Choline transport links macrophage phospholipid metabolism and inflammation

Shayne A. Snider*1,3, Kaitlyn D. Margison*1,3, Peyman Ghorbani1,3, Nicholas D. LeBlond1,3, Conor O’Dwyer1,3, Julia R.C. Nunes1,3, Thao Nguyen1,2,3, Hongbin Xu1,2,3, Steffany A.L. Bennett1,2,3, Morgan D. Fullerton1,3

From the 1University of Ottawa Centre for Infection, Immunity and Inflammation and Centre for Catalysis Research and Innovation; 2Ottawa Institute of Systems Biology and University of Ottawa Brain and Mind Institute; 3Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, Ottawa, ON, Canada

Running title: Macrophage inflammation and choline metabolism

To whom correspondences should be addressed: Dr. Morgan Fullerton, Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, 4109A Roger Guindon Hall, 451 Smyth Rd, Ottawa, Ontario, Canada, K1H 8M5, Telephone (613) 562-5800 x8310; E-mail: morgan.fullerton@uottawa.ca

*These authors contributed equally to this work.

Keywords: Choline, CTL1/Slc44a1, macrophage, inflammation, phosphatidylcholine, phospholipid, choline transport, de novo lipogenesis

ABSTRACT

Choline is an essential nutrient that is required for synthesis of the main eukaryote phospholipid, phosphatidylcholine (PC). Macrophages are innate immune cells that survey and respond to danger and damage signals. Although it is well known that energy metabolism can dictate macrophage function, little is known as to the importance of choline homeostasis in macrophage biology. We hypothesized that the uptake and metabolism of choline is important for macrophage inflammation. Polarization of primary bone marrow macrophages with LPS resulted in an increased rate of choline uptake and higher levels of PC synthesis. This was attributed to a substantial increase in the transcript and protein expression of the choline transporter-like protein-1 (CTL1) in polarized cells. We next sought to determine the importance of choline uptake and CTL1 for macrophage immune responsiveness. Chronic pharmacological or CTL1 antibody-mediated inhibition of choline uptake resulted in altered cytokine secretion in response to LPS, which was associated with increased the levels of diacylglycerol (DAG) and activation of protein kinase C (PKC). These experiments establish a previously unappreciated link between choline phospholipid metabolism and macrophage immune responsiveness, highlighting a critical and regulatory role for macrophage choline uptake via the CTL1 transporter.

Macrophages represent a diverse and plastic subset of phagocytic innate immune cells that are critically important for immune surveillance and tissue homeostasis. In vivo, macrophage phenotypes are almost exclusively driven by external stimuli that
dictate the activation of necessary cellular programs (1,2). In the context of infection or pro-inflammatory stimuli, macrophages evoke a wave of cellular responses including the production and secretion of cytokines, as well as membrane biogenesis and phagocytosis. These processes are intricately linked to phospholipid homeostasis; however, there are few papers linking phospholipid metabolism and macrophage biology.

Choline is a quaternary amine and essential nutrient that participates in acetylcholine synthesis (cholinergic neurons) and one-carbon metabolism (primarily thought to be in the liver and kidney). However, in eukaryotic non-neuronal cells, choline is predominantly used for the synthesis of phosphatidylcholine (PC), the major outer-leaflet phospholipid. Once inside the cell, choline is shuttled along the Kennedy pathway (3) and combined with diacylglycerol (DAG) to form PC at the ER membrane. Moreover, PC can also be degraded by numerous phospholipases to yield lipid intermediates that can then be recycled or further processed. Although under most conditions the CTP:phosphocholine cytidylyltransferase (CCT) or DAG availability have been shown to control flux through the Kennedy pathway (4), recent studies have begun to shed light on the potential regulation of this pathway by choline transporters (5-7).

Due to its positive charge, specialized transporters facilitate cellular choline uptake. High-affinity transport exists in cholinergic neurons (via CHT1/Slc5a7) (8,9), while the organic cation transporter family (OCT/Slc22a1-20) are generic transporters of heavy metals and organic cations that have a low rate and specificity for transporting choline (10,11). Another family of choline transporters has been identified and named the choline transporter-like proteins (CTL1-5). CTL1 is ubiquitously expressed and thought to be primarily responsible for mediating choline uptake in non-neuronal cells (5,6,12-16). Although few studies have investigated choline metabolism in immune cells, early experiments revealed that macrophage stimulation with the bacterial endotoxin lipopolysaccharide (LPS) acutely increased PC synthesis (17,18). When PC synthesis was compromised by myeloid-specific deletion of CCTα (the rate-limiting enzyme in PC synthesis), cells had a reduced capacity to secrete pro-inflammatory cytokines in response to LPS (19). These data suggest that in response to an inflammatory insult, choline availability and PC synthesis may be important.

In the present study we sought to investigate how macrophage LPS-polarization affects choline uptake and subsequent metabolism. We observed an increase in PC synthesis in response to LPS due to an up-regulation in the transcription of the CTL1 gene (Slc44a1), which facilitates an increased rate of uptake and synthesis of PC. Conversely, we also sought to ask the reciprocal question of how limiting the availability of choline might affect acute response to LPS stimulation. Pharmacological and antibody-mediated inhibition of CTL1 limited choline uptake and diminished PC content, which altered cytokine secretion. Interestingly, we found that inhibition of PC synthesis resulted in an increase in DAG accumulation and subsequent protein kinase C (PKC) activation. Restoring PC levels and inhibiting PKC activity were important for regulating the pro-inflammatory responses, linking macrophage phospholipid metabolism and inflammation.

RESULTS

Macrophage LPS stimulation increases choline uptake and alters PC homeostasis-LPS is known to increase choline incorporation into PC (17,18); however, the role of longterm exposure to LPS on choline uptake and metabolism remains unclear. Macrophages were treated for 48 hours with
LPS and the transcript expression of pro-inflammatory (Tnfa, Il1b, Il6, the ratio of iNos/Arg1) or anti-inflammatory (Tgfb1, Mrc1) markers were measured to confirm polarization (SI-Figure 1). The rate of choline uptake was markedly increased in M[LPS] compared to control M[0] (Figure 1A). Moreover, LPS-polarization increased the maximal transport of choline from 418 ± 49 to 821 ± 62 pmol/mg/min, where the apparent affinity remained unchanged (68.62 ± 20.07 to 72.66 ± 13.42 μM) (Figure 1B).

Following uptake in non-hepatic or renal tissue, choline is rapidly phosphorylated and shuttled along the CDP-choline pathway to produce PC. In keeping with an increased rate of uptake, the incorporation of $^3$H-choline into PC was higher in LPS-stimulated macrophages (Figure 1C). It has been shown previously that arachidonic acid (AA) liberated from PC via the actions of phospholipase A2 can have subsequent roles in inflammatory signaling via conversion to prostaglandin and lipid mediators following LPS stimulation (17,20,21). We reasoned that perhaps LPS-stimulation of PC synthesis might be accompanied by a coordinated increase in overall PC turnover to facilitate AA metabolism. However, pulse-chase experiments revealed no difference in the rate of PC degradation between M[LPS] and M[0] cells (SI-Figure 2A). There was also no difference in the sensitivity to the choline uptake inhibitor hemicholinium-3 (HC3) between naïve and LPS-treated cells (SI-Figure 2B). These data indicate that when chronically polarized with LPS, choline transport and subsequent incorporation into PC is up-regulated.

To validate these findings and address directly whether the levels of PC and/or the downstream lipid sphingomyelin (SM) was increase following LPS stimulation, we used a targeted lipidomic approach to profile and quantify total content and lipid species at the molecular level. A total of 35 PC and 27 SM species were detected by high-performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS). Corroborating recent work in murine immortalized macrophages (22), we found that in LPS-treated macrophages, total PC content was increased ~50% (Figure 2A) ($p=0.0817$, using a small sample size of n=3). There was a trend consistent with higher levels of SM in LPS-treated cells, although side chain composition was only different between treatments in SM(d18:1/16:0) (SI-Figure 3A-B). However, there was a significant increase in the most abundant PC molecular species, including PC(16:0/16:1), PC(16:0/18:1), PC(16:1/18:1) and PC(18:0/18:2) (Figure 2B), which represent potentially newly synthesized, desaturated and elongated fatty acids. The transcript expression of Elovl6 and Scd1, which encode for the elongase and desaturase activity, respectively were upregulated by 48 h LPS treatment (SI-Figure 3C). To further investigate this phenomenon, we measured LPS-stimulated de novo fatty acid synthesis by assessing the incorporation of $^3$H-acetate into all lipids, which was increased overall (Figure 2C) and confirms previously published work (23). Interestingly, in spite of the dramatic increase in $^3$H-acetate-derived fatty acids and an absolute increase in PC, the relative proportion of de novo fatty acids incorporated into the total phospholipid pool diminishes with LPS treatment compared to control (51.73% vs. 36.55%; $p<0.01$). As previously reported (24,25), LPS triggered an increased fatty acid incorporation into triglyceride (TG) (29.89% vs. 45.09%; $p<0.0001$), where fatty acid esterification onto DAG or cholesteryl esters remained consistent between naïve and LPS-treated macrophages (Figure 2C). Together these results demonstrate that chronic LPS polarization stimulates an increase in total PC and a remodeling of fatty acid side chains, potentially in keeping with augmented
lipogenesis observed with 48 h LPS-polarized cells.

The choline transporter CTL1 is up-regulated in LPS-stimulated macrophages. We hypothesized that the protein expression of choline transporters was increased in LPS-stimulated cells, which would explain the observed increase in the maximal rate of choline transport. We first assessed the transcript levels of the potential choline transport proteins (choline transporter-like proteins; \textit{Slc44a1-5}, organic cation transporters; \textit{Slc22a1-3} and the high-affinity choline transporter; \textit{Slc5a7}) and show that only \textit{Slc44a1}/CTL1 and \textit{Slc44a2}/CTL2 were expressed in bone marrow-derived macrophages (BMDM) (Figure 3 and data not shown). In response to chronic LPS stimulation the transcript expression of \textit{Slc44a1} and \textit{Slc44a2} were significantly increased compared to untreated cells (Figure 3A). There were no differences in transcript expression of the first two enzymes in the CDP-choline pathway, \textit{Chka} or \textit{Pcyt1} or the rate-limiting enzyme of the CDP-ethanolamine pathway, \textit{Pcyt2} (Figure 4A). Interestingly, although there was a corresponding up-regulation of the CTL1 protein with LPS stimulation, CTL2 protein expression was unchanged (Figure 3B). This was counter to the increased \textit{Slc44a2} transcript expression and suggests that CTL1 is the main choline transporter in BMDM. Taken together, these results demonstrate that in response to chronic stimulation with LPS, the transcript and protein expression of CTL1 is correspondingly higher and may facilitate the increase in choline metabolism fueled by inflammatory stimulation.

Chronic inhibition of choline uptake lowers PC.- Whole body choline deficiency manifests symptoms of fatty liver and muscle fatigue (26); however, the effects of choline depletion on macrophages is not known. To address this question we used a pharmacological and antibody occlusion approach to chronically inhibit general and CTL1-specific choline uptake. Following a chronic (48 h) incubation with either the choline uptake inhibitor hemicholinium-3 (HC3) or a CTL1-specific primary antibody (CTL1-Ab), choline uptake was significantly reduced (Figure 4A). Next, we treated macrophages with either HC3 or the CTL1-Ab in the presence of $^3\text{H}$-choline for 48 h to assess the endogenous pool of PC after the duration of choline uptake inhibition. In keeping with the choline uptake measurements, the amount of PC was significantly reduced in both HC3 and CTL1 Ab-treated cells (Figure 4B). HC3-mediated choline uptake inhibition was equal to that of the CTL1-specific antibody, suggesting that CTL1 is the primary transporter of choline in these cells. Chronic treatment at the indicated concentrations, HC3 had no affect on cell size and slightly, but significantly decreased cell viability (SI-Figure 4). These effects were not observed using the CTL1-Ab (data not shown).

Chronic inhibition of choline uptake alters cytokine secretion.- Previous work has shown that CCTα-deficient macrophages, which lack the rate-limiting enzyme in the CDP-choline pathway and have reduced PC content, secrete less TNFα and IL-6 in response to LPS. This is due to secretory defects at the level of the ER and Golgi. In our BMDM model of choline uptake inhibition, we used a cytokine array panel and determined that chronic HC3 treatment altered 6 h LPS-stimulated secretion of various cytokines and chemokines, including TNFα and IL-10 (SI-Figure 5). Contrary to the findings in CCTα-deficient cells, when we assessed LPS-stimulated primary macrophages that had experienced chronic choline uptake inhibition (6 h LPS), we observed a significant increase in the secretion of both TNFα and IL-6, along with significant reductions in IL-10 secretion (Figure 5A-C), while no differences in IL-1β...
were seen (data not shown). The affect of choline uptake inhibition was only noted in the presence of LPS (Figure 5A-C).

**Inhibition of choline uptake increases DAG and PKC activation** - The final step in the biosynthesis of PC involves the combination of CDP-choline and DAG in the ER. We next tested the hypothesis that the inhibition of choline uptake subsequently altered the levels or metabolism of DAG. We incubated BMDM with either HC3 or the CTL1-Ab in the presence of \(^3\)H-glycerol for 48 h and demonstrated that in response to both forms of inhibition, there was a marked increase in the amount of DAG (Figure 6A). In addition, there was a significant increase in TG levels (Figure 6B). As expected, a significant reduction in \(^3\)H-glycerol incorporation into PC (Figure 6C). In keeping with altered DAG metabolism, HC3 treatment resulted in an increase in the transcript expression of the final step of TG synthesis, Dgat2, but not Dgat1 (Figure 6D). This was associated with more defined lipid droplets in HC3-treated cells (Figure 6E). Increased cellular DAG is known to activate conventional and novel isoforms of PKC, which in turn has been linked to macrophage inflammation (27-29). HC3-mediated chronic choline inhibition was associated with an overall increase in macrophage PKC activity as assessed using a substrate motif antibody (Figure 7), where in cells treated with the choline uptake inhibitor, an increased abundance of phosphorylated PKC substrates was observed.

**Restoring PC levels but not inhibition of PKC rescues cytokine secretion** - Inhibiting choline uptake directly skewed the macrophage inflammatory profile. As a final interrogation we aimed to rescue the aberrant cytokine secretion via two independent approaches. We first supplemented macrophages with excess choline in the media (500 \(\mu\)M) in the presence of both HC3 and the CTL1-Ab. We hypothesized that excess choline would compete with the inhibitor and potentially restore choline uptake and metabolism. Interestingly, choline treatment rescued the levels of TNF\(\alpha\), IL-6 and IL-10 that were altered by HC3 (Figure 8A-C); however, in the presence of the CTL1-Ab, excess choline treatment was completely ineffective (Figure 8D-F). As a secondary approach, cells were treated with the conventional/novel PKC inhibitor bisindolylmaleimide I (BIM) (30), (20 \(\mu\)M) concurrently with HC3 and CTL1-Ab inhibition to potentially counter DAG-mediated PKC signaling. Independent of pharmacological or antibody-mediated choline inhibition, BIM treatment was partially able to correct the secreted levels of LPS-stimulated TNF\(\alpha\), IL-6 and IL-10 (Figure 8A-F). Finally, we confirmed that the supplementation of excess choline was able to dilute the effect of HC3-mediated inhibition on choline uptake and increased PC levels (Figure 9A), but had no effect in cells treated with the CTL1-Ab. As expected, BIM treatment had no effect on PC levels (Figure 9B).

**DISCUSSION**

The metabolism of immune cells is intimately linked to cellular responses and programs (31,32), but how phospholipid homeostasis is regulated in immunometabolism remains unclear. The current study looked to investigate the link between choline uptake, its subsequent metabolism and inflammation in primary murine macrophages.

Macrophage activation is coupled with changes in membrane composition and dynamics; however, unlike other immune cells such as T cell and natural killer cells, differentiated macrophages do not routinely undergo proliferative expansion. Hence the induction of phospholipid biosynthesis, mainly PC, has been mainly attributed to a need for processes such as phagocytosis,
Macrophage inflammation and choline metabolism

organelle biogenesis, secretory functions and endocytosis (33). Past work had established that acute LPS stimulation of elicited peritoneal macrophages increased the incorporation of \(^{3}H\)-choline into PC; however, the mechanisms responsible have remained unclear (18). Here, we corroborate past findings and also show a higher rate of PC synthesis in response to LPS (Figure 2 and 3). Coupled to this, we also demonstrate that the transport of choline via CTL1 is specifically up-regulated in LPS-stimulated macrophages (Figure 1-3).

Augmented flux through the CDP-choline pathway has also been previously demonstrated in LPS-stimulated B cells (34), THP-1 monocytes (17) and elicited peritoneal macrophages (18,19). Interestingly in B cells, this was due to the up-regulation of choline phosphotransferase and lipin-1 genes, as well as an enhanced function of CCT, the rate limiting enzyme in PC synthesis (34). A subsequent study by the same group using peritoneal macrophages revealed that LPS-stimulation increased the gene expression of both the choline/ethanolamine phosphotransferase and CCT gene (Pcyt1). In our LPS-stimulated BMDM model, transcript expressions of genes in the CDP-choline pathway were unaltered (Figure 3). However, it is possible that CCT function was augmented post-transcriptionally in response to higher levels of fatty acids (Figure 2) and increased availability of intracellular choline (Figure 1). While previous studies did not investigate the expression of choline transporters in their model systems, there remains the potential that choline transport was also augmented to facilitate the increased PC biosynthesis in LPS-stimulated B cells and peritoneal macrophages.

The increase in LPS-stimulated choline uptake was associated with higher levels of PC. However, coupled to this was an overall increase in \textit{de novo} lipogenesis. While it is well known that LPS and other pro-inflammatory stimuli polarize macrophages toward a cellular program that favours glucose uptake, glycolysis and diminished fatty acid \(\beta\)-oxidation (35,36), it was recently shown that this augmented glucose metabolism is at least partly responsible for the higher levels of fatty acid synthesis and TG storage (25). Moreover, during monocyte-macrophage differentiation, sterol regulatory element binding protein-induced fatty acid synthesis is also bolstered and directly facilitates increases in phospholipid synthesis (37). This presents a potential scenario whereby upon LPS stimulation, glucose uptake and transcriptional programs drive fatty acid and TG synthesis as well as choline uptake to facilitate PC synthesis. Whether diminishing the availability of choline or inhibiting choline uptake perturbs this LPS-derived increase in fatty acid production has yet to be investigated.

When human THP-1 monocytes underwent phorbol ester-induced differentiation into macrophages, this was accompanied by a similar disappearance of CTL1 from the cell surface and a presumed reduction in PC synthesis (although this was never measured) (5). In primary macrophages we now demonstrate that the only choline transporters expressed are CTL1 and CTL2 (Figure 3A and data not shown). Moreover, LPS-stimulation induces the transcript expression of both the CTL1 and CTL2 genes, while at the protein level, only CTL1 was increased (Figure 3B). The mechanism by which only the CTL1 protein is up-regulated is unclear. However, in subsequent experiments we used increasing amounts of a CTL1-specific antibody to show that the majority of macrophage choline uptake was CTL1-dependent, as PC synthesis decreased more than 80% and cell viability was compromised as antibody concentrations increased (Figure 4 and data not shown). A previous characterization of the CTL1 promoter uncovered a putative binding site for
Macrophage inflammation and choline metabolism

the NFκB transcription factor, which was validated in vitro by gel mobility shift assays (38). Future studies are needed to verify if NFκB signaling is required for the LPS-induced up-regulation of CTL1 and hence choline uptake.

While LPS-stimulation of macrophages increases choline uptake and metabolism, we next aimed to ask the reciprocal question as to the importance of choline uptake and metabolism for macrophage inflammation (in response to LPS). We initially hypothesized that choline uptake and subsequent incorporation into PC in the macrophage would be important to accommodate the increased burden of cytokine secretion and trafficking, which involve the ER and trans Golgi network (39,40). The Chinese hamster ovary cell line MT58 (containing a thermosensitive mutation in the Pcytl gene) has provided an excellent model to study PC depletion and have shown that reductions of PC levels lead to ER and Golgi dysfunction (41-43). Similar defects in protein trafficking were observed in peritoneal macrophages derived from CCTα-deficient mice, where reduced PC synthesis was accompanied by intracellular aggregation and reduced secretion of TNF and IL-6 (19).

Contrary to our hypothesis, when we inhibited choline uptake with HC3 or with a CTL1-specific antibody for a period of 48 h followed by a 6 h stimulation with LPS, the secretion of inflammatory cytokines such as TNFα and IL-6 was higher, where as IL-10 secretion was lower (Figure 5). Interestingly, HC3 or CTL1-Ab treatment effectively inhibited choline uptake and diminished PC levels in the cell (Figure 4); however no defects in cytokine secretion were observed. There remains the potential that experimental differences could be the root of the divergent phenotype between our model of choline depletion and the CCTα-deficient macrophages. Firstly, we used primary BMDM, whereas thioglycollate was used to elicit peritoneal macrophages from wild-type and CCTα-deficient mice (19). It is well-established that the phenotypic and metabolic differences between these two primary macrophage populations are many; therefore, it is unlikely that a direct comparison can be made (44). Moreover, we inhibited choline uptake, both pharmacologically and using antibody treatments for 48 h. This is in comparison to a genetic knockout, in which compensatory mechanisms (apparent or not) may have been at play. Interestingly, previous studies have shown an anti-inflammatory role for exogenous choline in primary macrophages from α7 nicotinic acetylcholine receptors null mice, whereby in these cells TNFα release was blunted (45). Moreover, PC supplementation release was blunted (45). Moreover, PC supplementation stemmed pro-inflammatory programming of intestinal epithelial cells (46). Taken together, these findings suggest that choline and PC supplementation may have anti-inflammatory implications that are both independent of the cholinergic system and extend beyond innate immune cells.

In our model, following the chronic inhibition of choline uptake, LPS-triggered TNFα and IL-6 were higher, where IL-10 secretion was lower, which is potentially indicative of a pro-inflammatory shift. Past work has demonstrated that choline deficiency can induce phospholipase C activity to lower sphingomyelin and increase ceramide levels (47), which has ties to apoptotic and pro-inflammatory signaling (48). In addition to providing the major membrane phospholipid component, PC is also considered an important reservoir for AA-derived lipid messengers such as prostaglandins and leukotrienes (20,21,49). Future studies could test the potential that the inhibition of PC synthesis alters downstream AA-derived signaling pathways to differentially affect cytokine release.

Phospholipid metabolism is intricately linked to FA and lipid homeostasis. There have been many examples in various cellular
systems whereby the disruption of either the CDP-choline or CDP-ethanolamine pathways, which produce PC and phosphatidylethanolamine, respectively, have resulted in a redirection of DAG and aberrant lipid homeostasis (19,47,50-52). In our studies, choline deficiency induced by pharmacological or CTL1-specific inhibition limited the availability of the initial substrate in PC biosynthesis and hence led to 1) a reduced flux through the CDP-choline pathway and 2) an accumulation of DAG and TG. Although the cellular consequence of increased TG remains unclear, HC3 treated cells had higher expression of Dgat2 and more defined lipid droplet formation, which is in keeping with a redirection of DAG toward TG. However, how TG metabolism may be altered under this condition warrants further investigation. We provide evidence for an upregulation of PKC activation that was associated with higher DAG levels. Previous works in macrophages have demonstrated that DAG-mediated PKC activation can increase signal transduction through the NF-κB pathway and treatment of macrophages with a PKC inhibitor reduces LPS-induced inflammatory signaling (27-29). Inhibition of PKC activity with BIM was partially effective at restoring normal cytokine secretion in our study, where TNFα and IL-6 was lower and IL-10 was higher (Figure 8). Although this points to the involvement of PKC signaling, 1) an exhaustive panel of isoform inhibitors was not used and 2) at the concentration used (20 µM), the specificity for conventional and novel PKC isoforms is reduced. Although pharmacological inhibition and antibody occlusion provide models of choline deficiency, it would be interesting to interrogate the role of CTL1-mediated choline uptake in primary macrophages from CTL1-deficient mice; however, to date, no mouse model has been described.

We demonstrate that pro-inflammatory polarization increased choline uptake and subsequently PC biosynthesis, which primes the macrophages to respond appropriately to immune stimuli (SI-Figure 6). Augmented choline uptake is likely CTL1-dependent and would provide necessary amounts of PC to membranes (organelle and plasma membranes) for the packaging and secretion of cytokines. Moreover, we show that modulating PC biosynthesis has the capacity to reprogram immune responsiveness in primary macrophages and highlights a reciprocal link between choline metabolism and macrophage inflammation.

**EXPERIMENTAL PROCEDURES**

### Animals

Mice (C57Bl/6J) were originally purchased from Jackson Laboratories (Stock no. 00064) and bred in a pathogen-free facility in the University of Ottawa animal facility. Mice were maintained on a 12-hour light dark cycle (lights on at 7:00 am) and housed at 23°C with bedding enrichment. Male and female mice ages 8-16 weeks were used for the generation of primary macrophages as described below. All animal procedures were approved by the University of Ottawa Animal Care Committee.

**Isolation, culturing and polarization of bone marrow macrophages**

BMDM were isolated and cultured as previously described (53,54). Bone marrow cells were obtained from the femur and tibia by centrifugation. Briefly, mice were euthanized and the bones were dissected free of all musculature and connective tissue. Bones from each leg were cut and placed inside a 0.5 ml tube with a hole punctured in the bottom by an 18-gauge needle, which was placed inside a larger 1.5 ml tube. To the 0.5 ml (bone-containing tube) 100 µl of DMEM media was added and cells were obtained by centrifugation at 4000 rpm. Bone marrow cells were re-suspended in 1 ml of DMEM and filtered through a 40 µm filter to remove any...
debris and made up in a final volume of 85 ml of complete DMEM (4.5 g/l glucose, with 1x L-glutamine and sodium pyruvate (Wisent)), supplemented with 10% FBS (Wisent) and 1% penicillin/streptomycin (Fisher). Cells were differentiated into macrophages using 15% L929-conditioned media as a source of macrophage colony-stimulating factor. Cells were plated into 10 or 15 cm dishes and allowed to differentiate for 7 days. On day 8, cells were lifted by gentle cell scraping, counted and seeded into culture plates for experiments (2.5x10^5 for 24-well, 4x10^5 for 12-well and 1x10^6 for 6-well). Cells were treated for 48 h with or without 100 ng/ml LPS (E. coli:B4, Sigma-Aldrich) to skew BMDM toward an LPS-polarized phenotype.

### Choline uptake experiments

The rate of choline uptake was determined by measuring \(^3\text{H}\)-choline chloride (Perkin Elmer) uptake over time. Cells were seeded into 24-well plates. One hour prior to uptake, media was removed and cells were washed with PBS before being incubated in Krebs-Ringer-HEPES buffer (KRH; 130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), 1.2 mM KH\(_2\)PO\(_4\), 10 mM HEPES, pH 7.4, and 10 mM glucose) to remove exogenous choline for 1 h. Immediately prior to uptake, cells were washed again followed by the addition of KRH containing 1 μCi/ml of \(^3\text{H}\)-choline and were incubated at 37°C for desired time point (1-30 minutes). Following incubation, cells were washed twice with ice-cold KRH buffer, lysed in 150 μl of 0.1 M NaOH and an aliquot was used to determine radioactivity by liquid scintillation counting (LSC). Total cellular protein was determined using a BCA protein assay (Thermofisher) according to the manufacturer’s instructions. Choline uptake was expressed as pmol choline per mg protein.

Choline transport kinetics and uptake inhibition to the choline analogue HC3 were performed as we have described previously (5).

### \(^3\text{H}\)-Choline, \(^3\text{H}\)-acetate and \(^3\text{H}\)-glycerol incorporation into lipids

Macrophages were seeded into 6-well plates. Cells were washed with PBS and treated with DMEM containing 1 μCi/ml \(^3\text{H}\)-choline, \(^3\text{H}\)-acetate or \(^3\text{H}\)-glycerol for the indicated time (2-48 h). Cells were then washed twice with PBS and lysed with a freeze thaw cycle at -80°C in 200 μl of PBS. Lipids were extracted using the method of Bligh and Dyer (55), the chloroform (lipid-containing) phase was evaporated to dryness under nitrogen gas and resuspended in 50 μl chloroform. Phospholipid and neutral lipids were separated using thin-layer chromatography as previously described (51) and the radioactivity associated with each lipid was measured using LCS. Total protein was measured by a BCA assay.

### PC degradation

For pulse-chase experiments to determine PC degradation, macrophages were seeded into 12 well plates and pulsed for 2 h with 1 μCi/ml of \(^3\text{H}\)-choline-containing DMEM. Radiolabeled medium was then removed, and the cells were washed with PBS and chased with DMEM containing an excess (500 μM) of unlabeled choline for 1, 2, and 4 h. For analyses, the cells were washed twice with ice-cold PBS and processed as described above.

### Chronic choline uptake inhibition and determination of PC content

Choline uptake was chronically inhibited by incubating BMDM with 250 μM of the high/intermediate affinity choline transport inhibitor hemicholinium-3 (HC3) for 48 h. To specifically test the role of CTL1, cells were incubated for 48 h in the presence of either a CTL1-specific antibody (a gift from Dr. Marica Bakovic at the University of Guelph) or an IgG isotype control (Jackson ImmunoResearch), both at a 1/250 dilution. In parallel experiments, macrophages were treated with HC3 and CTL1 antibody in the
presence of 500 μM choline chloride (Sigma) or 20 μM BIM (a gift from Dr. Marceline Côté; originally purchased from Cayman Chemical). To determine PC content, cells were plated in 6 well plates and treated as described above. Following 48 treatment, cells were washed with ice cold PBS and processed using a PC assay kit (Abcam) as per the manufacturer’s instructions. Duplicate wells were used for protein determination and PC content was expressed as nmol/mg protein.

**Determination of cytokine secretion**

Cells were plated in 24 well plates. Following chronic (48 h) choline uptake inhibition as described above, BMDM were stimulated with or without 100 ng/ml LPS for 6 h in 300 µl of media (HC3 or CTL1 Ab were replenished during this treatment). The media was collected and stored at -80°C. Cytokines were determined initially using the Mouse Cytokine Array Panel A (R&D Systems), according to the manufacturer’s instructions. For TNFα, IL-6, IL-10 and IL-1β, Duoset ELISA kits (R&D Systems) were used, again in accordance to the manufacturer’s instructions.

**Transcript expression**

Total RNA was isolated from BMDM using the TriPure reagent protocol (Roche Life Sciences). Isolated RNA was re-suspended in 30 µl of RNAse/DNase free water (Wisent). QuantiNova™ reverse transcription kit (Qiagen) was used to synthesize cDNA according to kit instructions. To determine transcript expression, the QuantiNova™ Probe PCR kit (Qiagen) was used in combination with TaqMan primer-probe sets. qPCR reactions were run on the Roto-Gene Q (Qiagen). Relative transcript expression was determined using the delta-delta Ct method and normalized to the average expression of β-actin and Tbp (56).

**Immunoblotting**

Following treatments, BMDM were washed twice with PBS and lysed in a denaturing lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5% NP-40, 100 μM sodium orthovanadate and protease inhibitor cocktail tablet; Roche) or for CTL1 immunoblotting, a non-denaturing native lysis buffer (identical to denaturing lysis buffer, but lacking Triton X-100 and NP-40) was used. Proteins were equally loaded onto an 8% SDS-PAGE gel, where samples being probed for CTL1 were not boiled prior to loading. We have found that although previous literature documents that full non-denaturing conditions are optimal for identifying a single CTL1 (~72 kDa) band via immunoblot (6), a combination of native lysis buffer and denaturing SDS-PAGE is ideal for cultured macrophages. Gels were transferred onto a PVDF membrane (17 minutes at 1.3 A) using the Trans-Blot® Turbo™ Transfer System (Biorad) with Bjerrum Schafer-Nielsen buffer (48 mM Tris, 39 mM glycine, 20% methanol). Membranes were blocked for 1 hour in 5% BSA then incubated at 4°C overnight in CTL1, CTL2 (Abcam #177877), protein kinase C (PKC) substrate motif (CST #6967) or β-actin rabbit (HRP conjugated, CST #5125). A 1/1000 dilution of stock antibodies in 5% BSA were used for primary antibody incubations. The following day, membranes were washed 4 times in TBS-T (20 mM Tris, 150 mM NaCl, 0.05% Tween® 20) and all blots except β-actin were incubated for 1 hour in HRP conjugated anti-rabbit IgG (CST #7074, 1/5000 dilution). Clarity™ Western ECL solution (BioRad) was applied to membranes which were visualized using LAS 4010 ImageQuant Imaging System (General Electric). Densitometry analysis was performed using ImageJ software (version 1.48), where CTL1 and CTL2 expressions were normalized to β-actin.

**High-performance liquid chromatography**

**electrospray ionization tandem mass**
Macrophage inflammation and choline metabolism

**Lipidomics**

Macrophages were treated as described above and extracted using a modified Bligh and Dyer protocol (55) previously described (57-59). Briefly, cells were collected, counted, washed with PBS, and pelleted at a concentration of 1x10^6 cells per sample. PBS was removed and pellets were stored at -80°C until extraction. Four milliliter acidified methanol (Fisher, cat# BP1105-4) containing 2% acetic acid (Fisher, cat# A38-212) were added to cell pellets and transferred using a glass Pasteur pipette into Kimble 10 ml glass threaded tubes (disposable; VWR, cat# 21020-640). MS grade lipid standards, PC(13:0/0:0) [90.7 ng Avanti LM-1600] and PC(12:0/13:0) [100 ng, Avanti LM-1000], were added to the homogenate at time of extraction. Chloroform (Fisher, cat# C298-500) and 0.1 M sodium acetate (J.T. Baker, cat#9831-03) were added to each sample at a final ratio of acidified methanol/chloroform/sodium acetate (2:1.9:1.6). Samples were vortexed and centrifuged at 600 x g for 5 min at 4°C. The organic phase was retained and the aqueous phase was successively back-extracted using chloroform three times. The four organic extracts were combined and evaporated at room temperature under a constant stream of nitrogen gas. Final extracts were solubilized in 300 µl of anhydrous ethanol (Commercial Alcohols P016EAAN), flushed with nitrogen gas and stored at -80°C in amber glass vials (Chromatographic Specialties C779100AW).

Lipid samples were analyzed using a triple quadrupole-linear ion trap mass spectrometer QTRAP 5500 equipped with a Turbo V ion source (AB SCIEX, Concord, Ontario). Samples were prepared for HPLC injection by mixing 5 µl of lipid extract with 5 µl of an internal standard mixture consisting of PC(O-16:0-d4/0:0) [2.5 ng, Cayman 360906], PC(O-18:0-d4/0:0) [2.5 ng, Cayman 10010228], PC(O-16:0-d4/2:0) [1.25 ng, Cayman 360900], PC(O-18:0-d4/2:0) [1.25 ng Cayman, 10010229], PC(15:0/18:1-d7) [1.25 ng, Avanti Polar Lipids 791637C], PC(18:1-d7/0:0) [1.25 ng, Avanti Polar Lipids 791643C], SM(d18:1/18:1-d9) [1.25 ng, Avanti Polar Lipids 791649C] in EtOH, and 13.5 µL of Solvent A (see below). HPLC was performed with an Agilent Infinity II system operating at a flow rate of 10 µl/min with 3 µL sample injections by an autosampler maintained at 4°C. A 100 mm x 250 µm (i.d.) capillary column packed with ReproSil-Pur 200 C18 (particle size of 3 µm and pore size of 200 Å, Dr. A. Maisch, Ammerbruch, Germany) was used with a binary solvent gradient consisting of water with 0.1% formic acid (Fluka, cat# 56302) and 10 mM ammonium acetate (OmniPur, cat# 2145) (solvent A) and ACN/IPA (J.T. Baker, cat# 9829-03 and Fisher, cat# A416-4) (5:2, v/v) with 0.1% formic acid and 10 mM ammonium acetate (Solvent B). The gradient started from 30% B, reached 100% B in 8 min and maintained for 37 min. The solvent composition returned to 30% B within 1 min and maintained for 14 min to re-equilibrate the column prior to the next sample injection. The PC and SM lipidome was first profiled using precursor ion scan in positive ion mode monitoring transitions from protonated molecular ions to m/z 184.1. Data acquisition for quantification was then performed in the positive ion mode using selected reaction monitoring (SRM), monitoring transitions from protonated molecular ions to m/z 184.1. Molecular identities were confirmed in a single high performance liquid chromatography-SRM-information dependent acquisition (IDA)-enhanced product ion (EPI) experiment in which SRM was used as a survey scan to identify target analytes and an IDA of EPI spectra were acquired in the linear ion trap. After acquisition, the EPI spectra were examined for structural determination. Instrument control and data acquisition were performed with Analyst software (v. 1.6.2, AB SCIEX). MultiQuant 3.0.2 software...
Macrophage inflammation and choline metabolism

(v3.0.8664.0, SCIEX) was used for processing of quantitative multiple reaction monitoring data. For quantification, raw peak areas were corrected for extraction efficiency and instrument response by normalization to internal standards added at time of extraction.

Lipid abundances were then expressed as pmol equivalents of the internal standard PC(13:0/0:0) per 1x10^6 cells.

**Flow cytometry**

Macrophages were plated in 6-well plates and treated with DMSO or HC3 (250 μM) for 48 h. After washing with PBS, cells were scraped and stained with Zombie Aqua dye (BioLegend) for 30 minutes on ice. Cells were washed and resuspended in DAPI-containing FACS buffer (1% BSA, 2 mM EDTA in PBS) and acquired using a FACSCelesta flow cytometer (BD). Viability and cell size were calculated from the frequency of live cells among singlet cell events and forward scatter, respectively.

**Confocal microscopy**

Macrophages were plated in 24-well plates containing #1.5 glass coverslips and treated with DMSO or HC3 (250 μM) for 48 h. After washing with PBS, coverslips were fixed with 1% PFA for 15 minutes at RT and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at RT. Cells were stained with DAPI and Nile Red for 15 min at RT and mounted using Prolong Antifade Gold (Thermo Fisher) on SuperFrost Plus slides (Thermo Fisher). Z-stack images were acquired using an LSM800 AxioObserverZ1 microscope (Zeiss) with a 63X (1.4 NA) oil objective. Images were equally adjusted and using FIJI software (NIH).

**Statistics**

All statistical analyses were performed using Prism 7 (GraphPad Software Inc.). Transcript and protein expression were compared using a non-paired Student’s t-test. Choline incorporation data was analyzed using a two-way ANOVA. Kinetic curves, HC3 IC_{50} curve and values were generated using the Prism 7 non-linear regression Michaelis-Menten curve fit and non-linear regression vs. response with variable slopes curve fit, respectively. Chronic choline inhibition experiments and cytokine secretion experiments were analyzed using a one-way or two-way ANOVA as indicated, where significant difference between groups were determined by Tukey’s post hoc test. For all comparisons, p value of less than 0.05 was considered significant. Quantification of PC and sphingomyelin abundances were analyzed by two-way ANOVA with Holm Sidak post hoc test. For all comparisons, p value of less than 0.05 was considered significant.

**Acknowledgments:** We thank Dr. Marica Bakovic (University of Guelph) for kindly contributing the CTL1 antibody. We also thank Dr. Subash Sad, Dr. Marceline Côté, Corina Warkentin, Graham Gould Maule and Tyler Smith for helpful discussions and reagents. This research was supported by a Discovery Grant from the Natural Science and Engineering Research Council (NSERC) of Canada awarded to MDF (RGPIN-2015-04004) and (RGPIN-2015-5377) and the Canadian Institutes of Health Research (MOP 311838) to SALB. MDF is supported by a Canadian Institutes of Health Research New Investigator award (MSH141981) and is recipient of an Ontario Ministry of Research, Innovation and Science Early Researcher Award. SAS was supported by an NSERC Canadian Graduate Scholarship and NDL was supported by an Ontario Graduate Scholarship.

**Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of this article
**Author contributions:** SAS, KDM and MDF planned the experiments. SAS, KDM, NDL, PG, JRCN and COD conducted the experiments and analyzed the results. TN, HX and SALB performed and analyzed lipidomic experiments. SAS, KDM and MDF wrote the manuscript and all authors had a part in editing the manuscript.

**REFERENCES**

1. Kawasaki, T., and Kawai, T. (2014) Toll-like receptor signaling pathways. *Front Immunol* **5**, 461

2. Bianchi, M. E. (2007) DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol* **81**, 1-5

3. Kennedy, E. P., and Weiss, S. B. (1956) The function of cytidine coenzymes in the biosynthesis of phospholipides. *The Journal of biological chemistry* **222**, 193-214

4. Cornell, R. B., and Ridgway, N. D. (2015) CTP:phosphocholine cytidylyltransferase: Function, regulation, and structure of an amphitropic enzyme required for membrane biogenesis. *Progress in lipid research* **59**, 147-171

5. Fullerton, M. D., Wagner, L., Yuan, Z., and Bakovic, M. (2006) Impaired trafficking of choline transporter-like protein-1 at plasma membrane and inhibition of choline transport in THP-1 monocyte-derived macrophages. *American journal of physiology. Cell physiology* **290**, C1230-1238

6. Michel, V., and Bakovic, M. (2009) The solute carrier 44A1 is a mitochondrial protein and mediates choline transport. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **23**, 2749-2758

7. da Costa, K. A., Corbin, K. D., Niculescu, M. D., Galanko, J. A., and Zeisel, S. H. (2014) Identification of new genetic polymorphisms that alter the dietary requirement for choline and vary in their distribution across ethnic and racial groups. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **28**, 2970-2978

8. Haga, T. (2014) Molecular properties of the high-affinity choline transporter CHT1. *J Biochem* **156**, 181-194

9. Okuda, T., Haga, T., Kanai, Y., Endou, H., Ishihara, T., and Katsura, I. (2000) Identification and characterization of the high-affinity choline transporter. *Nature neuroscience* **3**, 120-125

10. Gorboulev, V., Ulzheimer, J. C., Akhoundova, A., Ulzheimer-Teuber, I., Karbach, U., Quester, S., Baumann, C., Lang, F., Busch, A. E., and Koepsell, H. (1997) Cloning and characterization of two human polyspecific organic cation transporters. *DNA and cell biology* **16**, 871-881
Sweet, D. H., Miller, D. S., and Pritchard, J. B. (2001) Ventricular choline transport: a role for organic cation transporter 2 expressed in choroid plexus. *The Journal of biological chemistry* **276**, 41611-41619

Traiffort, E., O'Regan, S., and Ruat, M. (2013) The choline transporter-like family SLC44: properties and roles in human diseases. *Molecular aspects of medicine* **34**, 646-654

Fujita, T., Shimada, A., Okada, N., and Yamamoto, A. (2006) Functional characterization of Na+-independent choline transport in primary cultures of neurons from mouse cerebral cortex. *Neuroscience letters* **393**, 216-221

Inazu, M., Takeda, H., and Matsumiya, T. (2005) Molecular and functional characterization of an Na+-independent choline transporter in rat astrocytes. *Journal of neurochemistry* **94**, 1427-1437

Ishiguro, N., Oyabu, M., Sato, T., Maeda, T., Minami, H., and Tamai, I. (2008) Decreased biosynthesis of lung surfactant constituent phosphatidylcholine due to inhibition of choline transporter by gefitinib in lung alveolar cells. *Pharmaceutical research* **25**, 417-427

Wille, S., Szekeres, A., Majdic, O., Prager, E., Staffler, G., Stockl, J., Künthaler, D., Prieschl, E. E., Baumruker, T., Burtscher, H., Zlabinger, G. J., Knapp, W., and Stockinger, H. (2001) Characterization of CDw92 as a member of the choline transporter-like protein family regulated specifically on dendritic cells. *Journal of immunology* **167**, 5795-5804

Chu, A. J. (1992) Bacterial lipopolysaccharide stimulates phospholipid synthesis and phosphatidylcholine breakdown in cultured human leukemia monocyti c THP-1 cells. *The International journal of biochemistry* **24**, 317-323

Grove, R. I., Allegretto, N. J., Kiener, P. A., and Warr, G. A. (1990) Lipopolysaccharide (LPS) alters phosphatidylcholine metabolism in elicited peritoneal macrophages. *Journal of leukocyte biology* **48**, 38-42

Tian, Y., Pate, C., Andreolotti, A., Wang, L., Tuomanen, E., Boyd, K., Claro, E., and Jackowski, S. (2008) Cytokine secretion requires phosphatidylcholine synthesis. *The Journal of cell biology* **181**, 945-957

Lennartz, M. R. (1999) Phospholipases and phagocytosis: the role of phospholipid-derived second messengers in phagocytosis. *Int J Biochem Cell Biol* **31**, 415-430

Balsinde, J., Balboa, M. A., and Dennis, E. A. (1997) Inflammatory activation of arachidonic acid signaling in murine P388D1 macrophages via sphingomyelin synthesis. *The Journal of biological chemistry* **272**, 20373-20377

Zhang, C., Wang, Y., Wang, F., Wang, Z., Lu, Y., Xu, Y., Wang, K., Shen, H., Yang, P., Li, S., Qin, X., and Yu, H. (2017) Quantitative profiling of glycerophospholipids during
Macrophage inflammation and choline metabolism

23. Im, S. S., Yousef, L., Blaschitz, C., Liu, J. Z., Edwards, R. A., Young, S. G., Raffatellu, M., and Osborne, T. F. (2011) Linking lipid metabolism to the innate immune response in macrophages through sterol regulatory element binding protein-1a. Cell metabolism 13, 540-549

24. Huang, Y. L., Morales-Rosado, J., Ray, J., Myers, T. G., Kho, T., Lu, M., and Munford, R. S. (2014) Toll-like receptor agonists promote prolonged triglyceride storage in macrophages. The Journal of biological chemistry 289, 3001-3012

25. Feingold, K. R., Shigenaga, J. K., Kazemi, M. R., McDonald, C. M., Patzek, S. M., Cross, A. S., Moser, A., and Grunfeld, C. (2012) Mechanisms of triglyceride accumulation in activated macrophages. J Leukoc Biol 92, 829-839

26. Zeisel, S. H., Da Costa, K. A., Franklin, P. D., Alexander, E. A., Lamont, J. T., Sheard, N. F., and Beiser, A. (1991) Choline, an essential nutrient for humans. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 5, 2093-2098

27. Zhou, X., Yang, W., and Li, J. (2006) Ca2+- and protein kinase C-dependent signaling pathway for nuclear factor-kappaB activation, inducible nitric-oxide synthase expression, and tumor necrosis factor-alpha production in lipopolysaccharide-stimulated rat peritoneal macrophages. The Journal of biological chemistry 281, 31337-31347

28. Asehnoune, K., Strassheim, D., Mitra, S., Yeol Kim, J., and Abraham, E. (2005) Involvement of PKalpha/beta in TLR4 and TLR2 dependent activation of NF-kappaB. Cellular signalling 17, 385-394

29. Foey, A. D., and Brennan, F. M. (2004) Conventional protein kinase C and atypical protein kinase Czeta differentially regulate macrophage production of tumour necrosis factor-alpha and interleukin-10. Immunology 112, 44-53

30. Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., and et al. (1991) The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. The Journal of biological chemistry 266, 15771-15781

31. Peyssonnaux, C., Datta, V., Cramer, T., Doedens, A., Theodorakis, E. A., Gallo, R. L., Hurtado-Ziola, N., Nizet, V., and Johnson, R. S. (2005) HIF-1alpha expression regulates the bactericidal capacity of phagocytes. The Journal of clinical investigation 115, 1806-1815

32. Galvan-Pena, S., and O'Neill, L. A. (2014) Metabolic reprograming in macrophage polarization. Front Immunol 5, 420
33. Ecker, J., Liebisch, G., Englmaier, M., Grandl, M., Robenek, H., and Schmitz, G. (2010) Induction of fatty acid synthesis is a key requirement for phagocytic differentiation of human monocytes. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 7817-7822

34. Fagone, P., Sriburi, R., Ward-Chapman, C., Frank, M., Wang, J., Gunter, C., Brewer, J. W., and Jackowski, S. (2007) Phospholipid biosynthesis program underlying membrane expansion during B-lymphocyte differentiation. *The Journal of biological chemistry* **282**, 7591-7605

35. Norata, G. D., Caligiuri, G., Chavakis, T., Matarese, G., Netea, M. G., Nicoletti, A., O'Neill, L. A., and Marelli-Berg, F. M. (2015) The Cellular and Molecular Basis of Translational Immunometabolism. *Immunity* **43**, 421-434

36. Langston, P. K., Shibata, M., and Horng, T. (2017) Metabolism Supports Macrophage Activation. *Front Immunol* **8**, 61

37. Gordon, S., Pluddemann, A., and Martinez Estrada, F. (2014) Macrophage heterogeneity in tissues: phenotypic diversity and functions. *Immunol Rev* **262**, 36-55

38. Yuan, Z., Tie, A., Tarnopolsky, M., and Bakovic, M. (2006) Genomic organization, promoter activity, and expression of the human choline transporter-like protein 1. *Physiological genomics* **26**, 76-90

39. Manderson, A. P., Kay, J. G., Hammond, L. A., Brown, D. L., and Stow, J. L. (2007) Subcompartments of the macrophage recycling endosome direct the differential secretion of IL-6 and TNFalpha. *The Journal of cell biology* **178**, 57-69

40. Murray, R. Z., and Stow, J. L. (2014) Cytokine Secretion in Macrophages: SNAREs, Rabs, and Membrane Trafficking. *Front Immunol* **5**, 538

41. Marciniak, S. J., Yun, C. Y., Oyadomari, S., Novoa, I., Zhang, Y., Jungreis, R., Nagata, K., Harding, H. P., and Ron, D. (2004) CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes Dev* **18**, 3066-3077

42. Nishitoh, H. (2012) CHOP is a multifunctional transcription factor in the ER stress response. *J Biochem* **151**, 217-219

43. van der Sanden, M. H., Houweling, M., van Golde, L. M., and Vaandrager, A. B. (2003) Inhibition of phosphatidylcholine synthesis induces expression of the endoplasmic reticulum stress and apoptosis-related protein CCAAT/enhancer-binding protein-homologous protein (CHOP/GADD153). *The Biochemical journal* **369**, 643-650

44. Artyomov, M. N., Sergushichev, A., and Schilling, J. D. (2016) Integrating immunometabolism and macrophage diversity. *Semin Immunol* **28**, 417-424
45. Rowley, T. J., McKinstry, A., Greenidge, E., Smith, W., and Flood, P. (2010) Antinociceptive and anti-inflammatory effects of choline in a mouse model of postoperative pain. *Br J Anaesth* **105**, 201-207

46. Treede, I., Braun, A., Sparla, R., Kuhnel, M., Giese, T., Turner, J. R., Anes, E., Kulaksiz, H., Fullekrug, J., Stremmel, W., Griffiths, G., and Ehehalt, R. (2007) Anti-inflammatory effects of phosphatidylcholine. *The Journal of biological chemistry* **282**, 27155-27164

47. Yen, C. L., Mar, M. H., and Zeisel, S. H. (1999) Choline deficiency-induced apoptosis in PC12 cells is associated with diminished membrane phosphatidylcholine and sphingomyelin, accumulation of ceramide and diacylglycerol, and activation of a caspase. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **13**, 135-142

48. Schutze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K., and Kronke, M. (1992) TNF activates NF-kappa B by phosphatidylcholine-specific phospholipase C-induced "acidic" sphingomyelin breakdown. *Cell* **71**, 765-776

49. Dennis, E. A., and Norris, P. C. (2015) Eicosanoid storm in infection and inflammation. *Nature reviews. Immunology* **15**, 511-523

50. Selathurai, A., Kowalski, G. M., Burch, M. L., Sepulveda, P., Risis, S., Lee-Young, R. S., Lamon, S., Meikle, P. J., Genders, A. J., McGee, S. L., Watt, M. J., Russell, A. P., Frank, M., Jackowski, S., Febbraio, M. A., and Bruce, C. R. (2015) The CDP-Ethanolamine Pathway Regulates Skeletal Muscle Diacylglycerol Content and Mitochondrial Biogenesis without Altering Insulin Sensitivity. *Cell metabolism* **21**, 718-730

51. Fullerton, M. D., Hakimuddin, F., Bonen, A., and Bakovic, M. (2009) The development of a metabolic disease phenotype in CTP:phosphoethanolamine cytidylyltransferase-deficient mice. *The Journal of biological chemistry* **284**, 25704-25713

52. Leonardi, R., Frank, M. W., Jackson, P. D., Rock, C. O., and Jackowski, S. (2009) Elimination of the CDP-ethanolamine pathway disrupts hepatic lipid homeostasis. *The Journal of biological chemistry* **284**, 27077-27089

53. Fullerton, M. D., Ford, R. J., McGregor, C. P., LeBlond, N. D., Snider, S. A., Styopa, S. A., Day, E. A., Lhotak, S., Schertzer, J. D., Austin, R. C., Kemp, B. E., and Steinberg, G. R. (2015) Salicylate improves macrophage cholesterol homeostasis via activation of Ampk. *Journal of lipid research* **56**, 1025-1033

54. Galic, S., Fullerton, M. D., Schertzer, J. D., Sikkema, S., Marcinko, K., Walkley, C. R., Izon, D., Honeyman, J., Chen, Z. P., van Denderen, B. J., Kemp, B. E., and Steinberg, G. R. (2011) Hematopoietic AMPK beta1 reduces mouse adipose tissue macrophage inflammation and insulin resistance in obesity. *The Journal of clinical investigation* **121**, 4903-4915

55. Bligh, E. G., and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology* **37**, 911-917
56. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408

57. Whitehead, S. N., Hou, W., Ethier, M., Smith, J. C., Bourgeois, A., Denis, R., Bennett, S. A. L., and Figeys, D. (2007) Rapid identification and quantitation of changes in the platelet activating factor family of glycerophospholipids over the course of neuronal differentiation by high performance liquid chromatography electrospray ionization tandem mass spectrometry. Anal Chem 79, 8359-8548

58. Ryan, S. D., Whitehead, S. N., Swayne, L. A., Moffat, T. C., Hou, W., Ethier, M., Bourgeois, A. J. G., Rashidian, J., P Blanchard, A., Fraser, P. E., Park, D. S., Figeys, D., and Bennett, S. A. L. (2009) Amyloid-β42 signals tau hyperphosphorylation and compromises neuronal viability by disrupting alkylacylglycerophosphocholine metabolism. Proc Natl Acad Sci U S A 106, 20936-20941

59. Xu, H., Valenzuela, N., Fai, S., Figeys, D., and Bennett, S. A. L. (2013) Targeted lipidomics - advances in profiling lysophosphocholine and platelet-activating factor second messengers. The FEBS journal 280, 5652-5667

FOOTNOTE
Abbreviations: PC, phosphatidylcholine; CTL1, choline transporter-like protein 1; DAG, diacylglycerol; PKC, protein kinase C; CCT, phosphocholine cytidylyltransferase; SLC, solute carrier; CHT1, high affinity choline transporter; OCT, organic ion transporter; LPS, lipopolysaccharide; M[0], basal macrophage; M[LPS], LPS-stimulated macrophage; AA, arachidonic acid; SM, sphingomyelin; HPLC-ESI-MS/MS, high-performance liquid chromatography electrospray ionization tandem mass spectrometry; TG, triglyceride; BMDM, bone marrow-derived macrophages; CHK, choline kinase; Pcyt1, gene encoding phosphocholine cytidylyltransferase; Pcyt2, gene encoding phosphoethanolamine cytidylyltransferase; HC3, hemicholinium-3; Ab, antibody; TNF, tumour necrosis factor; IL, interleukin; BIM, bisindolylmaleimide I; LCS, liquid scintillation counting; SRM, selected reaction monitoring; IDA, information dependent acquisition; EPI, enhanced product ion
Figure 1. LPS-stimulation increases choline uptake and PC synthesis. BMDM were treated with LPS (100 ng/ml) for 48 hours prior to measuring choline uptake. (A) Choline uptake was measured at the indicated times by incubating cells in a solution of KRH buffer containing 1 μCi/ml of $^3$H-choline chloride (n= 6 separate bone marrow isolations, each performed in triplicate). (B) Choline uptake was measured over ten minutes by incubating cells in a solution of KRH buffer containing 1 μCi/mL of $^3$H-choline chloride and increasing amounts of non-radiolabeled ‘cold’ choline (n= 6-8 separate bone marrow isolations, each performed in triplicate). Choline uptake was determined by measuring intracellular radioactivity, which was plotted against the amount of protein. (C) BMDM were treated with LPS (100 ng/ml) for 48 hours prior to treatment with 1 μCi/ml $^3$H-choline chloride in DMEM for the indicated times and the incorporation into PC was determined (n= 6-9 separate bone marrow isolations, each performed in triplicate). Data are expressed as mean ± SEM. The rates of choline uptake (A) were determined via linear regression, where the p-value indicated represents significance between M[0] and M[LPS] cells. For choline uptake kinetics (B), data was fit to the Michaelis-Menten curve and statistical significance is represented by **** p<0.0001 as determined by a comparison of the curve fit using extra sum-of-squares F-test. For PC synthesis (C) statistical significance is represented by * p<0.05, ** p<0.01 and **** p<0.0001 compared between treatments, and ## p<0.01 compared to the 2 h time point determined by two-way ANOVA.
Figure 2. Macrophage LPS-polarization increases PC and de novo lipogenesis. BMDM were treated with or without LPS (100 ng/ml) for 48 h. HPLC-ESI-MS/MS lipidomics was used to assess (A) total PC content and (B) PC fatty acid composition (n=3 separate bone marrow isolations). Cells treated with and without LPS were incubated in the presence of \(^3\)H-acetate to measure of fatty acid synthesis, where (C) is the incorporation into all lipids, with the proportional incorporation of \(^3\)H-acetate-derived fatty acids into phospholipid (PL), diacylglycerol (DAG), triglyceride (TG) or cholesteryl ester (CE) shown to the right. Data for A-C are expressed as mean ± SEM where statistical significance is represented by * p<0.05 and **** p<0.0001 compared to M[0] treated cells as determined by two-way ANOVA as indicated. For D, M[LPS] is shown relative to M[0] where the log2 transformed relative difference is reflected by the size of the pie (n=4 separate bone marrow isolations, each performed in triplicate).
Figure 3. Choline transporters transcript and protein expression induced by LPS. BMDM were treated with LPS (100 ng/ml) for 48 hours. qPCR determination of the relative expression of (A) *Slc44a1*, *Slc44a2*, *Chka*, *Pcyt1a* and *Pcyt2*, which were normalized to the average expression of β-actin and *Tbp* (n= 4 separate bone marrow isolations, each performed in triplicate). Data are mean ± SEM, where statistical significance is represented by **** p<0.0001 compared to M[0] cells as determined by an unpaired two-tailed Student’s t-test. (B) Protein expression of CTL1 and CTL2 was determined and normalized to β-actin (n= 3 separate bone marrow isolations, each run in duplicate). Data are mean ± SEM of the densitometry quantification (Image J) and the blots are representative images of the biological replicates, where statistical significance is represented by ** p<0.01 compared to M[0] cells as determined by an unpaired two-tailed Student’s t-test.
Figure 4. Chronic pharmacological or CTL1-specific antibody inhibition lowers choline uptake and PC levels. BMDM were incubated for 48 h in the presence or absence of the choline uptake inhibitor HC3 (250 μM), where DMSO was used as a vehicle control or with a primary CTL1 antibody, where an isotype control IgG antibody was used (both at 1/250 dilution). (A) Acute \( ^3 \text{H} \)-choline uptake was assessed over ten minutes (n= 4 separate isolations, each performed in triplicate). (B) Chronic choline uptake inhibition was accompanied by chronic \( ^3 \text{H} \)-choline incorporation into PC (n= 4 separate isolations, each performed in triplicate). Data are mean ± SEM, where statistical significance is represented by *** \( p<0.001 \) compared to control treatment as indicated and assessed by one-way ANOVA.
Figure 5. Inhibiting choline uptake alters LPS-induced cytokine secretion. Cells were incubated in the presence or absence of HC3 (250 μM) or CTL1-Ab (1/250) for 48 h. The media was removed and cells were stimulated with 100 ng/ml of LPS for 6 h (with inhibitor treatments replenished) and from the supernatant, (A) TNFα, (B) IL-6 and (C) IL-10 secretion was determined (n= 5 separate isolations, each performed in triplicate). Data are mean ± SEM, where statistical significance is represented by **** p<0.0001 compared to LPS-stimulated control treatment as indicated and assessed by two-way ANOVA.
Macrophage inflammation and choline metabolism

Figure 6. Inhibiting choline uptake causes accumulation and redirection of DAG. BMDM were pulsed with $^3$H-glycerol for 48 h in the presence or absence of choline uptake inhibitors HC3 (250 μM) or the CTL1-Ab (1/250). Lipids were then extracted and separated via TLC to determine radioactivity of (A) PC, (B) DAG and (C) TG (n= at least 4 separate isolations). (D) qPCR determination of the relative expression of Dgat 1 and Dgat2 after 48 h treatment as indicated, which were normalized to the average expression of β-actin (n= 4 separate bone marrow isolations, each performed in triplicate). (E) Representative images of the maximum intensity projection confocal images of cells incubated with DMSO or HC3 (250 μM) for 48 h and stained with DAPI or Nile Red. Scale bars represent 10 μm. Corner insets are enlarged from the yellow boxes. Data are mean ± SEM, where statistical significance for A-C is represented by **p<0.01, *** p<0.001 and ****p<0.0001 compared to control treatment as indicated and assessed by one-way ANOVA and statistical significance for D is represented by * p<0.05 and ** p<0.01 compared to DMSO control, determined by Student’s t-test.
Figure 7. Inhibiting choline uptake is associated with higher PKC activity. Choline uptake was inhibited in BMDM for 48 h using HC3 (250 μM). Cell lysate was collected and immunoblotted using a PKC substrate motif antibody. Equal loading was confirmed by using β-actin, run on a duplicate gel (n= 4 separate isolations, each performed in duplicate). Blots are representative images of the biological replicates.
Figure 8. Extracellular choline or PKC inhibition rescues cytokine secretion profile. Cells were incubated in the presence or absence of HC3 (250 μM) or the CTL1-Ab (1/250) for 48 h and co-treated with excess choline chloride (500 μM) or the PKC inhibitor bisindolylmaleimide I (BIM; 20 μM). The media was removed and cells were stimulated with 100 ng/ml of LPS for 6 h, during which time the initial treatments were replenished. Secretion of (A and D) TNFα, (B and E) IL-6 and (C and F) IL-10 into the media was determined (n= 5 separate isolations, each performed in triplicate). Data are mean ± SEM, where statistical significance is represented by **** p<0.0001 compared to LPS-stimulated control or control IgG treatment and # p<0.05, ## p<0.01, ### p<0.001 compared to HC3 as indicated and assessed by one-way ANOVA.
Figure 9. PC levels in the presence of choline or PKC inhibitor. (A) Cells were incubated with or without HC3 (250 μM), excess choline chloride (500 μM) or BIM (20 μM) for 48 h to assess PC levels (n= 4 separate isolations performed in duplicate). (B) Cells were treated with the CTL1-Ab (1/250), excess choline (500 μM) or BIM (20 μM) to assess PC levels (n= 4 separate isolations performed in duplicate). Data are mean ± SEM, where statistical significance is represented by * p<0.05, ** p<0.01 and *** p<0.001 compared to indicated control and ## p<0.01 compared to HC3 (A) group as indicated and assessed by one-way ANOVA.
