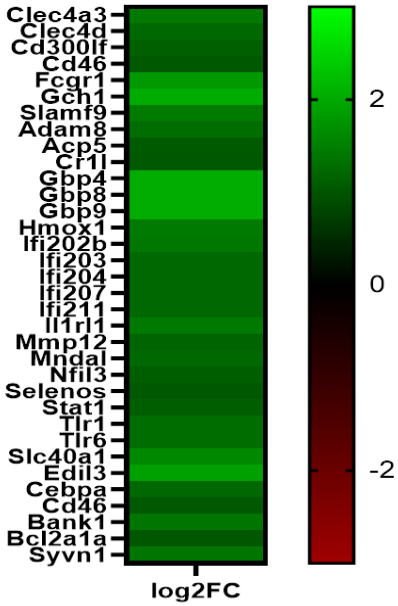

naive
6h LPS
TPEx02
TPEx05
TPEx01
TPEx06
TPEx03
PCo2(0.22%)
PCo1(99.78%)
Group
Control KO
PHF8 KO
TPEx18
TPEx17
TPEx15
TPEx16
TPEx14
TPEx13
PCo2(21.79%)
PCo1(74.14%)
Group
Control KO LPS 6h
PHF8 KO LPS 6h
−log10FDR
log2FC
Significance
FDR 0.001
FDR 0.01
FDR 0.05
Unchanged

Figure 8: RNA sequencing in Raw264.7 PHF8 overexpressing cells and Raw264.7 PHF8 K.O. cells at the naïve state and after LPS stimulation A)confocal microscopy depiction of PHF8 levels in Raw264.7 PHF8 overexpressing cells and Raw264.7 PHF8 K.O. cells B)Pco Analysis of Raw264.7 PHF8 overexpressing and K.O. cells at the naïve state and after LPS stimulation C)Heatmap of gene expression in Raw264.7 PHF8 overexpressing and K.O. cells at the naïve state and after LPS stimulation D)Pco Analysis and volcano plot of Raw264.7 PHF8 overexpressing cells E)Pco Analysis and volcano plot of Raw274.7 PHF8 K.O. cells

E.2 PHF8 overexpression attenuates the expression of LPS induced immune related genes but positively regulates IFN-β pathway genes

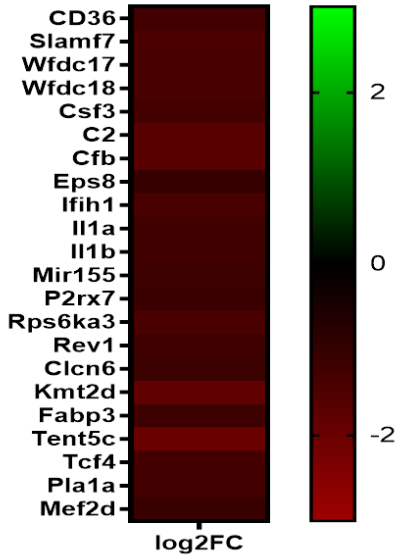
Gene Ontology analysis in our data set revealed differences in immune response related genes. After 6 hours of LPS stimulation PHF8 overexpressing cells exhibit reduced expression of genes involved in inflammatory response (figure 9, B). This cluster of genes include members involved in cytokine production such as TNF and IL6 production pathways and complement activation. IL-1α and IL-1β also show reduced expression as well as MiR155, a well -studied central regulator of inflammatory response. In agreement with these data RNA sequencing results in PHF8 knock out cells after 6 hours of LPS stimulation we detected upregulated proinflammatory genes such as IL6ra and NOS2 (figure 11, E). Interestingly at the naïve state PHF8 appears to positively regulate genes related to IFN-β response, including many IFN induced genes and Stat1(figure 9, A).

A)



Biological Processes	p- value
Cellular response to IFN-b	9.6E-7
Leukocyte activation	2.8E-2
Response to bacterium	1E-3

B)



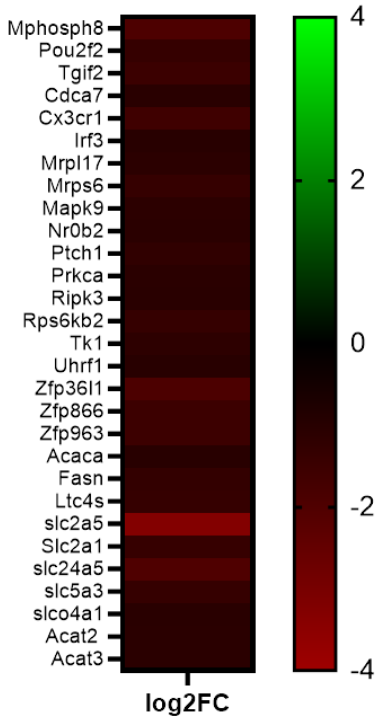
Biological Processes	p- value
Regulation of immune response	8.6E-5
Complement activation	8.5E-4
Positive regulation of cytokine production	3.0E-4
Positive regulation of IL-6 production	3.8E-5
Positive regulation of TNF-a production	2.5E-4

Figure 9: PHF8 regulates the expression of immune related genes. A)Genes upregulated at the naïve state in Raw264.7 PHF8 overexpressing cells. B)Genes downregulated after LPS stimulation inRaw264.7 PHF8 overexpressing cells

E.3 PHF8 regulates metabolic genes

Metabolism has a central role in the modulation of immune response. Different metabolic pathways characterize different macrophage functional phenotypes[88]. PHF8 overexpressing cells display different metabolic features. Gene Ontology analysis revealed differences in metabolic genes in Raw264.7 overexpressing (O/E) cells at the naïve state. In general genes involved in biosynthetic processes such as peptide and lipid biosynthesis were downregulated in Raw264.7 O/E cells. FasN, a master regulator of lipid synthesis activated downstream of mTORC1 was found downregulated in our list, as well as protein synthesis regulators such as ribosomal protein s6 kinase (Rsp6kb2) and mitochondrial ribosomal proteins (Mrps6, Mrpl17) (figure 10, A). To further study lipid metabolism in Raw264.7 O/E cells by RT-PCR we measured the RNA levels of CPT1a and Srebf1, two central regulators of fatty acid oxidation and fatty acid synthesis correspondingly. Although both were upregulated CPT1a has a greater and more significant difference, which in accordance with the RNA sequencing results suggests these cells possibly have higher rates of fatty acid oxidation (figure 10, B). Furthermore many transporters were found differentially expressed in our data set. Amino acid transporters, such as slc6a9 catalyzing glycine transport, slc1a4, which transfers neutral amino acids including alanine, threonine, cysteine, serine and slc3a2 were upregulated, while the glucose transporters glut1 and glut5 were downregulated at the naïve state in cells overexpressing PHF8 (figure 10, A).

A)



Biological Processes	p- value
Regulation of cell macromolecule biosynthetic process	7.3E-3
Lipid biosynthetic process	6.7E-3
Peptide biosynthetic process	5.9E-2

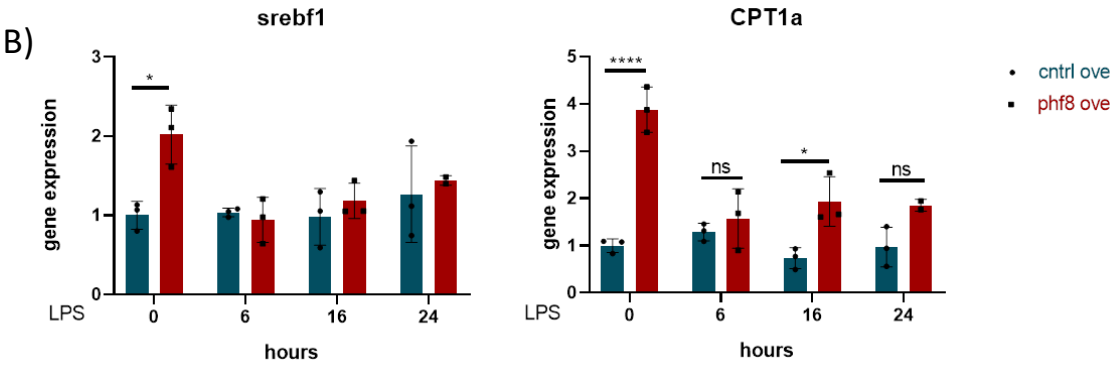
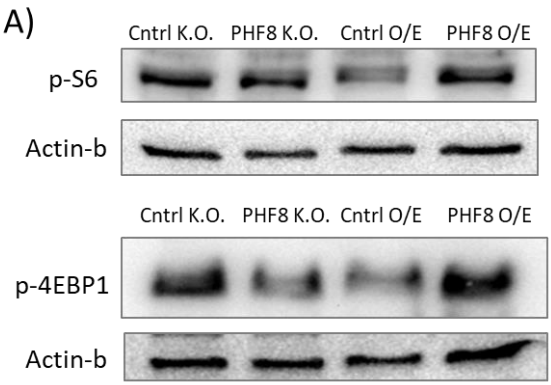


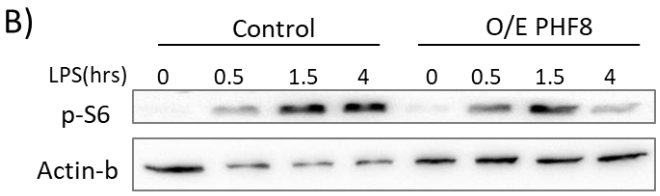
Figure 10: PHF8 controls metabolic genes. A)Downregulated metabolic genes at the naïve state in Raw264.7 PHF8 overexpressing cells .B) relative gene expression of srebf1 and cpt1a, hk3 and pfkp in Raw264.7 PHF8 O/E cells analyzed by q-PCR

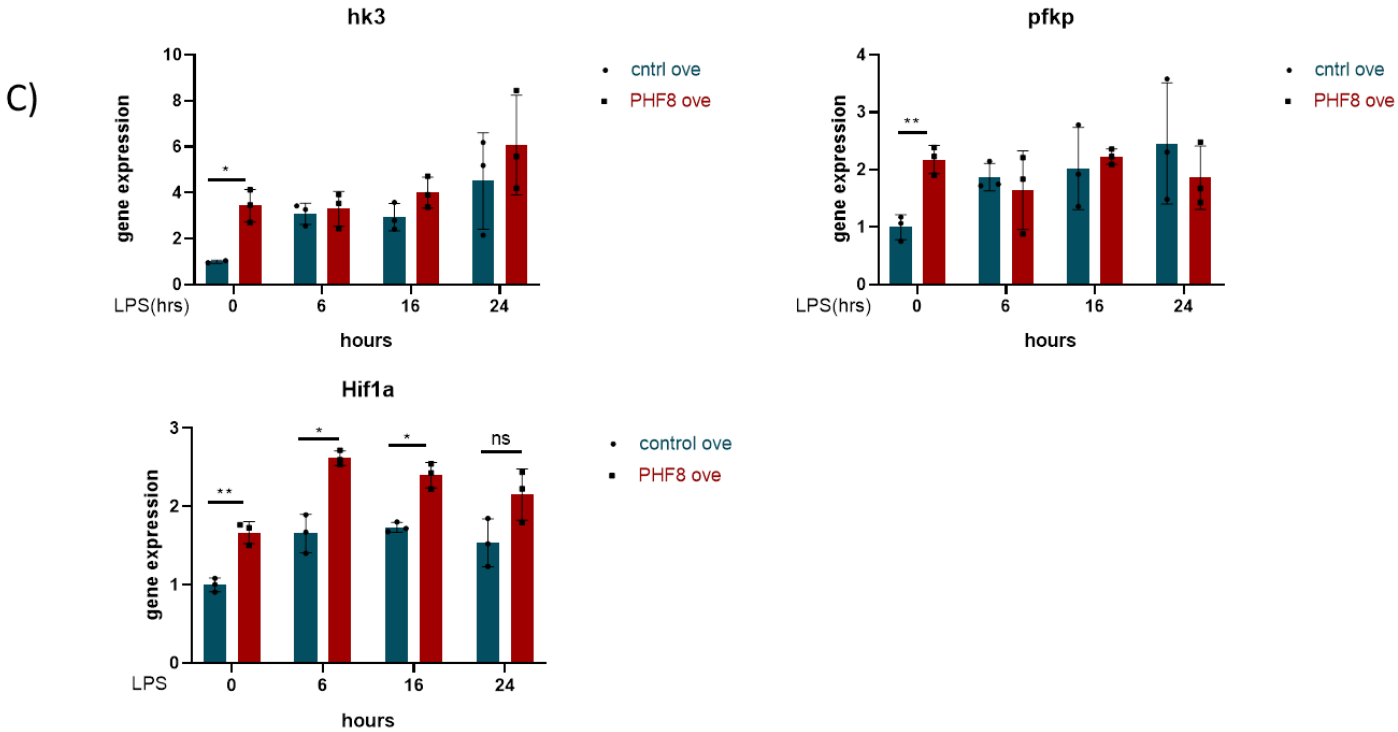
E.4 PHF8 positively regulates mTORC1 at the naïve state but impairs its activation after LPS stimulation

mTORC1 plays a pivotal role in macrophage activation. Polarizing signals impinge on mTORC1, which in turn regulates metabolic pathways and macrophage activation[164]. It has been reported that PHF8 regulates mTORC1 activity in neuronal cells[91]. To further investigate the role of PHF8 in the regulation of mTORC1 in innate immune response we analyzed by Western blot the protein levels of mTORC1 downstream targets p-S6 and p-4EBP1 in cells overexpressing PHF8 and K.O. cells at the naïve state and after LPS stimulation. At the naïve state in cells overexpressing PHF8 p-S6 and p-4EBP1 are upregulated. The opposite is observed in knock out cells (figure 11, A). Nonetheless Raw264.7 PHF8 overexpressing cells exhibit impaired mTORC1 activation after LPS stimulation (figure 11, B). This result is compatible with the hyporesponsive phenotype observed in cells overexpressing PHF8. M1 polarizing signals cause metabolic switch to glycolysis controlled by Hif1a, master regulator of glycolysis, which is activated by mTORC1. By q-PCR we measured the RNA levels of Hif1a and both pfkp and hk3, two key glycolytic genes at the naïve state and after 6, 16 and 24 hours of LPS stimulation. At the naïve state hk3, pfkp were found upregulated in PHF8 overexpressing cells compared to their controls, compatible with the enhanced mTORC1 activity at the basal state, while after LPS stimulation there is no statistically significant differences between control RAW264.7 and O/E PHF8 RAW264.7 (figure 11, C). As expected p-S6 and p-4EBP1 expression in control cells is induced after LPS stimulation, but PHF8 overexpressing cells show no further induction (figure 11, B). In a similar way hk3 and pfkp are induced in control cells after LPS stimulation, a response not observed in Raw264.7 PHF8 overexpressing cells (figure 11, D). Moreover RAW264.7 PHF8 overexpressing cells display increased Hif1a expression at all time points. These observations suggest that PHF8 overexpressing cells have enhanced glycolysis, but fail to further increase it in response to LPS stimulation. The lack of glycolytic program inducibility in the presence of LPS stimulus may account for the reduced responsiveness in PHF8 overexpressing cells. Consistent with these findings Gene Ontology analysis in PHF8 K.O. cells after 6 hours of LPS stimulation revealed upregulated genes involved in

carbohydrate and glucose metabolism. More specifically genes involved in Hif1a pathway and glycolysis had increased expression in PHF8 K.O. cells. , and may associate with their pronounced proinflammatory phenotype(figure 11, E).







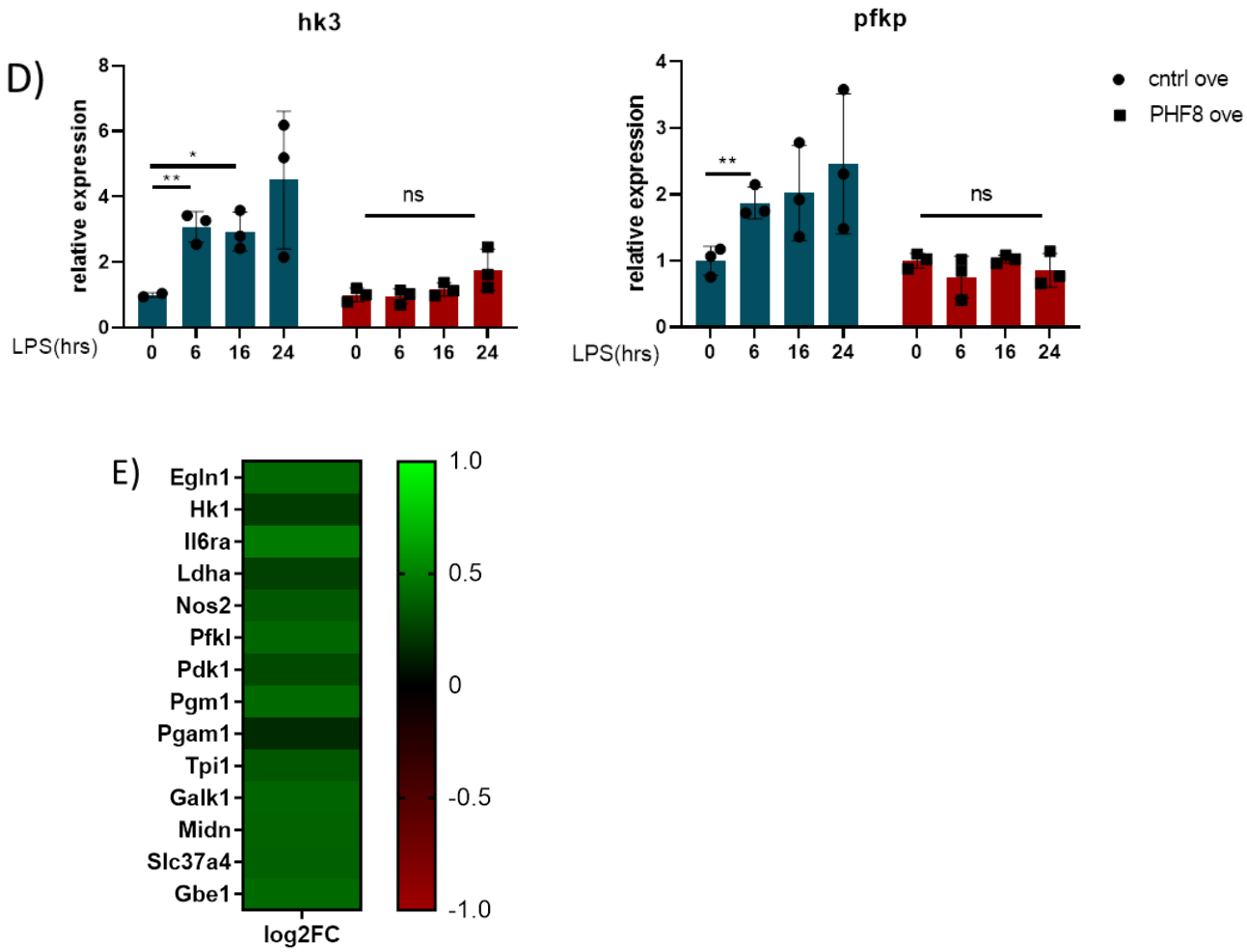
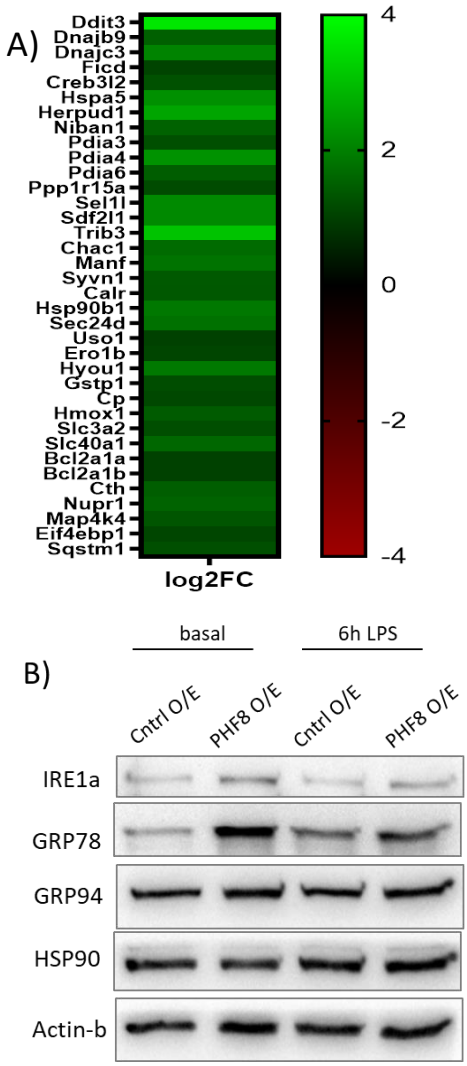


Figure 11: PHF8 positively regulates mTORC1 at the naïve state but impairs its activation after LPS stimulation. A) Protein levels of mTORC1 targets at the naïve state in Raw264.7 O/E cells and Raw264.7 K.O. cells. B) Protein levels of mTORC1 targets after LPS stimulation in Raw264.7 O/E cells C)hk3, pfkp and Hif1a relative expression analyzed by q-PCR D) hk3 and pfkp relative expression . Each genotype expression is normalized to their basal expression E)Heatmap of upregulated genes in PHF8 K.O. cells after 6 hours of LPS stimulation.

E.5 PHF8 positively regulates ER stress related genes

Gene Ontology analysis in our RNA sequencing data set from cells overexpressing PHF8 using the David bioinformatics tool revealed enrichment with genes involved in biological processes of Endoplasmatic Reticulum stress and Unfolded Protein Response with Ddit3, the gene that encodes CHOP, a C/EBP family of transcription factors member mainly induced under ER stress conditions being the highest expressed gene in our data set (figure 12, A). Also chaperones expression was enhanced, Hsp40 family members, HSP90b1(or GRP94) and Hspa5 gene, which encodes for BiP or GRP78, an ER located chaperone which upon E.R. stress dissociates from UPR regulators allowing the initiation of their signaling cascades. Additionally members of the ERAD (E.R. associated degradation) pathway were found to be upregulated at the naïve state. SQSTM1, the gene encoding for p62 protein was also

detected to be upregulated in our data set. p62 has a pivotal role in the two main protein degradation pathways, in the ubiquitin proteasome system and autophagy. To further validate our results regarding the positive regulation of E.R. stress related genes we analyzed by Western blot the protein levels of UPR related genes such as IRE1α, GRP78, GRP94 and HSP90 in RAW264.7 PHF8 overexpressing cell line. In accordance with the sequencing results IRE1α and GRP78 were found to be upregulated compared to controls, while no differences were observed at the protein levels of HSP90 and GRP94 (figure 12, B). These results imply that PHF8 positively regulates ER stress related genes and may epigenetically control the UPR response. To further test this hypothesis we knocked down PHF8 in mouse BMDMs and treated them with tunicamycin, an E.R. stress inducer, for 6 hours. Despite our hypothesis PHF8 knocked down BMDMs showed no differences in IRE1α, GRP78, GRP94 and HSP90 protein levels (figure 12, C).



Biological Processes	p- value
Response to ER stress	9.1E-16
Response to UPR	2.5E-8
ER UPR	4.3E-7
Negative regulation of DNA binding	9.6E-7
Ub dependent ERAD pathway	1.6E-6
Retrograde protein transport, ER to cytosol	4.8E-6

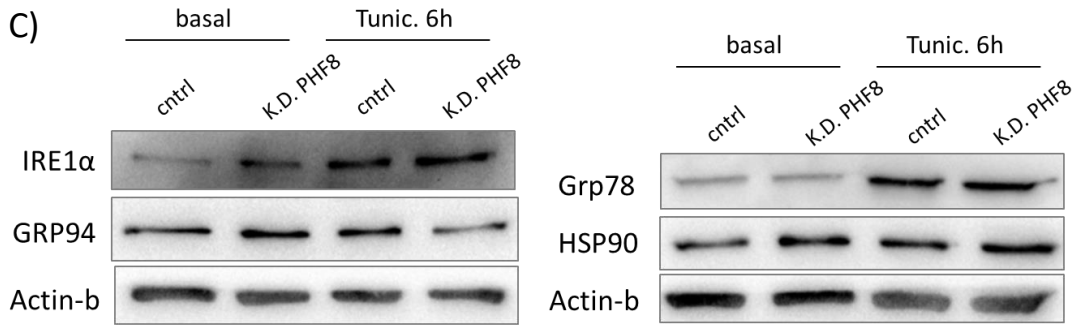


Figure 12: PHF8 positively regulates E.R. stress related genes. A) Genes upregulated at the naïve state of Raw264.7 O/E cells. B) Protein levels of E.R. stress related genes in Raw264.7 PHF8 O/E cells analyzed by Western Blot C)Protein levels of E.R. stress related genes after treatment with tunicamycin in control and PHF8 knock down BMDMs

E.6 Autophagy is upregulated in RAW264.7 PHF8 overexpressing cells

The two major functions of the UPR response include the upregulation of chaperones in order to improve protein folding capacity and the activation of degradation pathways to discard the accumulated misfolded proteins. As ER stress related genes were upregulated in cells overexpressing PHF8 we made the hypothesis that autophagy pathways could also be positively regulated. So we used Western blot analysis to measure the LC3-II/LC3-I protein ration in RAW264.7 PHF8 O/E cells both at the naïve state and at the LPS stimulated state. As expected LC3-II/LC3-I protein ration was higher in RAW264.7 PHF8 O/E cells both at the naïve state and after LPS stimulation (figure 13, A). In addition we performed RT-PCR in these cells to measure the mRNA levels of pink1 and parkin, two key proteins involved in mitophagy, which revealed significant upregulation of these genes in PHF8 overexpressing cells (figure 13, B). Consistent with this fact, mitochondrial mass was measured using the Mitotracker stain and green fluorescence intensity was quantified and found decreased in Raw264.7 PHF8 overexpressing cells (figure 13, C). Supporting these data sqstm1, a selective autophagy receptor was found to be upregulated in our RNA seq data set at the naïve state as previously mentioned, implying increased autophagy (figure 13, A).

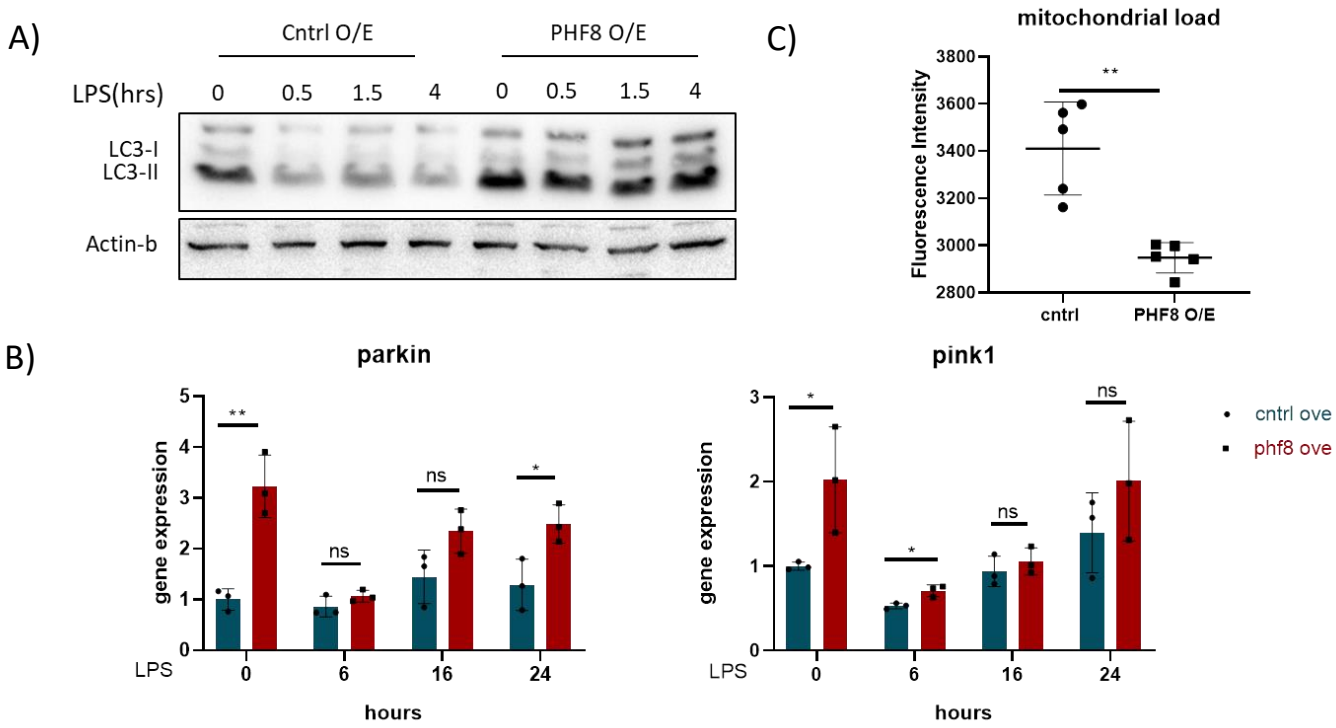
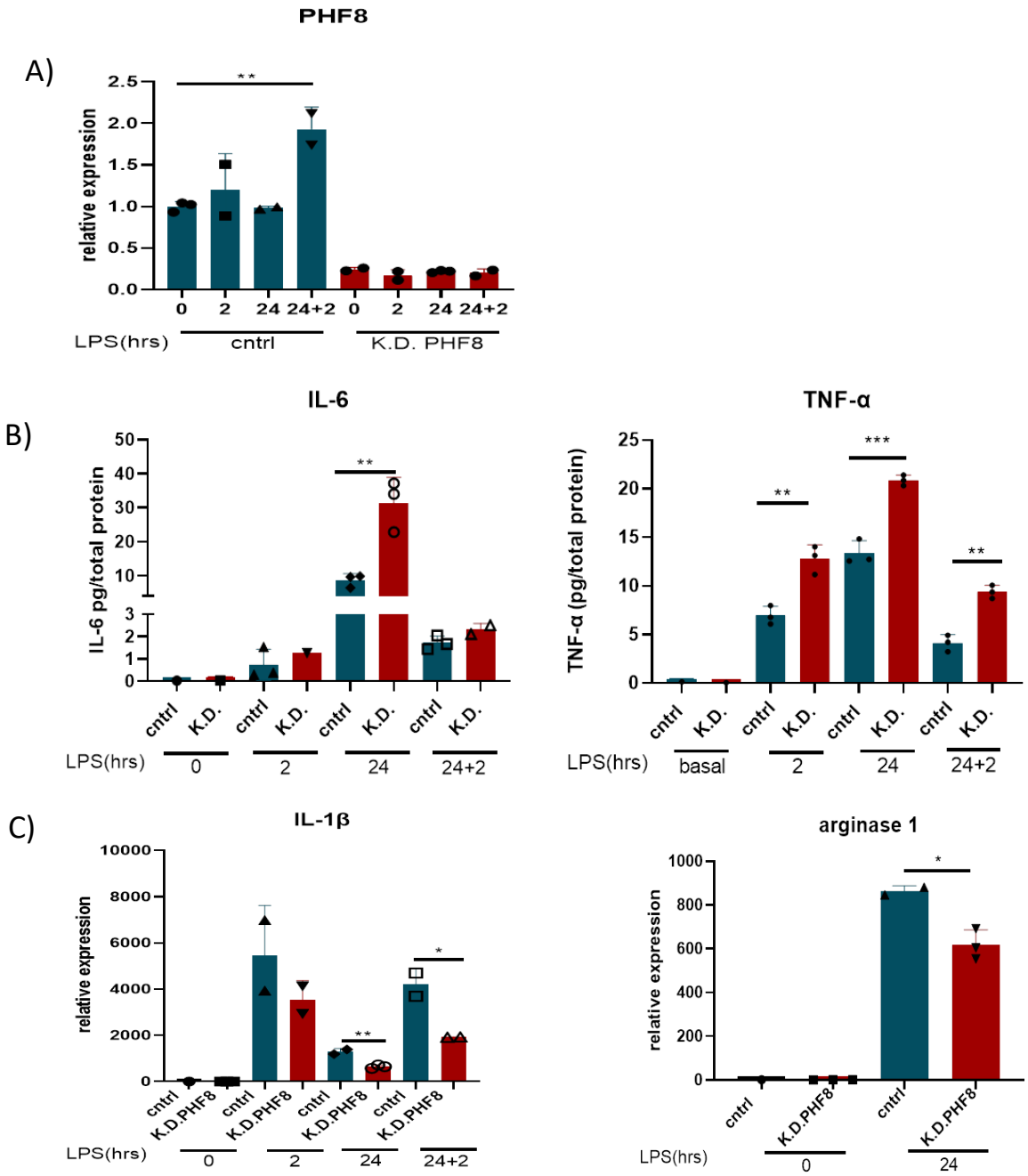


Figure 13: Autophagy is upregulated in Raw264.7 PHF8 O/E cells. A) LC3 protein levels in Raw264.7 PHF8 0/E cells B) parkin and pink 1 relative expression in Raw264.7 O/E cells analyzed by q-PCR C)Mitochondrial load in cntrl and Raw264.7 PHF8 overexpressing cells measured by fluorescence of mitotracher green

E.7 PHF8 negatively regulates macrophage classic activation in mouse BMDMs

In our cell lines we have already described that PHF8 negatively relates to the proinflammatory classic macrophage activation phenotype and causes a hyporesponsive phenotype in cells overexpressing PHF8 upon LPS stimulation, while cells knocked out for PHF8 display increased cytokine production after LPS stimulation. In order to further investigate the role of PHF8 in immune response in mouse primary cells we knocked down PHF8 by using specific siRNA in mouse BMDMs and measured the cytokine levels of IL6 and TNF after 24 hours of LPS stimulation. Both IL6 and TNF levels were increased in K.D. BMDMs after 24 hours of LPS stimulation (figure 14, B). Also arginase 1, a marker of M2 alternative macrophage polarization was less induced in K.D. BMDMs (figure 14, C). All these findings are consistent with the induction of a more pronounced proinflammatory phenotype in the absence of PHF8 which suggests that PHF8 plays a role in later stages of macrophage activation and contributes to the resolution of inflammation. Additionally epigenetic regulators have been shown to underlie innate immune memory phenotypes. In order to investigate whether PHF8 epigenetically regulates the transition to endotoxin tolerance in control and knocked down for PHF8 BMDMs we did a 24 hour stimulation with LPS followed by 2 hours resting and a secondary 2 hour stimulus. We measured by Elisa TNF and IL6 levels in the supernatants. Although BMDMs silenced for PHF8 became tolerant characterized by lower TNF production after the 24 hour LPS pretreatment compared to the sole 2 hour stimulus, they had statistically significant higher levels of TNF compared to their siScramble

counterparts (figure 14, B). Nevertheless IL-1β mRNA levels were decreased at all timepoints of LPS stimulation (2, 24) and at the tolerant state in knocked down for PHF8 BMDMs (figure 14, C). Interestingly PHF8 mRNA expression was upregulated at the tolerant state, suggesting that PHF8 plays a role in endotoxin tolerance (figure 14, A).
Another interesting finding was that BMDMs knocked down for PHF8 had increased mTORC1 activity as evidenced by increased protein levels of p-4EBP1 and p-S6 compared to controls. This may in part explain the hyperresponsive phenotype of BMDMs knocked down for PHF8 (figure 14, D).



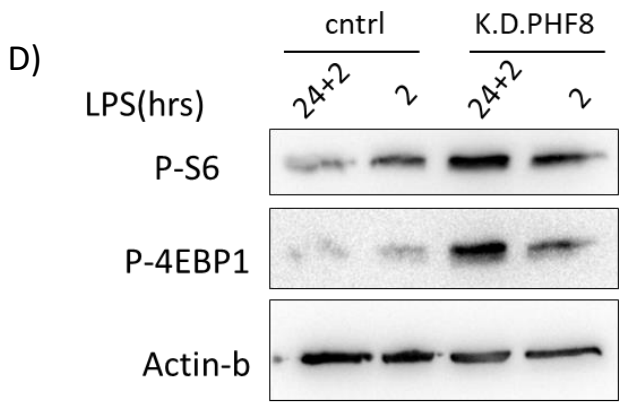


Figure 14: PHF8 negatively regulates proinflammatory cytokine expression A)PHF8 relative expression in cntrl and PHF8 knock down BMDMs B)IL-6 and TNF supernatant levels in cntrl and PHF8 knock down BMDMs analyzed by Elisa C)IL-1β and arginase 1 relative expression analyzed by q-PCR D) P-4EBP1 and P-S6 protein levels after 2 and 24+2 hours of LPS stimulation in cntrl and PHF8 knock down BMDMs analyzed by Western Blot

E.8 α-KG induces PHF8

Metabolites play a crucial role in metabolic rewiring process and have been shown to underscore metabolic and functional adaptations in macrophages[131]. PHF8 is an α-Ketoglutarate dependent demethylase, which has inhibitory role in proinflammatory gene expression. So we wanted to assess whether α-KG could induce PHF8, which consequently would lead to an immunosuppressive phenotype. We incubated Raw264.7 cells with α-KG of various concentrations and duration and assessed PHF8 protein levels by Western Blot. Indeed PHF8 levels were increased after 16 and 20 hours of α-KG incubation(figure 15).

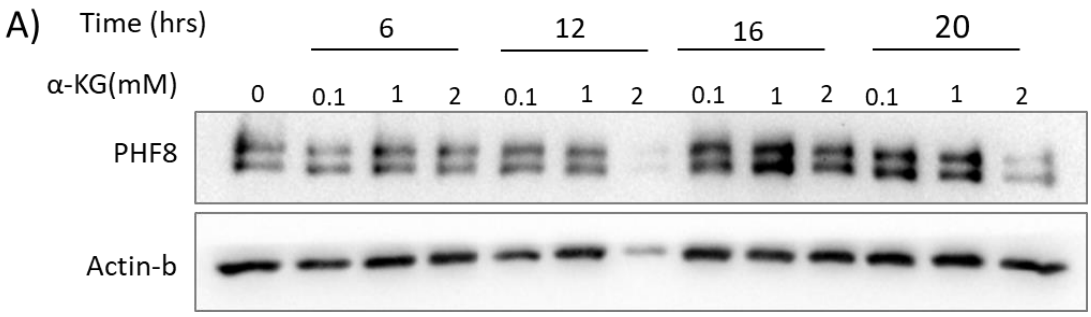


Figure 15: α-KG induces PHF8 expression in Raw264.7 cells A) Protein levels of PHF8 after treatment with α-KG in various concentrations and different durations analyzed by Western blot.

E.9 PHF8 does not epigenetically underlie the hyporesponsive phenotype observed in AKT2$^{-/-}$mice

In previous published work of the lab it has been described that AKT2$^{-/-}$ mice display hyporesponsiveness because of insulin resistance[187]. AKT2$^{-/-}$ macrophages, as well as other insulin resistance models have increased basal mTORC1 activity and glycolysis, responsible for the enhanced M2 markers production[187]. Our hypothesis was that in AKT2$^{-/-}$mice PHF8 is upregulated and controls epigenetically the hyporesponsive phenotype. Experimental analysis of PHF8 protein and RNA levels by Western blot and RT-PCR respectively in peritoneal macrophages from B6 and AKT2$^{-/-}$ mice after 6 and 24 hours of LPS stimulation showed lower levels of PHF8 in AKT2$^{-/-}$ mice opposing our original hypothesis. In B6 mice PHF8 increased after 24 hours of LPS stimulation, while it did not in AKT2$^{-/-}$ mice (figure 16).

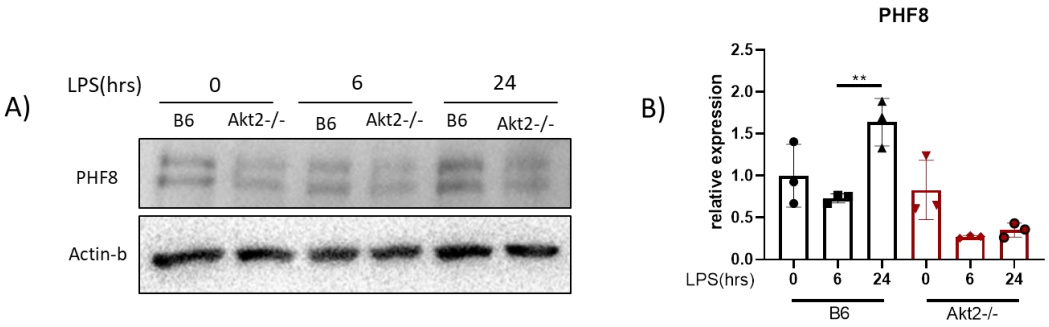


Figure 16: PHF8 does not epigenetically underlie the hyporesponsive phenotype observed in AKT2$^{-/-}$ mice A)protein levels of PHF8 in C57BL/6 and AKT2$^{-/-}$ mice after 6 and 24 hours of LPS stimulation B) PHF8 relative expression in C57BL/6 and AKT2$^{-/-}$ mice after 6 and 24 hours of LPS stimulation analyzed by q-PCR

F. Discussion

Epigenetic marks are dynamically regulated by polarizing signals, which impinge on transcriptional and epigenetic regulators to modulate macrophage activation[134]. In this study, we focused on the role of PHF8, a jumonji C containing domain histone lysine demethylase, in modulating the immune response and the mechanisms by which PHF8 regulates macrophage response. PHF8 appears to have a negative role in classic macrophage activation as evidenced by the enhanced cytokine expression in BMDMs knocked down for PHF8 after 6 and 24 hours of LPS stimulation. Also RAW264.7 PHF8 overexpressing and knock out cell lines display reduced and enhanced cytokine production respectively after LPS challenge. Enhanced TNF production is also observed at the endotoxin tolerance state in PHF8 knock down BMDMs, while Arginase 1, an M2 polarization marker has lower induction after 24 hours of LPS stimulation in these cells. These data suggest that PHF8 plays a role in late LPS response and in M2 polarization. Nevertheless the exact role of PHF8 in immune response remains to be defined. For instance it is not clear whether PHF8 mediates the transition to the M2 resolving phenotype or whether PHF8 is an epigenetic regulator of endotoxin tolerance, a phenotype characterized by altered epigenetic profile regarding proinflammatory mediators. Enhanced PHF8 expression in the tolerant state is in agreement with this hypothesis.

Cellular metabolism underlies functional macrophage activation. RNA sequencing data already showed that PHF8 negatively regulates the expression of genes involved in peptide and lipid biosynthesis pathways. Biosynthetic metabolism is predominantly induced upon classic macrophage activation, while alternatively activated or inflammatory resolving macrophages rely mostly on oxidative metabolism[95]. This is in accordance with our proposing role for PHF8 in immune response. A suggested mechanism by which PHF8 could control alternative macrophage polarization or transition to an anti-inflammatory phenotype is by epigenetically regulating genes of metabolic pathways that functionally support this activation state. PHF8 has primarily activating role in transcription by demethylating the repressive marks H3K9m2 and H3K9me3. So in case of downregulated genes it could mediate the activation of suppressive genes. Whether PHF8 regulatory effect is direct or indirect requires further investigation. For example a ChIP-seq experiment for PHF8 would help delineate this question.

A protein complex known to incorporate different signals and to impact metabolic rewiring is mTORC1. mTORC1 regulates protein and lipid biosynthesis and activates Hif1a, which in turn induces glycolytic switch, a primary metabolic pathway in proinflammatory macrophages. Regulation of mTORC1 activity could comprise a mechanism by which PHF8 regulates immune response. **Chen, X., et al.** in neuronal cells have already showed that PHF8 can impact on mTORC1 activity by regulating the expression of the mTOR activator Rps6ka1. Mechanistically PHF8 inhibits the expression of Rps6ka1by $H_4K_{20}me_1$ demethylation from the promoter of Rps6ka1[91]. In our experiments knock down of PHF8 in BMDMs resulted in enhanced mTORC1 activity especially at the tolerant state, observation compatible with the increase cytokine production in K.D. PHF8 BMDMs. Nonetheless mTORC1 activity is also increased at the naïve state of PHF8 overexpressing cells, along with genes involved in the glycolytic program and Hif1a, but shows reduced activation after LPS stimulation. Correspondingly Hk3 and pfkp exhibit no further induction in contrast with control cells, in which as expected they are induced after LPS stimulation. This lack of mTORC1 activation and glycolytic genes further increase may at least in part account for the reduced responsiveness of Raw264.7 PHF8 overexpressing cells. Consistent with this notion Raw264.7 PHF8 K.O. cells display elevated expression of glycolysis and Hif1a pathway at the LPS stimulated state suggesting that PHF8 has a regulatory role in switch to glycolysis.

Another finding is that PHF8 is a positive regulator of E.R. stress related genes. It causes the expression of many genes associated with the unfolded protein response, as well as the ER associated degradation pathway. Also by Western Blot we measured elevated expression of the autophagy marker LC3-II and higher mRNA levels of two mitophagy markers, pink 1 and parkin. Consequently it is possible that regulation of response to E.R. stress is a mechanism by which PHF8 mediates its effect in immune response. Several studies highlight the E.R. stress role in immune response. E.R. stress promotes inflammation, while prolonged E.R. stress associates with imbalance in M1 and M2 polarization and has been implicated in the pathophysiology of many inflammatory and autoimmune diseases. Also a study in C. elegans highlights the requirement of jmjd1.2, the PHF8 homolog in C. elegans for the longevity effect of mitochondrial unfolded protein response[188]. Yet tunicamycin treatment in control and knock down for PHF8 BMDMs didn't show any differences regarding the E.R. stress genes induction. In several settings prolonged E.R. stress has been shown to promote immunosuppression, as in cancer, atherosclerosis, aging, Altzheimer's and pulmonary fibrosis[189]. In all these cases PHF8 could be an epigenetic positive modulator of the E.R. stress response underlying the tolerogenic phenotype of immune cells. How PHF8 mediated control of the unfolded protein response relates with immune response requires further investigation.

Bibliographically it is known that α-KG can induce tolerance supported by its function as cofactor of immunomodulatory epigenetic enzymes[120]. Another interesting observation was that PHF8 could be induced by α-KG. α-KG is a cofactor of the jmjd family of demethylases including PHF8. Mechanistically PHF8 activation by α-KG could at least partially provide a mechanistic insight of α-KG effect in macrophage phenotype. Additionally metabolite use as a means of metabolic rewiring towards tolerance would be a desirable effect in cases of inflammatory disorders and autoimmunities, where loss of tolerance is observed.

G. Future Perspectives

In our experiments we have found that PHF8 negatively regulates proinflammatory cytokine production. In order to better understand the role of PHF8 in immune response we could employ IL4 stimulation in control and PHF8 knock down BMDMs to examine the expression of M2 markers and assess the role of PHF8 in M2 transition. Additionally we have observed that α-KG, a metabolite known to regulate tolerance, induces PHF8 production. We could further assess the role of PHF8 as an epigenetic mediator of α-KG dependent immunosuppressive phenotype. An approach to this question would be to pretreat control and knocked down for PHF8 BMDMs with α-KG followed by LPS or IL4 stimulation. We would expect higher levels of proinflammatory cytokines and lower of anti-inflammatory mediators to support this hypothesis. Moreover we would like to further investigate the connection between E.R. stress and immune function and whether E.R. stress is a mechanism through which PHF8 exerts its immunomodulatory role. We have observed lower levels of CHOP at the endotoxin tolerance state of PHF8 knock down BMDMs, which are additionally characterized by higher cytokine expression compared to controls. E.R. stress could be a mechanism regulated by PHF8 to promote endotoxin tolerance. Further assessment of E.R. stress related genes at the endotoxin tolerance state is needed. Additionally endotoxin tolerance protocol simultaneously with E.R. stress induction in PHF8 knock down BMDMs would help address this question. Finally $PHF8^{-/-}$ mice are available. As a proof of concept we could consider their response to sepsis model compared with C57BL/6 mice, in which PHF8 induction could also be addressed. We would expect worse survival, while deletion of PHF8 may exhibit protective effect in pathogen infection models.

H. References

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