Malonylation of GAPDH is an inflammatory signal in macrophages

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Macrophages undergo metabolic changes during activation that are coupled to functional responses. The gram negative bacterial product lipopolysaccharide (LPS) is especially potent at driving metabolic reprogramming, enhancing glycolysis and altering the Krebs cycle. Here we describe a role for the citrate-derived metabolite malonyl-CoA in the effect of LPS in macrophages. Malonylation of a wide variety of proteins occurs in response to LPS. We focused on one of these, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In resting macrophages, GAPDH binds to and suppresses translation of several inflammatory mRNAs, including that encoding TNFα. Upon LPS stimulation, GAPDH undergoes malonylation on lysine 213, leading to its dissociation from TNFα mRNA, promoting translation. We therefore identify for the first time malonylation as a signal, regulating GAPDH mRNA binding to promote inflammation.
Post-translational modifications (PTMs) are key to expanding the functional diversity of proteins and have an important impact on protein function in health and disease\(^1\). Malonylation is a recently identified, evolutionarily conserved modification\(^2,3\), wherein malonyl-CoA is used as a substrate to add a malonyl group to the amino acid lysine\(^4,5\), changing its charge from +1 to −1. This change is predicted to disrupt electrostatic interactions with other amino acids and alter protein conformation and binding to targets\(^2\). Malonylation remains a poorly understood modification, with very few studies having investigated its functional impact. It has been shown to be present across various metabolic pathways, including fatty acid synthesis and oxidation\(^6,9,10\), mitochondrial respiration\(^1,6\) and glycolysis\(^6,7\), as well as being capable of modifying histones\(^5\). It has also been recently linked to angiogenesis in endothelial cells by modifying mTOR complex 1 (mTORC1) kinase activity\(^9\). However, no further physiological function has yet been attributed to malonylation.

Several studies have recently highlighted the role of metabolic reprogramming in determining the function of immune cells (reviewed in ref. \(^9\)). Metabolites have been a particular focus in this regard. These front line cells of innate immunity, inflammation, and tissue repair\(^11\), display different metabolic features depending on their function. Pro-inflammatory macrophages, such as those activated by lipopolysaccharide (LPS), are highly glycolytic with a disrupted Krebs cycle\(^12,13\). Succinate has been shown to accumulate and drive production of reactive oxygen species, leading to the activation of hypoxia-inducible factor-1α and the induction of target genes, such as that encoding IL-1β\(^14,15\). Another Krebs cycle intermediate, citrate, accumulates, driving the production of inflammatory mediators, such as nitric oxide and prostaglandins\(^16\), as well as the anti-inflammatory metabolite itaconate\(^17\). The role of the malonylation substrate and downstream metabolite of citrate, malonyl-CoA, is yet to be explored in immune cells and inflammation. Malonyl-CoA is synthesised in the cytosol from acetyl-CoA by acetyl-CoA carboxylase (ACC)\(^18\) or in the mitochondria from malonate by ACSF3\(^3\). There are two different ACC isoforms; ACC1 is reported to be responsible for the production of malonyl-CoA in tissues with high levels of lipid synthesis and when knocked out in mice, it is embryonically lethal\(^19\). ACC2 is reported to be mostly expressed in oxidative tissues, where it can inhibit fatty acid oxidation via malonyl-CoA. ACC2 KO mice do not display embryonic lethality, but are resistant to obesity and diet-induced diabetes\(^20\). Interestingly, ACC1 has been recently shown to play a role in the differentiation of human CD4+ T cells into effector cells\(^21\), as well as in the mechanism of defense of Th1 cells against *Mycobacterium tuberculosis* infection\(^22\).

Here, we characterise malonylated proteins in an immune cell population. The malonylation response occurs in bone marrow-derived macrophages (BMDMs) following cell activation and relies on ACC1-dependent malonyl-CoA production. We found that malonylation of the glycolytic enzyme GAPDH in particular, has an impact on pro-inflammatory cytokine production, by modulating both its enzymatic activity and RNA-binding capacity. This novel finding reveals a hitherto unknown mechanism in LPS signalling that regulates the expression of central pro-inflammatory mediators, while further emphasising the importance of metabolic reprogramming in macrophage activation.

**Results**

**MalonylCoA alters cytokine production in macrophages.** To determine the role of malonyl-CoA in macrophages, we first set out to measure its production in LPS-activated BMDMs, and found it to be significantly increased following 24 h of LPS treatment (Fig. 1a). In order to identify the source of malonyl-CoA and be able to manipulate its levels, expression of the three existing malonyl-CoA-synthesising enzymes was analysed. We found the ACC1 isoform to be the highest expressed in BMDMs, followed by ACSF3, while no expression of the ACC2 isoform was detected. (Fig. 1b). We sought to compare our qPCR expression data with the existing RNAseq data from different immune cell types from the ImmGen consortium. The available RNAseq data from macrophages supports our results, with no ACC2 (acacb) expression detected and with ACC1 (acaαa) being the highest expressed enzyme of the three (Supplementary Fig. 1). Furthermore, to our surprise, macrophages appear to have up to 10 times higher ACC1 expression than any other immune cell type (Supplementary Fig. 1a). Similarly, while ACC2 appears to be expressed in bone marrow immune stem cell precursors, it is not expressed in most immune cells, with the exception of some B cell populations, CD8− T cells and FoxP3+ Treg cells (Supplementary Fig. 1b).

We sought to manipulate malonyl-CoA levels in BMDMs by knocking down ACC1 or ACSF3 using two independent siRNAs. ~50% knockdown (KD) of each enzyme was obtained as measured by gene expression and confirmed at the protein level in the case of ACC1 (Fig. 1c, d). ACC1 and ACSF3 KD resulted in a 30–50% reduction in basal malonyl-CoA and a 70% reduction in the LPS-elevated malonyl-CoA (Fig. 1e). Under these conditions, we evaluated cytokine production in LPS-treated BMDMs in order to assess what the functional consequences of reduced malonyl-CoA might be. We found that both ACC1 and ACSF3 KD decreased production of the pro-inflammatory cytokine IL6 (Supplementary Fig. 2a, 2b), whilst simultaneously boosting the production of the anti-inflammatory cytokine IL10 (Supplementary Fig. 2c, d). TNFα production was also reduced but only in the ACC1 KD, and interestingly, the inhibitory effect was only observed at the protein level (Fig. 1f), as TNFα transcription was unaffected (Fig. 1g), both following 6 and 24 h LPS treatment (Supplementary Fig. 2e). Next, to confirm that the distinct effects on TNFα were malonyl-CoA dependent, we sought to increase the metabolite’s levels within the cells by treating them with malonyl-CoA. Treatment of BMDMs with malonyl-CoA resulted in an increase in intracellular malonyl-CoA levels (Supplementary Fig. 2g). Pre-treatment of ACC1 KD BMDMs with malonyl-CoA was able to recover the decrease in TNFα (Fig. 1h), thus confirming that malonyl-CoA can act as a modulator of cytokine production in macrophages.

**Activation of macrophages results in protein malonylation.** Given the increase in malonyl-CoA and the observed functional consequences of inhibiting its production, we next investigated protein malonylation in response to LPS, as this would be a likely outcome of malonyl-CoA accumulation. As shown in Fig. 2a, LPS increased overall lysine malonylation (mal-K) on multiple proteins. Having tested the specificity of the antibody through peptide competition (Supplementary Fig. 3), we proceeded to validate this increase and identify the substrates of the LPS-induced malonylation through mass spectrometry. Malonylated proteins from untreated and LPS-treated macrophages were affinity purified and analysed via LC–MS/MS (Supplementary Fig. 4a, Supplementary Data 1). More than 80% of proteins identified were found to have a maximum of two malonylated sites on each protein (Supplementary Fig. 4b). Label-free quantification\(^23\) was applied to the 412 quantifiable proteins and 843 quantifiable sites were identified on a range of proteins. Two hundred and eighty sites were found to be upregulated by LPS in total. Seventy eight of these were only found in LPS-treated samples, while 202 sites were found to be upregulated by more than 1.5 fold by LPS
Comparison to untreated, 98 of which were found to be statistically significant (Supplementary Data 1, Supplementary Fig. 4b). The majority of LPS-induced malonylated proteins are cytosolic, with 31% nuclear proteins, and only 7% mitochondrial proteins (Supplementary Fig. 5a). Unlike previous studies, where malonylation has mainly been shown to in

LPS-induced malonylation in macrophage activation.

The proteins identified in the analysis was glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This is a critical enzyme in glycolysis but can also bind RNAs directly and regulate translation within the interferon-γ-activated inhibitor of translation (GAIT) complex. Given the role of glycolysis in LPS action in macrophages, as well as its RNA-binding capacity, we chose GAPDH as a good candidate to investigate the role of LPS-induced malonylation in macrophage activation.

**LPS induces malonylation of GAPDH at lysine 213.** We immunoprecipitated GAPDH from BMDM lysates and showed that in agreement with the mass spectrometry results, LPS strongly increased its malonylation by western blotting with an anti-mal-K antibody (Fig. 2c, lower panel, compare lane 5 to lanes 6–8). GAPDH LPS-induced malonylation was observed no earlier...
than 6 h and seemed to gradually increase, with a much more noticeable increase at 24 h. Furthermore, we also used the malonylation chemical probe, MalAMyne (Fig. 2d), and methodology by X. Bao et al. 27, to label malonlated lysines in untreated and LPS-treated BMDMs. This additional method further confirmed that LPS strongly induced malonylation of GAPDH (Fig. 2e, compare lane 4 to lane 3). In addition, we found that GAPDH in vitro is highly sensitive to malonylation by low concentrations of malonyl-CoA. K-malonylation was assessed by western blotting. All data shown are representative of three independent experiments.

Seven malonylated sites had been originally identified on GAPDH (Supplementary Data 1). We next sought to determine which of these would be the most abundant and physiologically relevant by immunoprecipitating endogenous GAPDH from BMDMs and samples probed with an anti-mal-K antibody (lower panel). GAPDH expression in the immunoprecipitated (upper panel) samples was also examined. d Chemical formula of the MalAMyne probe. e Untreated and LPS-treated (100 ng/mL, 24 h) BMDMs were labelled with MalAMyne (10 μM) or vehicle control. Copper-catalysed click chemistry was performed on the lysates using biotin, followed by immunoprecipitation using streptavidin (strept.). Samples were probed for GAPDH via western blotting. f Purified GAPDH (100 μg/mL) was incubated in the presence of TCEP and malonyl-CoA (or buffer as control) for 1 h at 37 °C, pH 7.5. K-malonylation was assessed by western blotting. g Identification of malonylated sites in immunoprecipitated trypsin-digested GAPDH peptides from untreated and LPS-treated BMDMs (10^6 cells). Peptides were analysed via LC-MS. h Purified GAPDH was preincubated with TCEP and 25 mM iodoacetamide, 80 mM methyl methanethiosulfonate (MMTS), 5 μM heptelidic acid (HA) or buffer for 30 min at 37 °C, followed by 500 μM malonyl-CoA. K-malonylation was assessed by western blotting. All data shown are representative of three independent experiments.

Fig. 2 Activation of macrophages with LPS increases protein malonylation, with GAPDH as a substrate. a Western blot analysis of lysine malonylation (mal-K) in lysates from BMDMs treated with LPS (100 ng/mL) for 24 h. b Most enriched functions associated with LPS-induced malonlated proteins (FDR < 0.05). c Immunoprecipitated GAPDH from untreated and LPS-treated (100 ng/mL, 6, 12 and 24 h) BMDMs and samples probed with an anti-β-actin antibody (lower panel). GAPDH expression in the immunoprecipitated (upper panel) samples was also examined. d Chemical formula of the MalAMyne probe. 

Western blot analysis of lysine malonylation

| Lanes | IgG | GAPDH |
|-------|-----|-------|
| 1     |     |       |
| 2     |     |       |
| 3     |     |       |
| 4     |     |       |
| 5     |     |       |
| 6     |     |       |
| 7     |     |       |
| 8     |     |       |

IP: GAPDH

LPS: 0 h 6 h 12 h 24 h 37 kDa

IB: GAPDH

IB: Mal-K

Fig. 2a shows the Western blot analysis of lysine malonylation (mal-K) in lysates from BMDMs treated with LPS (100 ng/mL) for 24 h. Fig. 2b shows the most enriched functions associated with LPS-induced malonlated proteins (FDR < 0.05). Fig. 2c shows the immunoprecipitated GAPDH from untreated and LPS-treated (100 ng/mL, 6, 12 and 24 h) BMDMs and samples probed with an anti-β-actin antibody (lower panel). GAPDH expression in the immunoprecipitated (upper panel) samples was also examined. Fig. 2d shows the chemical formula of the MalAMyne probe. Fig. 2e shows the Western blot analysis of lysine malonylation.
rapid GAPDH in vitro malonylation could be prevented by the alkylating agents iodoacetamide and methylmethanethiosulfonate, and more importantly, by the GAPDH inhibitor heptelidic acid (HA), which selectively alkylates the active site cysteine of GAPDH\(^{15}\) (Fig. 2h). These findings suggest that K213 is particularly sensitive to malonylation in response to the elevation of cytosolic malonyl-CoA upon LPS treatment, and might indicate that GAPDH can perhaps catalyse its own malonylation under certain conditions.

**GAPDH is needed for cytokine production in macrophages.** We next explored the role of GAPDH malonylation on K213 during LPS activation in detail, first exploring the effect of malonylation on GAPDH activity. Consistent with previous reports showing that pro-inflammatory macrophages have increased glycolytic flux\(^{12,36}\), the activity of GAPDH in LPS-treated macrophages was increased after 24 h (Fig. 3a). No changes in protein expression were detected (Supplementary Fig. 8a), however, we had previously shown malonylation of GAPDH to be highest at this time. Furthermore, inhibiting GAPDH enzymatic activity with HA\(^{37}\) (Supplementary Fig. 8b), reduced transcription of the pro-inflammatory cytokine pro-IL\(1\beta\), to the same extent as another glycolytic inhibitor, 2-deoxyglucose (2-DG), as shown previously\(^{14}\) (Supplementary Fig. 8c). The induction of pro-IL\(1\beta\) protein was also inhibited by HA (Supplementary Fig. 8d). IL6 mRNA and protein (Supplementary Fig. 8e, f) were also inhibited by HA. Intriguingly, a different result was obtained with TNF\(\alpha\). A clear inhibitory effect was observed at the protein level with HA but not 2-DG (Fig. 3b). HA did not inhibit the induction of TNF\(\alpha\) mRNA however (Fig. 3c), while 2DG boosted TNF\(\alpha\) mRNA production. We further explored the role of GAPDH in cytokine production using siRNA to KD GAPDH in BMDMs (Fig. 3d, Supplementary Fig. 9a). Consistent with the inhibitory effect of HA, KD of GAPDH with two independent siRNAs resulted in a decrease in pro-IL\(1\beta\) (Fig. 3d, Supplementary Fig. 9b) and IL6 production (Supplementary Fig. 9c, d). GAPDH KD had however, the opposite effect on TNF\(\alpha\), the production of which was boosted (Fig. 3e), while transcript levels remained unchanged (Fig. 3f). These findings confirm a glycolysis-dependent regulation of pro-IL\(1\beta\) and IL6, as shown by both glycolytic inhibitors and GAPDH KD having the same effect. On the other hand, a glycolysis-independent, post-transcriptional mechanism of regulation of TNF\(\alpha\) production mediated by GAPDH is indicated by the lack of effect of 2DG on TNF\(\alpha\). The inhibitory effect of HA on TNF\(\alpha\) protein production was intriguing, indicating that targeting of GAPDH by HA would perhaps boost mRNA binding by GAPDH, thereby repressing its post-transcriptional processing. A possible mechanism is HA preventing GAPDH malonylation, implying that malonylation could be a signal to dissociate GAPDH from TNF\(\alpha\) mRNA, allowing for its post-transcriptional processing. The differing effects of 2DG, HA and GAPDH KD are depicted in Supplementary Fig. 9e. By attenuating glycolysis, induction of IL\(1\beta\) is blocked, as expected. Glycolysis per se has no role in TNF\(\alpha\) production, however, GAPDH represses TNF\(\alpha\), possibly because of RNA binding blocking translation. LPS relieves this repression by causing the dissociation of GAPDH from the mRNA, which requires GAPDH malonylation.

**GAPDH regulates TNF\(\alpha\) production via RNA-binding.** To test the possibility that GAPDH may be post-transcriptionally...
regulating TNFα production directly, we turned our attention to RNA-binding by GAPDH. GAPDH has been previously reported to bind mRNA transcripts containing AU-rich elements, which are present in TNFα, as well as IFNγ mRNAs. It has also been shown to be part of the GAIT complex, which mediates translational repression of GAIT element-containing transcripts.

We first immunoprecipitated GAPDH from BMDMs and examined bound RNAs by qPCR. GAPDH in resting macrophages was found to bind the RNA of TNFα (Fig. 4a). Following 6 h activation with LPS, there was a significant reduction in binding to TNFα RNA, and following 24 h, GAPDH binding could no longer be detected (Fig. 4a). Similarly, in resting macrophages GAPDH bound to mRNA for the GAIT-element-containing death-associated protein kinase-1 (DAPK1) and dissociated following LPS treatment (Supplementary Fig. 10a). Enhanced translation appeared likely to be a key mechanism for TNFα and DAPK1 production, since there was a clear discrepancy between mRNA and protein induction for both genes. Both TNFα and DAPK1 mRNA levels were low when protein was high (Fig. 4b, Supplementary Fig. 10b). The 3′-untranslated region (UTR) of both genes was also repressive as they both inhibited reporter expression from luciferase vectors expressing DAPK1 and TNFα 3′-UTRs (Supplementary Fig. 10c). To validate that BMDMs are actively translating and secreting TNFα, we next sought to determine whether malonylation of GAPDH might be involved in GAPDH dissociation from target mRNAs. GAPDH protein expression is not affected by LPS treatment (Fig. 4d), suggesting that PTMs might indeed be the mechanism by which LPS could alter GAPDH activity. Treatment of BMDMs with HA prior to LPS, which would prevent GAPDH malonylation, resulted in a profound increase in binding of GAPDH to TNFα (Fig. 4e) and DAPK1 mRNA (Supplementary Fig. 12b), as shown by GAPDH RNA-immunoprecipitation. These results therefore might explain why HA can block TNFα production, as it will maintain GAPDH in its unmalonylated state on the TNFα 3′-UTR and suppress its translation.

Malonylation of GAPDH controls the enzyme’s activities. Having identified GAPDH as a two-way regulator of cytokine production, as it will maintain GAPDH in its unmalonylated state on the TNFα 3′-UTR and suppress its translation.
production in activated macrophages, we next set out to identify the mechanism behind GAPDH activities. Our data in Fig. 1e supported a role for GAPDH malonylation in TNFα production, since reduced malonyl-CoA levels in ACC1 KD cells also resulted in reduced TNFα production in response to LPS. GAPDH expression was unaffected in ACC1 KD and ACSF3 KD BMDMs (Supplementary Fig. 13a, b), however the activity of GAPDH was reduced in LPS-treated ACC1 KD cells (Fig. 5a). This implied that malonylation of GAPDH frees the enzyme from bound mRNA enabling it to enhance glycolytic flux. The same was not the case in ACSF3 KD, where activity of GAPDH was unaffected (Supplementary Fig. 13c). ACSF3-derived malonyl-CoA and subsequent malonylation are confined to the mitochondria4, while GAPDH is present in the cytosol, thus explaining the observed differences. Consistent with our results demonstrating that LPS both increased GAPDH enzymatic activity while decreasing GAPDH RNA-binding, these activities have been previously demonstrated to be mutually exclusive38,39. The evidence therefore indicates that ACC1-derived malonyl-CoA is needed for GAPDH malonylation, which in turn is needed for

**Fig. 5** K213 malonylation regulates GAPDH enzymatic activity and RNA-binding. a GAPDH enzymatic activity measured in lysates from ACC1 and ACSF3 KD BMDMs (LPS treated, 100 ng/mL, 24 h; mean + SD). b WT, K213Q and K213E GAPDH mutants were overexpressed in HEK293T, affinity purified, and the enzymatic activity measured (mean + SEM, n = 3). c GAPDH was knockdown using siRNA (48 h, 10 nM) followed by overexpression of WT, K213Q and K213E GAPDH mutants and expression assessed via western blotting (a, overexpressed GAPDH; b, endogenous GAPDH). d GAPDH mutants were immunoprecipitated following 48 h overexpression and fixing in HEK293T cells. Bound TNFα mRNA in the IP relative to IgG was assessed via qPCR. Mean ± 5D, representative of three independent experiments. Unpaired t-test, *p < 0.05; **p < 0.01. e In resting macrophages, GAPDH binds to the 3′-UTR of various pro-inflammatory mediators, such as TNFα, and prevents their translation. Following activation of macrophages with LPS, there is an accumulation of citrate that can be converted into malonyl-CoA in an ACC1-dependent manner. Malonyl-CoA can then in turn mediate an increase in lysine malonylation. One of the substrates of this modification is GAPDH, which after undergoing malonylation, releases the bound RNAs which can now be translated. At the same time, GAPDH enzymatic activity increases, which allows for an increased glycolytic flux, required for the activated macrophage to carry out its pro-inflammatory functions.
TNFα production via dissociation from its 3'-UTR, as well as for its increased enzymatic activity.

To address the role of GAPDH K213 malonylation specifically in these two activities, a K213 glutamate mutant (K213Q) was generated. K213Q acts both as an acetylation mimic and as a control, being unable to undergo malonylation. We also generated a K213 glutamate (K213E) mutant, to act as a malonylation control, being unable to undergo malonylation. We also generated in these two activities, a K213 glutamine mutant (K213Q) was important, while ACC2 is associated with tissues with pre- alcoholic changes and PTMs remains a very poorly understood function in recent years, and yet the connection between metabolic and function across cellular systems, and despite their different functional impacts, the great majority of them share their origins in metabolic intermediates. Metabolic reprogramming in immune cells has repeatedly been shown to be key for immune function in recent years, and yet the connection between metabolic changes and PTMs remains a very poorly understood area. Our study reports the role of the post-translational modification malonylation in macrophage function. We have identified multiple proteins with a wide range of functions ranging from metabolism, to cell death, to immune responses, as undergoing malonylation following activation of cells with LPS. This effect is dependent on malonyl-CoA production by one of the two ACC isoforms, ACC1, which is highly expressed in macrophages. We propose a model whereby the previously reported accumulation of citrate in activated macrophages exits the mitochondria and following conversion into acetyl-CoA, it is used to generate malonyl-CoA by ACC1 in the cytosol which in turn, can act as a substrate for malonylation of multiple substrates. LPS does not have any effect on ACC1 expression levels, indicating that the increase in malonyl-CoA production observed is likely the result of LPS modulating the enzyme's activity. Citrate has been shown to be able to allosterically regulate ACC activity directly, so the reported accumulation of citrate in activated macrophages might be the mechanism for the observed effects. Interestingly, we find no expression at all of the second ACC isoform, ACC2, an observation which is based on the currently available RNAseq data, and might not be restricted to just macrophages but to most immune cells. ACC1 is generally found in tissues where fatty acid synthesis is important, while ACC2 is associated with tissues with predominant oxidative metabolism. Their expression pattern thus fits with the existing literature showing fatty acid synthesis is upregulated and needed for function in macrophages, den- dritic cells, T cells, and macrophages, which is a hallmark of activated pro-inflammatory dendritic cells and macrophages, which might explain the absence in ACC2 expression.

While we have presented ACC1 as the source of malonyl-CoA for the malonylation reaction, the precise molecular details of the modification remain to be elucidated. The only known regulator of malonylation is sirtuin 5, which can act not only as a demalonylase, but also remove similar acylations, such as succinylation and glutarylation. It is usually the case that deacetylases and acyl-transferases, as with sirtuin 5, can catalyse the removal of malonylation following activation of cells with LPS. This effect is dependent on malonyl-CoA production by one of the LPS-induced malonylated substrates in macrophages, enabling the production of pro-inflammatory cytokines in response to LPS. GAPDH is needed for IL1β production because of the role of glycolysis in the process, and it must also dissociate from the TNFα mRNA in order for TNFα translation to occur. We therefore report a role for GAPDH malonylation on K213 in the translation of LPS-induced genes, notably that encoding TNFα. This scenario is depicted in Fig. 5e.

Discussion

PTMs play an essential role in the regulation of protein activity and function across cellular systems, and despite their different functional impacts, the great majority of them share their origins in metabolic intermediates. Metabolic reprogramming in immune cells has repeatedly been shown to be key for immune function in recent years, and yet the connection between metabolic changes and PTMs remains a very poorly understood area. Our study reports the role of the post-translational modification malonylation in macrophage function. We have identified multiple proteins with a wide range of functions ranging from metabolism, to cell death, to immune responses, as undergoing malonylation following activation of cells with LPS. This effect is dependent on malonyl-CoA production by one of the two ACC isoforms, ACC1, which is highly expressed in macrophages. We propose a model whereby the previously reported accumulation of citrate in activated macrophages exits the mitochondria and following conversion into acetyl-CoA, it is used to generate malonyl-CoA by ACC1 in the cytosol which in turn, can act as a substrate for malonylation of multiple substrates. LPS does not have any effect on ACC1 expression levels, indicating that the increase in malonyl-CoA production observed is likely the result of LPS modulating the enzyme's activity. Citrate has been shown to be able to allosterically regulate ACC activity directly, so the reported accumulation of citrate in activated macrophages might be the mechanism for the observed effects. Interestingly, we find no expression at all of the second ACC isoform, ACC2, an observation which is based on the currently available RNAseq data, and might not be restricted to just macrophages but to most immune cells. ACC1 is generally found in tissues where fatty acid synthesis is important, while ACC2 is associated with tissues with predominant oxidative metabolism. Their expression pattern thus fits with the existing literature showing fatty acid synthesis is...
hypermalonylated, which includes increased malonylation of K213. Targeting GAPDH through HA has also been shown to be beneficial in mouse models of breast cancer. Furthermore, a recent study indicating that dimethylfumarate targets the active site cysteine in GAPDH and thereby elicits its effects as therapy in multiple sclerosis, further emphasises the importance of GAPDH for inflammation. Further characterisation of this post-translational modification is likely to advance our understanding of underlying processes in infection and inflammation, and potentially indicate new therapeutic strategies to limit inflammation in disease.

**Methods**

**Reagents.** LPS was from Alexa, A/G plus agarse beads, streptavidin beads and biotin azide were obtained from Thermo Fisher. Sequencing grade modified trypsin was purchased from Promega. Heptelicidic acid was bought from Abcam. Lipo- fectamine RNAiMax and Lipofectamine 2000 transfection reagents were obtained from Invitrogen. Malonyl-CoA, 2-deoxyglucose, anti-FLAG M2 antibody and 50 μL of A/G beads were added overnight at 4 °C. Lysates were centrifuged for 3 min at 4 °C, the liquid was removed, and the beads were washed three times with low stringency buffer. Immune complexes were eluted by adding Protein samples from cultured cells were prepared by direct lysis of cells in 5X –PAGE and transferred to nitrocellulose or PVDF membranes via wet or iBlot (Invitrogen) transfer. Membranes were probed with the respective antibodies and visualised using LumiGLO enhanced chemiluminescent (ECL) substrate (Cell Signalling) or the Odyssey system. All primary antibodies were used in a 1:10,000 dilution. Secondary anti-mouse, anti-rabbit and anti-goat secondary antibodies were purchased from Jackson Immunoresearch. Odyssey anti-mouse, anti-rabbit and anti-goat secondary antibodies were obtained from LiCOR Biosciences. HEK293T cell line was obtained from ATCC. All other reagents, unless otherwise specified, were purchased from Thermo Fisher. Sequencing grade modifications were performed using the Maxquant LFQ algorithm, by comparing the abundance of the same peptides across runs, with both ion intensities and spectral counts used for this purpose.

**GAPDH PTMs mass spectrometry.** For the identification of PTMs present in GAPDH, GAPDH was immunoprecipitated from 1 mg of BMDM lysates as previously described, and IP samples were separated on 4–12% SDS–PAGE gels, Coomassie stained, and bands of interest, together with their respective control bands, excised from the gel. Gel pieces were trypsin-digested and peptides dissolved in 1% TFA and injected onto a C18 spray tip and analysed by Q Exactive-Orbitrap mass spectrometer. The raw data was searched using Maxquant. Trypsin was specified as the cleavage enzyme allowing up to four missed cleavages. Main search range was set to 5 ppm and 0.02 Da for fragment ions. Carbamidomethyl on Lys was specified as fixed modification. Lys and malonyllysine on Met were specified as variable modifications. Label-free quantification was performed using the Maxquant LFCQ algorithm, by comparing the abundance of the same peptides across runs, with both ion intensities and spectral counts used for this purpose.

**Mass spectrometry analysis.** Mass spectrometry analysis was performed blindly by PTM Biolabs.

**ELISAs and MSDs.** Cell culture supernatants were assayed for TNFα, IL6 and IL10 by ELISA (R&D) or by multiplex Mesoscale Discovery (MSD). MalonylCoA from lysates was assayed by ELISA (Cusabio).

**RNA extraction, reverse-transcriptase PCR and qPCR.** RNA was extracted using an RNaseasy kit (Qiagen) and 250 ng of total RNA was reverse-transcribed into cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems). For qPCR, Taqman gene expression primers were used (Applied Biosystems), with expression of the target gene normalised to the geometric mean of the expression of β-actin, 18S and GAPDH.

**siRNAs, mutagenesis and plasmids.** GAPDH siRNAs (sc24341 and s103461), ACC1 siRNAs (hs9880 and s98862) and ACSF3 siRNAs (s110944 and s11095) together with a silencer® select negative control (4390843), were obtained from Ambion. They were transfected into BMDMs seeded at 0.5 × 10⁶ cells/mL at a concentration of 10 nM using Lipofectamine RNAiMax.

The following stealth siRNA duplex was used to KD human GAPDH: Sense: CAUGAUCAACUCAAUAAAGUCUG Antisense: GAGGGUGUUAAUGUGGUAACUG

The following primers were used for mutagenesis reactions using the Quickchange II site-directed mutagenesis kit (Agilent Technologies):

GAPDH K213Q F: CTTCTTGGTGCCCGGGGCTGTTGC CTTTGGTGCCCGGGGCTGTTGC

The myc-DKK-GAPDH plasmid from Origene Technologies (Rockville, MD) was transfected into BMDMs seeded at 0.5 × 10⁶ cells/mL at a concentration of 10 nM using Lipofectamine RNAiMax.
(1 μg/mL, Promega) was added to 20 μL of lysate and read immediately on a luminometre to measure the renilla luciferase activity. Forty microtubes of 1X luciferase mix (20 μL: Triton: 2.67 μM MgSO₄·7H₂O: 0.1 μM EDTA: 33 mM DTT; 530 μM ATP; 270 μM acetyl CoA; 470 μM d-Luciferin; 5 mM NaOH; 267 μM magnesium carbonate hydroxide) was added to 20 μL of lysate and the plate read immediately to measure the firefly luciferase activity.

RNA-IP. BMDMs (10–20 × 10⁶ cells/condition) were fixed with 1% formaldehyde and then neutralized with 1 M glycine. Cells were washed with PBS and lysed in polysome lysis buffer (0.1 M Hepes, 0.1 M KCl, 5 mM MgCl₂, 0.5% NP40, 1 mM DTT, 1X Protease Inhibitors). Five micrograms of GAPDH antibody or IgG control were pre-fixed to 50 μL of A/G beads and added to lysates. The samples were left rotating at 4 °C for 6 h. The beads were then centrifuged and washed with 1 M tris (HCl pH 8, 0.01 M EDTA, 1% SDS) and samples incubated at 37 °C twice. The eluates were then removed from the beads and 3 μL 5 M NaCl and 10 μg Proteinase K added. Samples were then incubated at 42 °C for 45 min to digest cross-linked peptides and then incubated for 1 h at 65 °C to reverse formaldehyde cross-links. The RNA was then extracted using phenol:chlorform extraction.

GAPDH affinity purification and enzyme assay. The chromatography columns were first rinsed with TBS (50 mM Tris–HCl, 150 mM NaCl, pH 7.4) twice, and the columns were then packed with 1 mL ANTI FLAG M2 affinity gel. The column was washed with 1 mL 0.1 M glycine HCl pH 3.5, three times, and then washed with 1 mL TBS five times.

A total of 10⁶ HEK293T cells overexpressing FLAG-GAPDH were lysed using 1 mL lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton) and 1X protease inhibitor. Lysates were run through the column up to five times. The column was then washed 15 times with TBS. FLAG-GAPDH was eluted off the column by adding 5 mL of a 100 μg/mL 1X FLAG peptide solution. GAPDH enzymatic activity was assayed using a colorimetric GAPDH activity assay (Abcam).

Polysemic profiling. Prior to lysis, cells were treated with cycloheximide (100 mg/mL) 10 min at 37 °C 5% CO₂. Cells were washed three times with ice cold PBS and lysed in ice cold buffer A (0.5% NP40, 20 mM Tris–HCl pH 7.5, 100 mM KCl) and 10 mM MgCl₂. Lysates were passed through three times a 23 g needle and incubated on ice for 7 min. Extracts were then centrifuged at 10 k rpm for 7 min at 4 °C. The supernatant was collected as crude cytosolic extract. Cytosolic extracts were overlaid on 10–50% sucrose gradients prepared in 20 mM Tris–HCl pH 7.5, 100 mM KCl and 10 mM MgCl₂ buffer (prepared using the Gradient Station, Bioequip Instruments). Gradients were then ultracentrifuged at 40 k rpm for 1 h 20 min at 4 °C using an SW41 in a Beckman ultracentrifuge. Individual polyribosome fractions were subsequently purified using a Gradient Station (Bioequip Instruments). Total cellular RNA from BMDM cell lines or tissues was isolated using the Direct-zol™ RNA Miniprep Kit (Zymo Research) according to manufacturer’s instructions. RNA was quantified and controlled for purity with a nanodrop spectrometer. (Thermo Fisher). For RT-qPCR, 500–1000 ng were reversely transcribed (Script Reverse Transcription Supermix, Biorad) followed by RT-PCR (iQ SYBRgreen Supermix, Biorad) using the cycling conditions as follows: 50 °C 2 min, 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 45 s. The melting curve was graphically analysed to control for nonspecific amplification reactions. Quantitative RT-PCR analysis was performed with the following primers listed below:

Neat1 f: TTTGAGCAGTGCGTGGTG
Neat1 r: TCAATGTCAGCCAGACAGCA
Gadph f: CCAATGGTCGGCTGGTATC
Gadph r: GTGATGATTCCAGGACACAA
Tfna f: CAGTCTATGGCAGACCT
Tfna r: CGGACTCCGGAATCTAAG

Statistical analysis. Statistical tests were performed using GraphPad prism. A value of p < 0.05 was considered statistically significant.

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Any further data not included in the manuscript is available from the corresponding author on reasonable request.

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33. Kaczmarkska, Z. et al. Structure of p300 in complex with acyl-CoA variants. Nat. Chem. Biol. 13, 21–29 (2017).
34. James, A. M. et al. Non-enzymatic N-acetylation of lysine residues by acetylCoA often occurs via a proximal S-acetylated thiol intermediate sensitive to glyoxalase II. Cell Rep. 18, 2105–2112 (2017).
35. Kato, M., Sakai, K. & Endo, A. K oncic acid (heptelidic acid) inhibition of glyceraldehyde-3-phosphate dehydrogenases from various sources. Biochem. Ecol. 11, 130–138 (1992).
36. Hard, G. C. Some biochemical aspects of the immune macrophage. Br. J. Exp. Pathol. 51, 97–105 (1970).
37. Endo, A., Hasumi, K., Sakai, K. & Kanbe, T. Specific inhibition of glyceraldehyde-3-phosphate dehydrogenase by koningic acid (heptelidic acid). J. Antibiot. 38, 920–925 (1985).
38. Neri, F. & Rigby, W. F. Glyceraldehyde-3-phosphate dehydrogenase selectively binds AU-rich RNA in the NAD(+)-binding region (Rommel fold). J. Biol. Chem. 270, 2755–2763 (1995).
39. Nagy, E. et al. Identification of the NAD(+)-binding fold of glyceraldehyde-3-phosphate dehydrogenase as a novel RNA-binding domain. Biochem. Biophys. Res. Commun. 275, 253–260 (2000).
40. White, M. R. et al. A dimer interface mutation in glyceraldehyde-3-phosphate dehydrogenase regulates its binding to AU-rich RNA. J. Biol. Chem. 290, 1770–1785 (2015).
41. Millot, P., Vachharajani, V., McPhail, L., Yooa, B. & McCalla, C. E. GAPDH binding to TNF-alpha mRNA contributes to posttranscriptional repression in monocytes: a novel mechanism of communication between inflammation and metabolism. J. Immunol. 196, 2541–2551 (2016).
42. Chang, C. H. et al. Posttranscriptional control of T cell effector function by aerobic glycolysis. Cell 153, 1239–1251 (2013).
43. Donald, B. & Martin, P. R. V. The mechanism of tricarboxylic acid cycle regulation of fatty acid synthesis. J. Biol. Chem. 237, 1787–1792 (1962).
44. Black, R. T. et al. Regulation of fatty acid synthesis is a key requirement for phagocytic differentiation of human monocytes. Proc. Natl Acad. Sci. USA 107, 7817–7822 (2010).
45. Carroll, R. G. et al. An unexpected link between fatty acid synthase and cholesterol synthesis in proinflammatory macrophage activation. J. Biol. Chem. 293, 5509–5521 (2018).
46. Everts, B. et al. TLR-driven early glycolytic reprogramming via the kinases TBK1-IKKvarepsilon supports the anabolic demands of dendritic cell activation. Nat. Immunol. 15, 323–334 (2014).
47. Lee, J. et al. Regulator of fatty acid metabolism, acetyl coenzyme a carboxylase 1, controls T cell immunity. J. Immunol. 192, 3190–3199 (2014).
48. Berod, L. et al. De novo fatty acid synthesis controls the fate between regulatory T and T helper 17 cells. Nat. Med. 20, 1327–1333 (2014).
49. Everts, B. et al. Commitment to glycolysis sustains survival of NO-producing inflammatory dendritic cells. Blood 120, 1422–1431 (2012).
50. Munder, M., Eichmann, K. & Modolell, M. Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by CD4+ T cells correlates with Th1/Th2 phenotype. J. Immunol. 160, 5347–5354 (1998).
51. Tan, M. et al. Lysine glutarylation is a protein posttranslational modification regulated by SIRT5. Cell. Metab. 19, 605–614 (2014).
52. Bonafe, N. et al. Glyceraldehyde-3-phosphate dehydrogenase binds to the AU-Rich 3 untranslated region of colony-stimulating factor-1 (CSF-1) messenger RNA in human ovarian cancer cells: possible role in CSF-1 posttranscriptional regulation and tumor phenotype. Cancer Res. 65, 3762–3771 (2005).
53. Qian, L. et al. Global profiling of protein lysine malonylation in Escherichia coli reveals its role in energy metabolism. J. Proteome Res. 15, 2060–2071 (2016).
54. Takaoka, Y. et al. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) prevents lipopolysaccharide (LPS)-induced, sepsis-related severe acute lung injury in mice. Sci. Rep. 4, 5204 (2014).