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Article
The short chain fatty acid butyrate prevents intracellular replication of *Legionella* by regulating cysteine levels in macrophages

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**Keywords**: lung infection, immunometabolism, amino acids, nutrients, transporter
Abstract

Macrophages can prevent infections from intracellular pathogens by restricting access to essential nutrients, termed nutritional immunity. With the exception of tryptophan depletion, it is unclear if other amino acids are similarly regulated in infected macrophages. Here, we show that the expression of nutrient transporters in Legionella-infected macrophages is modulated by the short chain fatty acid butyrate. Butyrate prevented the upregulation of the cystine/glutamate exchanger, Slc7a11, in macrophages infected with L. pneumophila, which decreased cellular cysteine levels. Butyrate and the Slc7a11 inhibitor erastin impaired intracellular Legionella replication in macrophages in vitro, with these being restored by exogenous supplementation with cysteine. Butyrate caused increased histone acetylation in infected macrophages, and pan- and class II HDAC inhibitors also restricted intracellular Legionella growth in a cysteine-dependent manner. Intranasal administration of butyrate reduced L. pneumophila lung burdens in mice. Our data suggest that butyrate alters the metabolism of macrophages to promote nutritional immunity by decreasing cysteine levels and that this can be harnessed to treat bacterial lung infections.

Introduction

Macrophages are front line immune cells capable of sensing and eliminating invading microbes. The delivery of microbes to hydrolytic lysosomes promotes cell-intrinsic immunity (1). The induction of additional anti-microbial mechanisms are aimed at pathogens that are able to survive in macrophages (2). This includes nutritional immunity whereby macrophages limit the availability of essential nutrients to prevent intracellular microbial replication. Nutritional immunity is best understood in the case of iron and zinc which can be selectively depleted in pathogen-containing vacuoles and thus prevent microbial survival (3-5). Similarly, sensing of microbes induces the expression of indoleamine 2,3-dioxygenase 1 (IDO1) in macrophages, which depletes cellular tryptophan levels and thus restricts
replication of tryptophan-dependent microbes (6-8). Besides tryptophan, intracellular pathogens are likely reliant on other host-derived amino acids to satisfy their metabolic demands. The intriguing possibility that macrophages are able to deplete other amino acids to prevent infections remains largely unexplored.

*Legionella pneumophila* is a facultative intracellular bacterium that replicates in a range of protozoan hosts. Within lungs of susceptible individuals, *L. pneumophila* primarily grows in alveolar macrophages causing severe pneumonia due to uncontrolled bacterial burdens and inflammation (9). Intracellular *L. pneumophila* must gain access to sufficient levels of host-derived amino acids. This is partly because *L. pneumophila* is unable of de-novo synthesis of several amino acids, but also because glucose is primarily used for the generation of storage carbohydrates rather than as energy or carbon source (10). Depending on its type 4 secretion system, *L. pneumophila* forms a unique vacuole within host cells and avoids the otherwise nutrient rich lysosome (11). *L. pneumophila* escapes other anti-microbial host responses including autophagy known to provide nutrients to some pathogens (12, 13). Changes in host-derived nutrients are sensed by intracellular *L. pneumophila* to induce developmental programs. For instance, sufficient levels of amino acids within the *Legionella* containing vacuole trigger the differentiation to the replicative form, whereas amino acid starvation switches *Legionella* to the transmissive form that ceases replication and induces virulence factors (14). How intracellular *L. pneumophila* ensures sufficient access to amino acids is not fully understood. The inhibition host protein synthesis by *L. pneumophila* effectors and dependency on the host proteasome suggest that intracellular bacteria rely on protein turnover to gain access to amino acids (15-17). Despite this, earlier reports identified that the host amino acid transporter Slc1a5 enables *L. pneumophila* growth in monocytes, suggesting that intracellular bacteria gain access the host cell environment (18).
During infections, macrophages rewire their metabolic pathways to induce anti-microbial and inflammatory responses, referred to as immunometabolism (19). There is limited information on how the changes in host metabolism affect intracellular pathogens that replicate preferentially in macrophages, such as *Legionella* (20, 21). Furthermore, the nutritional environment within infection sites affect macrophage responses, as exemplified by the microbial short chain fatty acids (SCFAs) acetate, propionate and butyrate, which reach millimolar concentrations in the gut due to bacterial fermentation of fibre or consumption of SCFA-rich foods (22). Butyrate dampens inflammation in the gut by reducing the expression of inflammatory cytokines in macrophages likely by inhibiting histone deacetylase activity (23). As a fatty acid, butyrate is utilized by gut macrophages and other cells as a carbon source, augmenting metabolic and immune pathways that dampen inflammation (24). Furthermore, sensing of butyrate by cell surface receptors, such as G protein-coupled receptors (GPRs), stimulates the differentiation of immune cells to control gut inflammation (25). Intake of butyrate or fibre increases serum butyrate levels and thus affects macrophage and immune responses in other organs, including in the lung (24, 26-28). Because of the anti-inflammatory effects of butyrate, the SCFA is currently pursued in preclinical and clinical trials to improve outcomes in inflammatory diseases, such as Crohn’s disease, type-1 diabetes, allergy, and asthma (29, 30). The physiological consequence of SCFAs in infections, however, has resulted in contrasting outcomes, as butyrate is beneficial in some bacterial and viral infections (27, 31-34), but detrimental in other infectious diseases (35).

Here, we reveal that butyrate affects the metabolism of macrophages and thus prevents intracellular replication of *Legionella*. We show that butyrate controls the expression of cell surface solute carriers, such as Slc7a11 (also known as cystine/glutamate exchanger, xCT) thereby controlling amino acid levels in infected macrophages. Intranasal butyrate administration reduced bacterial lung burdens, suggesting that SCFAs increase the ability of
macrophages to control infections by restricting amino acid availability as a potential new therapeutic option.

Results

Butyrate decreases intracellular *L. pneumophila* burdens in macrophages.

To test whether short chain fatty acids affect intracellular *Legionella* survival, we infected bone marrow-derived macrophages (BMDMs) with *L. pneumophila* deficient in flagellin expression (∆flaA). Unlike wild type *L. pneumophila*, which induces rapid macrophage cell death due to the activation of the NLRC4/caspase-1 inflammasome (36), ∆flaA was able to replicate in BMDMs as determined by colony forming units 48 hours post infection (Fig 1a). *L. pneumophila* growth under these conditions is restricted to BMDMs, as GFP-expressing bacteria were primarily detected inside macrophages but not in the culture media (Fig. 1b). The supplementation of the macrophage culture media with the short chain fatty acids, acetate, propionate, and butyrate, during macrophage infections markedly reduced *L. pneumophila* burdens in BMDMs at 48 hours post infection (Fig 1a). Intracellular bacterial burdens remained unaffected at 6 hours post infection (Fig 1a), suggesting that SCFAs do not markedly affect bacterial uptake and elimination, but rather prevent subsequent bacterial growth. To identify which short chain fatty acid affects intracellular *Legionella*, BMDMs were exposed to acetate, propionate and butyrate separately. While acetate had no effect on ∆flaA burdens, butyrate and propionate prevented increased *Legionella* numbers at 48 hours post infection (Fig 1a). In the following experiments, we focused on butyrate to probe specific mechanisms. Live-cell imaging of GFP-expressing *L. pneumophila* confirmed that butyrate had little effect on initial BMDM infections, but prevented subsequent increased fluorescent signals, consistent with a lack of intracellular growth (Fig 1b).
Propionate and acetate can prevent axenic *Legionella* growth in rich culture media by acting as signalling molecules (37). Similarly, 4 mM and higher concentrations of butyrate markedly prevented axenic ∆flaA replication, which was not observed with *Salmonella* or *E. coli* (Fig S1a, b and c). Butyrate concentrations of 1mM and lower only marginally reduced axenic *L. pneumophila* growth rates, but prevented intracellular growth in BMDMs, suggesting that the SCFA may not directly affect intracellular bacteria (Fig S1a and Fig S1d). In contrast to BMDMs, 1mM butyrate failed to prevent *L. pneumophila* growth in the human macrophage cell line THP1 (Fig S1e) but also in immortalized mouse macrophages (iBMDMs) (Fig S1f), suggesting altered responses in cell lines rather than species differences. Recent studies indicated that butyrate can affect macrophage differentiation from bone marrow progenitor cells or blood monocytes (27, 33). The presence of butyrate during BMDM differentiation resulted in reduced intracellular *L. pneumophila* numbers, similar to when butyrate was administered at the time of BMDM infections (Fig S1g). Butyrate still caused reduced intracellular *L. pneumophila* burdens when the SCFA was used to pre-treat BMDMs but was removed during infections (Fig 1c). Butyrate also affected intracellular *L. pneumophila* numbers when applied after infections were established (post-infection) (Fig 1c). Butyrate also reduced intracellular *L. pneumophila* numbers in isolated mouse alveolar macrophages (Fig 1d). As reported previously, butyrate can stimulate autophagy as evidenced by decreased p62 levels and increased lipidation of the autophagosome LC3 member GABARAP in uninfected BMDMs (Fig S1h). *L. pneumophila*, however, inhibits autophagy to ensure intracellular survival (12). Consistent with this notion, butyrate failed to markedly reduce p62 levels in infected BMDMs (Fig S1h). Inhibiting autophagy with Bafilomycin A did not increase bacterial burdens in butyrate treated BMDMs (Fig S1i). Taken together, out data shows that butyrate treatment of BMDMs and alveolar macrophages causes reduced intracellular *L. pneumophila* numbers independent of autophagy.
Butyrate controls the expression of nutrient transporters during infections

To understand how butyrate affects macrophage responses in *Legionella* infections, we next performed RNAseq analysis of butyrate-treated BMDMs. Consistent with previous studies using purified LPS, butyrate prevented the upregulation of cytokines and mediators of inflammation during *L. pneumophila* infection, although some cytokines such as CSF3 were induced (Fig 2a and b). Genes that were up- or downregulated in butyrate treated and infected BMDMs were associated with innate immune responses, LPS and inflammation based on GO-term analysis (Fig 2a). In addition, we noticed that genes associated with metabolism were similarly regulated by butyrate. These included several solute carriers that were upregulated in *L. pneumophila*-infected BMDMs compared to uninfected macrophages (Fig S2a), but markedly reduced in butyrate-treated macrophages, such as the cysteine/glutamate exchanger (Slc7a11), importer of organic anions (Slco3a1), lysosomal copper (Slc31a2) and glucose (Slc2a6) and mitochondrial amino acids (Slc25a44) (Fig 2b). Butyrate treatment upregulated additional solute carriers that were otherwise largely unaffected by *L. pneumophila* infections, such as Slc45a4 an alternative sugar transporter (Fig 2b). Besides transporters, butyrate also regulated the expression of metabolic enzymes, such as IRG1 (Acod1) which was highly upregulated in infected BMDMs and generates the metabolite itaconate known to inhibit *L. pneumophila* growth (38). While Glut1 (Slc2a1) was upregulated in infected BMDMs, independent of butyrate, other glycolytic enzymes remained relatively unchanged during infections, including glycolytic (Hk1, Tpi1, Gpi1, Pdha1, Pgm2, Pfkb3) and TCA enzymes (Mdhl, Aco1) (Fig 2b). Butyrate stimulated the expression of hexokinase 1 (Hk1) as well as TCA cycle enzymes malate dehydrogenase 1 and aconitase 1, regardless of infection (Fig 2b). This suggests that butyrate affects multiple cellular metabolic pathways in macrophages. Butyrate also modulated gene expression in uninfected BMDMs, but the effects were not as pronounced as in infected BMDMs, further suggesting that butyrate may primarily affect pathways associated with infections in macrophages (Fig S2b).
To gain insights into how metabolic pathways are regulated by butyrate and whether this affects intracellular *L. pneumophila* replication, we focused on Slc7a11 as this transporter was the most highly upregulated gene in infected BMDMs and its expression was abrogated by butyrate treatment (Fig 2b). We confirmed by qRT-PCR and immunoblot analysis that Slc7a11 is induced in *L. pneumophila* infection but not in the presence of butyrate (Fig 2c and d). The Slc7a11 inhibitor, erastin, reduced intracellular *L. pneumophila* burdens in BMDMs (Fig 2e), suggesting that the effects of butyrate may be connected to Slc7a11 downregulation. Inhibition of Slc7a11 via erastin can trigger the programmed cell death pathway of ferroptosis (39). Treatment with the ferroptosis inhibitor, ferrostatin, however, failed to restore *L. pneumophila* burdens during erastin treatment and ferrostatin by itself did not affect *L. pneumophila* infections (Fig 2e). Butyrate treatment can also induce apoptosis (40), which does abrogate *Legionella* replication (41). Consistent with this notion, we noticed that butyrate reduced the expression of the anti-apoptotic BCL-2 member, MCL-1, and induces caspase-3 expression (Fig 2a). Immunoblot analysis confirmed that butyrate, but not propionate, reduced MCL-1 levels in uninfected BMDMs, but not infected BMDMs (Fig S2c). However, butyrate did not cause caspase-3 cleavage, including in *L. pneumophila* infected BMDMs (Fig S2c). Inhibition of caspase activity, using the pan-caspase inhibitor QVD, failed to restore intracellular *L. pneumophila* burdens after butyrate treatment, although QVD restored *L. pneumophila* replication in control treatment with ABT-737 which induced apoptosis (Fig S2d). Finally, butyrate failed to trigger increased cell death in infected macrophages, suggesting that the decreased CFUs are not due to macrophage cell death (Fig S2e). Besides regulating cell death pathways, Slc7a11 promotes glutathione synthesis which depends on the import of cystine in exchange for glutamate and reduction to cysteine. However, butyrate still prevented intracellular survival of *L. pneumophila* in the presence of membrane permeable glutathione and glutathione synthesis inhibitor (BSO) did not affect.
intracellular \textit{L. pneumophila} numbers (Fig 2f). Taken together, our data shows butyrate attenuates infection-induced expression of Slc7a11 and limits \textit{L. pneumophila} intracellular survival in a manner that is independent of established anti-microbial pathways including the induction of macrophage cell death and oxidative stress.

**Butyrate affects cysteine metabolism in infected macrophages.**

Given our observation that butyrate prevented the upregulation of Slc7a11 in \textit{Legionella}-infected macrophages, we determined whether butyrate affected intracellular cysteine and glutamate levels in BMDMs. To detect the unstable cysteine in macrophages, we performed a targeted metabolomic analysis, finding that this amino acid was indeed decreased in \textit{L. pneumophila}-infected BMDMs treated with butyrate (Fig 3a). Similarly, there was a trend towards lower levels of the cysteine-related metabolites glutathione and $\gamma$-glutamylcysteine in butyrate-treated macrophages, including those infected with \textit{L. pneumophila} (Fig S3a and b).

Besides Slc7a11-mediated cystine uptake, macrophages can utilise cysteine directly via different transporters. We thus tested whether the addition of cysteine in the culture media would affect \textit{L. pneumophila} intracellular survival, particularly in the presence of butyrate. Cysteine supplementation restored \textit{L. pneumophila} burdens in butyrate-treated BMDMs (Fig 3b). Imaging of GFP-expressing \textit{L. pneumophila} demonstrated that the green fluorescent signal increased within macrophages over time, whereas extracellular bacterial growth was not observed (Fig 3c). The addition of cysteine 24 hours after butyrate treatment of infected BMDMs similarly restored intracellular \textit{L. pneumophila} numbers (Fig 3d). Conversely, cysteine supplementation of butyrate pre-treated BMDMs enabled \textit{L. pneumophila} to survive in the absence of the SCFA (Fig 3d). Furthermore, intracellular \textit{L. pneumophila} loads were decreased in BMDMs cultured in cysteine/cystine-free media, with bacterial replication being restored by cysteine supplementation (Fig 3e). Macrophage viability was not affected in cysteine/cystine-free media (data not shown). In addition, supplementation of cystine-
containing culture media with the reducing agent 2-mercaptoethanol increased intracellular *L. pneumophila* burdens in butyrate-treated BMDMs (Fig 3f). Collectively, these findings support a model in which butyrate limits *L. pneumophila* replication in macrophages by restricting cysteine availability.

Besides Slc7a11, butyrate affected additional nutrient transporters and metabolic enzymes in *Legionella* infected macrophages (Fig 2b). Thus, we also measured intracellular metabolites using unbiased metabolite profiling which identified 28 metabolites with high confidence that were significantly altered between *L. pneumophila* infected BMDMs treated with or without butyrate (Fig 3g). Butyrate was enriched in butyrate-treated macrophages, validating the approach (Fig 3g). Similarly, glutamate levels were increased in butyrate-treated macrophages, consistent with the reduced expression of Slc7a11 which exports glutamate in exchange for cystine. Conversely, glutamate was the most highly decreased amino acid in infected BMDMs compared to uninfected cells (Fig S3d). Glutamine was similarly decreased, suggesting assimilation to glutamate (Fig S3d). Intriguingly, cysteine-dependent metabolites such as hypotaurine and taurine showed reduced levels in infected BMDMs, which may further increase free cysteine levels (Fig S3d). Conversely, butyrate treatment increased hypotaurine and taurine levels in infected macrophages, thus further depleting available cysteine (Fig 3g). Similarly, serine levels were increased in infected BMDMs and reduced in the presence of butyrate, although this did not reach statistical significance (Fig S3d). *L. pneumophila* infections affected the levels of other amino acids as well as glycolytic and TCA-cycle metabolites and nucleotides (Fig S3a). For instance, itaconate was one of the most highly increased metabolites in infected macrophages and its levels were decreased in the presence of butyrate (Fig 3g, S3d), consistent with the dampened expression of IRG1 (Fig 2b). Unlike cysteine supplementation, however, the addition of high concentrations of serine and other amino acids to the macrophage culture media failed to rescue intracellular *L. pneumophila*.
pneumophila survival after butyrate treatment (Fig S3f). Taken together, our data suggest butyrate treatment affects intracellular metabolite levels in infected macrophages which support immune and antimicrobial responses.

Conserved control of intracellular Legionella strains by butyrate

L. pneumophila clinical isolates contain different sets of virulence factors to establish infections in macrophages (42). We therefore tested additional Legionella strains and species to determine whether butyrate broadly affects infections. In contrast to L. pneumophila strain 130b described above, L. pneumophila Lp02 (Philadelphia isolate) is not markedly affected by butyrate (Fig 4a) or by erastin (Fig 4b) in either establishing infections or for intracellular replicating in BMDMs. This suggests access to cysteine via a different mechanism, although inhibition of the 26S proteasome, which has been implicated previously to generate amino acids for intracellular Legionella, similarly failed to prevent L. pneumophila Lp02 replication (Fig 4c) (15). The combination of erastin and MG132 did reduce L. pneumophila Lp02 numbers in BMDMs (Fig 4d). In contrast, L. pneumophila 130b was sensitive to the 26S proteasome inhibitors MG132 and bortezomib and cysteine supplementation restored intracellular bacterial burdens (Fig 4e and f). Similarly, butyrate and erastin reduced intracellular L. longbeachae burdens in BMDMs, but not when cysteine was supplemented in the macrophage media (Fig 4g and h). In cysteine/cystine-free macrophage media, L. longbeachae failed to increase bacterial burdens, unless cysteine was supplemented (Fig 4i). These data support the notion that intracellular growth of Legionella strains and species depends on sufficient access to amino acids, particularly cysteine.

Butyrate acts as a HDAC inhibitor to control intracellular Legionella replication

The effect of butyrate on mammalian cells can be mediated either by signalling via G protein-coupled receptors (GPCRs) or by inhibiting histone deacetylases (HDACs) (23, 40). We
initially tested whether GPCRs, which have previously been identified to sense butyrate and other SCFAs (43), affect intracellular *L. pneumophila* numbers. For this, we generated BMDMs from mice deficient in either GPR35, 41, 43, 65 or 109 and determined intracellular *L. pneumophila* numbers in the presence of butyrate. We observed reduced bacterial burdens in all GPCR-deficient BMDMs after butyrate treatment (Fig S4a), suggesting redundancy between GPCR signalling or that other mechanisms are involved in reducing bacterial numbers.

To assess whether butyrate inhibits host HDAC activity and thus prevents intracellular bacterial replication, we next treated BMDMs with the pan-HDAC inhibitor trichostatin A (TSA). Similar to butyrate treatment, TSA affected the expression of inflammatory mediators in *L. pneumophila* infected BMDMs, but also nutrient transporters, including Slc7a11 (Fig 5a). TSA prevented intracellular *L. pneumophila* replication to a similar extent as butyrate treatment (Fig 5b). Consistent with HDAC inhibition by butyrate in this experimental system, lysine acetylation of histone H3 was markedly increased after butyrate treatment in uninfected and *L. pneumophila* infected BMDMs, even more so than was apparent with TSA treatment (Fig 5c). We next wanted to identify the specific HDAC that enables intracellular *L. pneumophila* replication in macrophages. For this we initially used RGF966, which inhibits HDAC3, as this class I HDAC has been shown to prevent inflammatory and antimicrobial responses in macrophages (33, 44). Consistent with the notion that *Legionella* can evade antimicrobial responses, HDAC3 inhibition via RGF966 did not reduce intracellular bacterial burdens (Fig 5d). While loss of HDAC3 reduced the expression of several solute transporters in LPS-treated BMDMs, it did not prevent Slc7a11 upregulation (Fig S4b) (44). In contrast, the HDAC inhibitor tubastatin A, which targets class IIb HDAC6 and 10 (45), mimicked butyrate treatment in preventing intracellular *L. pneumophila* replication (Fig 5e) and cysteine supplementation restored intracellular burdens (Fig 5f). Consistent with these phenotypes, tubastatin A prevented the upregulation of Slc7a11 in *L.
pneumophila infected BMDMs (Fig 5g). L. pneumophila was able to replicate in HDAC6-
deficient BMDMs to the same degree as in WT macrophages (Fig 5h), suggesting a role of
HDAC10 or additional HDACs. LMK235, which inhibits multiple HDACs but has greatest
potency against class IIa HDACs, also markedly reduced intracellular L. pneumophila
numbers (Fig 5i). Taken together, this suggests that butyrate likely acts by inhibiting one or
more HDACs to attenuate Slc7a11-dependent cystine import, thus preventing intracellular L.
pneumophila replication in macrophages.

Butyrate controls L. pneumophila lung infections
We have previously shown that high-fiber diets increase serum butyrate levels, which then
affect immune responses in several tissues (28). Mice fed on high fiber diets, however, did
not display any changes in their ability to control L. pneumophila lung burdens compared to
no-fiber fed control animals (Fig 6a). In contrast, intranasal administration of the mixture of
SCFAs, acetate, propionate, and butyrate, reduced L. pneumophila lung burdens (Fig 6b).
Finally, butyrate alone was sufficient to reduce L. pneumophila lung burdens after intranasal
administration (Fig 6c). This shows that intranasal administration of butyrate promotes
immune responses that control Legionella lung infections.

Discussion
HDACs control macrophage function by regulating their development and metabolic
programs that underpin their activation (46). The short chain fatty acid butyrate inhibits the
activity of most HDACs or controls their expression (47). Consequently, butyrate affects
macrophage responses to pathogenic and commensal bacteria. This is best understood in the
gut, whereby butyrate alters immune response in gut macrophages resulting in reduced
inflammation to tolerate commensal bacteria and increased antimicrobial responses to protect
from invading pathogens (24, 32, 33, 48). The effects on gut macrophages are primarily
driven by inhibiting class I HDAC3 (33, 44). Here, we have shown that intranasal butyrate administration reduced *L. pneumophila* lung infections in mice. We have identified that butyrate regulated the expression of nutrient transporters during *Legionella* infections via epigenetic modification that likely involves class IIa and b HDACs. In particular, butyrate prevented the upregulation of the cystine/glutamate exchanger Slc7a11 during *Legionella* infections. Butyrate treatment reduced cellular cysteine levels in infected macrophages. Slc7a11 expression promoted intracellular replication of *Legionella* as these pathogens are auxotrophic for cysteine and depend on host-derived amino acids as carbon source. We thus propose that butyrate promotes nutritional immunity to protect against intracellular infections by *Legionella*.

Butyrate has a well-established activity in dampening inflammation in macrophages. For instance, butyrate prevents the expression of inflammatory cytokines after LPS exposure (23) and this is also true in *Legionella* infections as shown here. This would suggest that increased levels of butyrate may predispose to certain infections, an important consideration for current efforts to treat unrelated diseases with SCFA supplementation. Butyrate also affects the metabolism of host cells such as macrophages by promoting oxidative phosphorylation (24) and reducing glycolysis (33), thus controlling additional immune responses. However, we did not detect marked changes in core mitochondrial and glycolytic enzymes in butyrate treated macrophages after *Legionella* infections. This may relate to our observation that *Legionella* infections only marginally affected the expression of core metabolic enzymes (Fig 2b), in stark contrast to other infections which upregulate the expression of metabolic genes (49, 50). *Legionella* infections, nevertheless, triggered metabolic changes that are consistent with the concept of immunometabolism, including increased levels of glycolytic metabolites, such as PEP, 2P glycerate and DHAP, and mitochondrial intermediates, such as succinate and itaconate. This suggests that intracellular *Legionella* interfere with host metabolic pathways
to promote their own survival. Studies on cultured *L. pneumophila* have highlighted unique metabolic programs that drive bacteria growth whereby amino acids such as serine and cysteine are used as major carbon sources, even in the presence of glucose (10). Despite this notion, intracellular *L. pneumophila* inhibit mitochondrial function and promote glycolysis of macrophages (51). The latter is dependent on the degradation of host glycogen via a *L. pneumophila* effector that contains amylase activity, which evolved to prevent amoeba cyst formation rather than to generate glucose as carbon source (8). Intriguingly, our analysis indicates the upregulation of the pentose phosphate pathway in infected macrophages which depends on glucose. It is tempting to speculate that the *Legionella* depend on host pentose phosphate to generate NADPH which enables the reduction of cystine to cysteine. Whether *L. pneumophila* specifically regulates host glycolysis and the pentose phosphate pathway awaits further clarification. It is clear, however, that intracellular *L. pneumophila* target host pathways that regulate amino acid levels, including the proteasome and host protein synthesis (15, 17, 52) (53). Our data demonstrates that cystine import via Slc7a11 provides an additional important pathway to fulfil the nutritional demands of intracellular *Legionella*. The activity of Slc7a11 causes decreased glutamate and glutamine levels in infected macrophages, which likely do not directly affect *Legionella*. Other amino acids are similarly decreased such as leucine and isoleucine which are essential for *Legionella* growth. Whether this is due to bacterial activity of host factors remains unknown.

Slc7a11 is highly upregulated in *Legionella* infected macrophages likely as part of the oxidative burst that occurs during phagocytosis. This is because cystine import via Slc7a11 enables glutathione synthesis, which is important to prevent cellular damage during oxidative stress. Consistent with previous reports, we find that glutathione itself plays only a minor role in *Legionella* infections (54, 55). Treatments that prevent the expression of Slc7a11 or its activity, such as butyrate and erastin, reduce intracellular *Legionella* burdens in macrophages,
unless the culture media is supplemented with cysteine. Cysteine supplementation also
enabled intracellular *Legionella* growth after 24 hours of butyrate treatment, suggesting that
the decreased cysteine levels in macrophages primarily affect bacterial replication not so
much their survival. Commonly used culture media contain cystine as it is more stable that
cysteine. Media that contain reducing agents to preserve cysteine and other supplements alter
the effects of butyrate on intracellular *Legionella*, highlighting that subtle changes to culture
conditions affect experimental outcomes. In addition, Slc7a11 expression is tightly regulated.
In contrast to antimicrobial responses, Slc7a11 expression is independent of HDAC3 activity
(44) but dependent on tubastatin A. This raises the possibility that butyrate or HDAC specific
inhibitors can be harnessed to treat infectious diseases to dampen hyperinflammation and
prevent microbial growth. Such an approach has been demonstrated in human macrophages,
whereby tubastatin A promoted clearance of intracellular *Salmonella* (56). To further this
idea, it will be important to understand the roles of butyrate *in vivo*. In animal models,
butyrate prevents inflammation and infections in the gut (24, 31, 33). In addition, increased
serum butyrate levels affect other organs and their immune cells and thus prevent detrimental
lung inflammation in flu infections (27). Similar approaches using high fibre diets or oral
butyrate failed to affect *L. pneumophila* lung burdens, suggesting that alveolar macrophages
may have limited access to serum butyrate levels or that serum levels are insufficient to
reduce *L. pneumophila* lung burdens. This is in line with recent reports showing that alveolar
macrophages have a different metabolic capacity and reduced access to serum-derived
metabolites such as glucose (57). Therefore, we have administered butyrate intranasally and
show that this is sufficient to reduce *L. pneumophila* lung burdens. Previous studies have
further identified an important role of Slc7a11 in alveolar macrophages, as it is upregulated in
patients containing active *Mycobacteria tuberculosis* lung infections (58). Slc7a11-deficient
mice are protected from *M. tuberculosi*s infections depending on increased clearance by
macrophages (58). This suggests that intranasal butyrate administration may also be beneficial in other bacterial lung infections.

In conclusion, our results demonstrate that butyrate treatment enables macrophages to control the intracellular growth of *Legionella* independent of inflammation. The underlying mechanism depends on HDAC inhibition by butyrate, resulting in attenuated expression of the cystine/glutamate exchanger Slc7a11, decreased intracellular cysteine levels and prevention of cysteine-dependent growth of *Legionella*. Butyrate thus controls the two major detrimental outcomes in lung infections, namely hyperinflammation and bacterial growth.

**Material and Methods**

**Ethics statement**

Animal experiments were performed in accordance with the National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals and were approved by the Monash University Animal Ethics Committee (approval number 14252). All mice were maintained under specific pathogen-free conditions. Age- and sex-matched mice were used in the treatment groups without randomization or blinding of the experimenter.

**Bacterial strains**

*Legionella pneumophila* 130b serogroup 1 (ATCC BAA-74) is a spectinomycin-resistant clinical isolate from the Wadsworth Veterans Administration Hospital, Los Angeles, CA. The flagellin-deficient Δ*flaA* is a deletion mutants of *L. pneumophila* 130b. GFP was expressed constitutively using the plasmid pMIP::GFP as described previously (Speir et al). The *L. longbeachae* NSW-150 strain is a serogroup 1 clinical isolate from Australia. *L. pneumophila LpO2 ΔflaA* is a derivative of *L. pneumophila* Philadelphia-1 that is thymidine auxotroph.
Salmonella enterica subsp. enterica serovar Typhimurium SL1344 and E. coli DH5α were used in this study.

Legionella strains were grown at 37 °C from –80 °C frozen glycerol (60 %) stocks on buffered charcoal-yeast extract (BCYE) agar for 48 h before each infection. Thymidine was added at 100 µg. mL⁻¹ for the thymidine auxotroph strains. To determine bacterial numbers before infection, an inoculation loop full of Legionella was scraped from the plate, re-suspended in 1 mL phosphate-buffered saline (PBS), and the optical density at 600 nm (OD₆₀₀) determined. An OD₆₀₀ of 1 was taken to be equal to 10⁹ bacteria/ml. Macrophages were infected with a multiplicity of infection (MOI) of 10, or as indicated.

To determine axenic growth rates, L. pneumophila, Salmonella and E. coli were grown overnight with continuous shaking at 37°C in buffered yeast extract (BYE) broth (1% yeast extract, 1%ACES at Ph 6.9, 3mM l-cysteine and 0.3mM ferric nitrate) or LB media. Bacterial density was estimated by absorbance at 600nm. Bacteria were then diluted to the OD of 0.1 in BYE broth and the optical density was taken at 600nm for every 2 hours for 24 hrs at 37°C in Tecan plate reader.

**Mice infections**

C57BL/6 wild type mice were bred at the Monash Animal Research Platform (Monash University) and infected in the morning (9-11am). 6-8-week-old male or female mice were anesthetised by 5 % isofluorane inhalation for 3-5 min to induce a moderate state of unconsciousness. Mice were then infected intra-nasally using a P200 pipette to drip 50 µL of sterile PBS containing either 2.5 × 10⁶ L. pneumophila ∆flaA onto the nose whilst holding the mouse loosely by the scruff of the neck in a vertical position. In some mice, butyrate (1mM) or vehicle (PBS) was included in bacterial inoculum without increasing the volume. Mice were
fed ad libidum regular chow or high-fiber or no-fiber (control) diets at least 1 week prior to infections. Mice were monitored until fully recovered. To determine bacterial lung loads, mice were euthanised via inhalation of CO$_2$ at the indicated time points. Both lung lobes were removed and homogenised for 20-30 s in 2 mL of PBS at 30 000 rpm, using the Omni Tissue Master homogeniser. Serial dilutions of the lung homogenates were plated onto BCYE agar plates. Bacterial colonies were counted after incubation at 37 °C for 72 hours.

Cell culture

Murine bone marrow-derived macrophages (BMDMs) were obtained from femur and tibia of female/male 6-8-week-old C57BL/6 WT or HDAC6$^{-/-}$ mice (59). Macrophages were cultured in RPMI 1640 medium supplemented with 15 % fetal bovine serum (Bovogen), 15% L-cell-conditioned medium (containing macrophage colony-stimulating factor), and 100 U/mL of penicillin-streptomycin (Sigma) in bacteriological dishes for 7 days, at 37 °C + 5 % CO$_2$. For infections, BMDMs were gently scraped from plates using a cell scraper (BD Falcon) and washed 3 times in PBS, before seeding into tissue culture-treated plates.

For the isolation of alveolar macrophages, lungs were snipped into small fragments and digested for up to 1 hour in lung digestion media (300 U/mL Collagenase type I (Worthington) and 50 U/mL DNAse I (Sigma-Aldrich) in RPMI-1640) in a 37°C water bath with occasional agitation using a pipette. The digested lung samples were passed through a 70-micron cell strainer and centrifuged. The cell pellet was resuspended in red blood cell lysis buffer, washed, enumerated and resuspended in FACS buffer. Lung cells were labelled with biotinylated antibody to CD45, followed by anti-biotin magnetic beads (Miltenyi Biotec). The cells were then subjected to a magnetic separator, and the positive fraction containing CD45$^+$ cells were collected. Cells were then labelled with Streptavidin (BD Biosciences) and a panel of antibodies targeting CD11b (clone M1/70; Biolegend), CD11c (clone HL3; BD Biosciences),
Siglec F (clone E50-2440; BD Biosciences) and Ly6G (clone 1A8; BD Biosciences). FcR
block (clone 2.4G2; WEHI) was added to reduce non-specific binding. Propidium iodide
(Sigma-Aldrich) was used to distinguish live cells from dead cells. Alveolar macrophages were
sorted on a BD Influx™ Cell Sorter (BD Biosciences).

Human THP-1 monocytes, originally isolated from a male patient, were cultured in RPMI 1640
medium supplemented with 12.5 mM HEPES, 10% FBS, and 100 U/mL penicillin-
streptomycin. Cells were differentiated to macrophages by supplementing 300 nM phorbol 12-
myristate 13-acetate (PMA, Sigma, P8139) into the media for 24 h followed by recovery for
24 h in fresh media without PMA. Differentiation to macrophages was confirmed by evaluating
cell adhesion and spreading under a dissection microscope.

**Quantification of *Legionella* burdens in vitro**

To determine bacterial burdens, macrophages were seeded at a density of $5 \times 10^5$ cells/mL into
24-well tissue culture plates and infected with *Legionella* strains at a MOI of 10 with or without
treatments. After 2 h, medium was replaced, cells washed with PBS and then further incubated
in media with or without treatment. At the indicated time points, cells were lysed in 0.05 %
digitonin for 5 min at room temperature and serial dilutions of the cell lysates and the
corresponding culture media were plated on BCYE agar plates, and bacterial colonies counted
after 48 h at 37 °C.

**Live-cell imaging**

Cells were seeded at a density of $5 \times 10^5$ cells/mL in 96-well tissue culture plates and cell death
was determined essentially as described previously (41). Cells were stained with 1 µM Cell
Tracker Green (CTG) (Invitrogen) for 20 min in serum-free RPMI 1640. Medium was then
replaced with RPMI 1640 supplemented with 15 % FBS and 15 % L-cell-conditioned medium.
Cells were stained with 0.6 µM Draq7 (Abcam). Cells were infected at a MOI between 10 (or as indicated) in triplicate biological repeats. Alternatively, unstained BMDMs were infected with GFP-expressing L. pneumophila ΔflaA (MOI = 10). Cells were imaged every 60 minutes on Leica AF6000 LX or DMi8 epi-fluorescence microscope containing a heated 5% carbon dioxide incubator and motorized stage with a 10× objective (NA: 0.8). Images were processed and analysed using MetaMorph, Excel and GraphPad Prism.

**Immuno-blot analysis**

2.5 × 10^5 cells were lysed in 120 µL SDS-loading dye, boiled for 5 min, and samples analyzed by 12–15% SDS-PAGE. After transfer to nitrocellulose membranes (Millipore), membranes were blocked with 5% skim milk in T-BST (Tween-20, Tris-buffer) or 3% BSA for 1 h at room temperature. Membranes were probed with anti-cleaved caspase-3 antibody (CST #9964), anti-BCL-XL antibody (CST #2764), anti-MCL-1 antibody (CST #5453), anti-xCT/Slc7a11 (CST# 98051), anti-acetyl histone H3(lys9) antibody (Millipore# 07-352), anti-histone H3 (abcam ab1791), anti-β-actin antibody (CST#8457), anti-GABARAP (CST#13733), anti-p62 (CST,5114) and anti-tubulin (CST#3873) re-suspended in T-BST and 5% skim milk or 3% BSA overnight at 4 °C. After washing, membranes were probed with secondary goat anti-rabbit IgG (Life Technologies) and goat anti-mouse IgG (Life Technologies) antibodies conjugated to HRP (1:10,000 dilution in T-BST + 5% skim milk or 3%BSA). Membranes were developed with the luminol-based enhanced chemiluminescence (ECL) and exposed to film (Kodak). Scanned images were processed in Photoshop Adobe.

**Quantitative RT-PCR**

Total RNA was isolated using the TRIZOL Reagent (Ambion). Equal amounts of cDNA were synthesized using Superscript III (Invitrogen). Quantitative PCR (qPCR) was performed on Agilent AriaMx Real-TIime PCR system using the FastStart Universal SYBR Green
Master Rox (Roche) master mix using the following conditions: 95°C, 10min; 95°C, 20sec; 60°C, 20sec (45x); 72°C, 20sec; 95°C, 15sec; 60°C, 15sec; slow increase of temperature to 95°C within 20min; 95°C, 15sec; 12°C. The specificity of the reaction was verified by melt curve analysis. The threshold crossing value was noted for each transcript and normalized to the internal control. The relative quantitation of each mRNA was performed using the comparative Ct method. Experimental data processing was performed using AriaMx (Agilent System). The following qPCR primers were used for this study.

| Slc7a11 F | agcaggttccacagegaagt |
| Slc7a11 R | tggccagctcgcgaatgaa |
| Actin F | cattgtgacaggatgcagaagg |
| Actin R | tgcttgaaggggacagtgagg |

RNA Seq

For RNA-seq experiment, 2 x 10^6 BMDMs/ml were uninfected or infected with *L. pneumophila* ΔflaA at a MOI of 10 for 3 hrs and treated with or without butyrate (1mM) or Trichostatin A (TSA, 10nM). Cells were washed in PBS and treated with Trizol reagent to isolate total RNA. The quality of total RNA was verified with the Bioanalyzer. One microgram of total RNA was used for library preparation by the PAT-seq method (60). In brief, a biotinylated PAT-anchor primer cagacgtgtgctcttccgatctttttttttttttttttttt compatible with Illumina adaptor sequences was used to generate a 3′ tag on RNA ensuring that reverse transcription is only possible from true 3’ends. A limited RNA digest was performed using 10 units of RNase T1 for 2 minutes, followed by chloroform/phenol extraction. Streptavidin magnetic beads were used to collect the 3′ fragments and 5′ ends were phosphorylated with T4 Polynucleotide Kinase. To ligate a 5′ splinted linker, the PAT-seq Splint A 5′-ccctacagcagctctcgrA(rC)(rT)-3′ and PAT-seq Splint B 3′-
gggatgtgctgcgagaaggctagannnn-5’ were pre-annealed and then ligated to the 5’ end of the 3’ fragments with T4 RNA ligase 2. Excess splint was removed by washing the magnetic beads. Reverse transcription was performed with SuperScript III utilizing the PAT-seq end-extend primer on the magnetic matrix. The cDNA was eluted from the beads in 2x formamide gel loading buffer and size selected (~150 – 400bp) on a 6% urea-PAGE. After excision from the gel, the cDNA was eluted by the “crush and soak” method followed by ethanol precipitation. One-third of the purified cDNA was used as input for 16 cycles of amplification with PAT-seq Universal forward sequencing primer 5’-

aatgatacgccgacccgccgacctcactctttccctacacgacgctcttccg-3’ and ScriptSeq Index PCR reverse primer and AmpliTaq Gold 360 Master Mix. PAT-seq libraries were sequenced with the Illumina Hiseq1500 platform using 150 base rapid chemistry according to the manufacturer’s instructions. To assign reads to a specific genome, reads were first clipped of poly(A) tail and low-quality sequence using Tail Tools (60). Clipped reads shorter than 20 bases were discarded. The analysis of the gene expressions was done using Degust software and the data is available online (https://degust.erc.monash.edu/degust/version/4.1/compare.html?code=01485e0b3f4264ada6f
c6090ecka83a4#/).

Metabolomics

The BMDMs were plated at cell density of 4x10^6 cells/10 cm dish overnight. Next day, BMDMs were infect the L. pneumophila ΔflaA at MOI of 10 for 6hr with or without butyrate (1mM). Media was removed, cells washed in ice cold (4°C) PBS and dish placed on ice for the remaining of the extraction. Metabolites were extracted with 750uL of extraction solvent (with or without 25 mM NEM in 80% methanol 20% 10mM ammonium formate, pH 7) at 4°C. Cells extracts were removed from the dish with a plastic cell scraper and thoroughly vortexed for 60 min at 4°C in Eppendorf tubes. After centrifugation at 20,000 x g for 10 min
4°C, supernatant was transferred to new tubes and evaporated under nitrogen stream at 20-25°C. Extract was stored at -80°C and prior to analysis solubilized in 120 µL of 80% methanol. After centrifugation at 20,000 x g for 10 minutes at 4°C, supernatant was transferred into liquid-chromatography and mass spectrometry (LC-MS) vials. Samples were analysed by LC-MS as and analysed with IDEOM software as described previously (61). The identified metabolites are listed in Table 1.

List of Inhibitors Used

| Name                                      | Company,catalogue | Concentration |
|-------------------------------------------|-------------------|---------------|
| TSA(Trichostatin A)                       | T8552, Sigma      | 10nM          |
| Tubastatin                                 | SML0044, Sigma    | 10µM          |
| RGFP966                                    | SML1652, Sigma    | 10µM          |
| Bortezomib                                 | Ab142123, Abcam   | 5nM           |
| Ferrostatin                                | SML0583, Sigma    | 1mM           |
| BSO(L buthionine sulfoximine)              | B2515, Sigma      | 10µM          |
| Erastin                                    | E7781, Sigma      | 5µM           |
| Sodium Butyrate                            | 303410, Sigma     | 1mM           |
| Cysteine(L-cysteine hydrochloride monohydrate) | C6852 ,Sigma   | 1mM           |
| Beta Mercapto ethanol                      | M6250, Sigma      | 50µM          |
| Mg132                                      | S2619, Selleckchem | 100nM        |
| GSH                                        | G1404, Sigma      | 50µg/ml       |

Statistical analysis

All statistical analyses were performed on GraphPad Prism 8. Statistically significant results were reported with exact $P$ values. Any non-significant comparisons are indicated by the absence of $P$ values. For comparison of two groups, unpaired two-tailed $t$-tests were used.
**Author contribution**

GA, AS, CKB, TNN, TB performed experiments. RK, MJS, EM generated material and analyzed data. TSPH, ML, AT, DJC, THB conceptualized experiments and analyzed data, GA, RK, MJS, AT, EM and TN wrote the manuscript.

**Acknowledgements**

We are grateful to Yusun Jeon, Tricia Lo and Dr Jiyoti Verma (Monash University) for assistance with qRT-PCR, Dr Patrick Matthias (Friedrich Miescher Institute, Switzerland) for providing HDAC6−/− mice, and the Monash Micro Imaging, Monash Animal Research Platform and Monash Bioinformatics Platform for support with imaging, animal studies and data analysis, respectively. The work was supported by funds from the Australian Research Council (TN, Future Fellowship) and National Health and Medical Research Council (TN).

**References**

1. Flannagan RS, Cosio G, Grinstein S. 2009. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. Nat Rev Microbiol 7:355-66.
2. MacMicking JD. 2012. Interferon-inducible effector mechanisms in cell-autonomous immunity. Nat Rev Immunol 12:367-82.
3. Nunez G, Sakamoto K, Soares MP. 2018. Innate Nutritional Immunity. J Immunol 201:11-18.
4. Subramanian Vignesh K, Landero Figueroa JA, Porollo A, Caruso JA, Deepe GS, Jr. 2013. Granulocyte macrophage-colony stimulating factor induced Zn sequestration enhances macrophage superoxide and limits intracellular pathogen survival. Immunity 39:697-710.
5. von Pein JB, Stocks CJ, Schembri MA, Kapetanovic R, Sweet MJ. 2021. An alloy of zinc and innate immunity: Galvanising host defence against infection. Cell Microbiol 23:e13268.
6. Murray HW, Szuro-Sudol A, Wellner D, Oca MJ, Granger AM, Libby DM, Rothermel CD, Rubin BY. 1989. Role of tryptophan degradation in respiratory burst-independent antimicrobial activity of gamma interferon-stimulated human macrophages. Infect Immun 57:845-9.
7. Ganesan S, Roy CR. 2019. Host cell depletion of tryptophan by IFNgamma-induced Indoleamine 2,3-dioxygenase 1 (IDO1) inhibits lysosomal replication of *Coxiella burnetii*. PLoS Pathog 15:e1007955.
8. Price C, Jones S, Mihelic M, Santic M, Abu Kwaik Y. 2020. Paradoxical Pro-inflammatory Responses by Human Macrophages to an Amoebae Host-Adapted Legionella Effector. Cell Host Microbe doi:10.1016/j.chom.2020.03.003.

9. Brown AS, Yang C, Fung KY, Bachem A, Bourges D, Bedoui S, Hartland EL, van Driel IR. 2016. Cooperation between Monocyte-Derived Cells and Lymphoid Cells in the Acute Response to a Bacterial Lung Pathogen. PLoS Pathog 12:e1005691.

10. Eylert E, Herrmann V, Jules M, Gillmaier N, Lautner M, Buchrieser C, Eisenreich W, Heuner K. 2010. Isotopologue profiling of Legionella pneumophila: role of serine and glucose as carbon substrates. J Biol Chem 285:22232-43.

11. Qiu J, Luo ZQ. 2017. Legionella and Coxiella effectors: strength in diversity and activity. Nat Rev Microbiol 15:591-605.

12. Rolando M, Escoll P, Nora T, Botti J, Boitez V, Bedia C, Daniels C, Abraham G, Stogios PJ, Skarina T, Christophe C, Dervins-Ravault D, Cazalet C, Hilbi H, Rupasinghe TW, Tull D, McConville MJ, Ong SY, Hartland EL, Codogno P, Levade T, Naderer T, Savchenko A, Buchrieser C. 2016. Legionella pneumophila S1P-lyase targets host sphingolipid metabolism and restrains autophagy. Proc Natl Acad Sci U S A 113:1901-6.

13. Choy A, Dancourt J, Mugo B, O’Connor TJ, Isberg RR, Melia TJ, Roy CR. 2012. The Legionella effector RavZ inhibits host autophagy through irreversible Atg8 deconjugation. Science 338:1072-6.

14. Fonseca MV, Swanson MS. 2014. Nutrient salvaging and metabolism by the intracellular pathogen Legionella pneumophila. Front Cell Infect Microbiol 4:12.

15. Price CT, Al-Quadan T, Santic M, Rosenshine I, Abu Kwaik Y. 2011. Host proteasomal degradation generates amino acids essential for intracellular bacterial growth. Science 334:1553-7.

16. De Leon JA, Qiu J, Nicolai CJ, Counihan JL, Barry KC, Xu L, Lawrence RE, Castellano BM, Zoncu R, Nomura DK, Luo Z-Q, Vance RE. 2017. Positive and Negative Regulation of the Master Metabolic Regulator mTORC1 by Two Families of Legionella pneumophila Effectors. Cell Reports 21:2031-2038.

17. Hempstead AD, Isberg RR. 2015. Inhibition of host cell translation elongation by Legionella pneumophila blocks the host cell unfolded protein response. Proc Natl Acad Sci U S A 112:E6790-7.

18. Wieland H, Ullrich S, Lang F, Neumeister B. 2005. Intracellular multiplication of Legionella pneumophila depends on host cell amino acid transporter SLC1A5. Mol Microbiol 55:1528-37.

19. O'Neill LA, Pearce EJ. 2016. Immunometabolism governs dendritic cell and macrophage function. J Exp Med 213:15-23.

20. Price JV, Vance RE. 2014. The macrophage paradox. Immunity 41:685-93.

21. Traven A, Naderer T. 2019. Central metabolic interactions of immune cells and microbes: prospects for defeating infections. EMBO Rep 20:e47995.

22. Louis P, Hold GL, Flint HJ. 2014. The gut microbiota, bacterial metabolites and colorectal cancer. Nat Rev Microbiol 12:661-72.

23. Chang PV, Hao L, Offermanns S, Medzhitov R. 2014. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. Proc Natl Acad Sci U S A 111:2247-52.

24. Scott NA, Andrusaite A, Andersen P, Lawson M, Alcon-Giner C, Leclaire C, Caim S, Le Gall G, Shaw T, Connolly JPR, Roe AJ, Wessel H, Bravo-Blas A, Thomson CA, Kästele V, Wang P, Peterson DA, Bancroft A, Li X, Grencis R, Mowat AM, Hall LJ, Travis MA, Milling SWF, Mann ER. 2018. Antibiotics induce sustained dysregulation of intestinal T cell immunity by perturbing macrophage homeostasis. Science Translational Medicine 10:eaa04755.
25. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, Nakanishi Y, Uetake C, Kato K, Kato T, Takahashi M, Fukuda NN, Murakami S, Miyauchi E, Hino S, Atarashi K, Onawa S, Fujimura Y, Lockett T, Clarke JM, Topping DL, Tomita M, Uetake C, Kato K, Kato T, Takahashi M, Fukuda NN, Murakami S, Miyauchi E, Hino S, Atarashi K, Onawa S, Fujimura Y, Lockett T, Clarke JM, Topping DL, Tomita M. 2013. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature 504:446-50.

26. Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, Blanchard C, Junt T, Nicod LP, Harris NL, Marsland BJ. 2014. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nat Med 20:159-66.

27. Trompette A, Gollwitzer ES, Pattaroni C, Lopez-Mejia JC, Riva E, Pernot J, Ubags N, Fajas L, Nicod LP, Marsland BJ. 2018. Dietary Fiber Confers Protection against Flu by Shaping Ly6c – Patrolling Monocyte Hematopoiesis and CD8 + T Cell Metabolism. Immunity 48:992-1005.e8.

28. Marino E, Richards JL, McLeod KH, Stanley D, Yap YA, Knight J, McKenzie C, Kranich J, Oliveira AC, Rossello FJ, Krishnamurthy B, Nezger CM, Macia L, Thorburn A, Baxter AG, Morahan G, Wong LH, Polo JM, Moore RJ, Lockett T, Clarke JM, Topping DL, Harrison LC, Mackay CR. 2017. Gut microbial metabolites limit the frequency of autoimmune T cells and protect against type 1 diabetes. Nat Immunol 18:552-562.

29. Richards JL, Yap YA, McLeod KH, Mackay CR, Marino E. 2016. Dietary metabolites and the gut microbiota: an alternative approach to control inflammatory and autoimmune diseases. Clin Transl Immunology 5:e82.

30. Thorburn AN, McKenzie CI, Shen S, Stanley D, Macia L, Mason LJ, Roberts LM, Wong CH, Shim R, Robert R, Chevalier N, Tan JK, Marino E, Moore RJ, Wong L, McConvile MJ, Tull DL, Wood LG, Murphy VE, Maths J, Gibson PG, Mackay CR. 2015. Evidence that asthma is a developmental origin disease influenced by maternal diet and bacterial metabolites. Nat Immunol 16:552-562.

31. Fachi JL, Felipe JS, Pral LP, da Silva BK, Correa RO, de Andrade MCP, da Fonseca DM, Basso PJ, Camara NOS, de Sales ESEL, Dos Santos Martins F, Guima SES, Thomas AM, Setubal JC, Magalhaes YT, Forti FL, Candreva T, Rodrigues HG, de Jesus MB, Consonni SR, Farias ADS, Vargas-Weisz P, Vinolo MAR. 2019. Butyrate Protects Mice from Clostridium difficile-Induced Colitis through an HIF-1-Dependent Mechanism. Cell Rep 27:750-761 e7.

32. Rivera-Chavez F, Zhang LF, Faber F, Lopez CA, Byndloss MX, Olsan EE, Xu G, Velazquez EM, Lebrilla CB, Winter SE, Baumler AJ. 2016. Depletion of Butyrate-Producing Clostridia from the Gut Microbiota Drives an Aerobic Luminal Expansion of Salmonella. Cell Host Microbe 19:443-54.

33. Schulthess J, Pandey S, Capitan M, Rue-Albrecht KC, Arnold I, Franchini F, Chomka A, Ilott NE, Johnston DGW, Pires E, McCullagh J, Sansom SN, Arancibia-Carcamo CV, Uhlig HH, Powrie F. 2019. The Short Chain Fatty Acid Butyrate Imprints an Antimicrobial Program in Macrophages. Immunity 50:432-445 e7.

34. Antunes KH, Fachi JL, de Paula R, da Silva EF, Pral LP, Dos Santos AA, Dias GBM, Vargas JE, Puga R, Mayer FQ, Maito F, Zarate-Blades CR, Ajami NJ, Sant’Ana MR, Candreva T, Rodrigues HG, Schmiele M, Silva Clerici MTP, Proenca-Modena JL, Vieira AT, Mackay CR, Mansur D, Caballero MT, Marzee J, Li J, Wang X, Bell D, Polack FP, Kleeberger SR, Stein RT, Vinolo MAR, de Souza APD. 2019. Microbiota-derived acetate protects against respiratory syncytial virus infection through a GPR43-type 1 interferon response. Nat Commun 10:3273.

35. Prow NA, Hirata TDC, Tang B, Larcher T, Mukhopadhyay P, Alves TL, Le TT, Gardner J, Poo YS, Nakayama E, Lutzky VP, Nakaya HI, Suhrbier A. 2019.
Exacerbation of Chikungunya Virus Rheumatic Immunopathology by a High Fiber Diet and Butyrate. Front Immunol 10:2736.

36. Ren T, Zamboni DS, Roy CR, Dietrich WF, Vance RE. 2006. Flagellin-deficient Legionella mutants evade caspase-1- and Naip5-mediated macrophage immunity. PLoS Pathog 2:e18.

37. Edwards RL, Dalebroux ZD, Swanson MS. 2009. Legionella pneumophila couples fatty acid flux to microbial differentiation and virulence. Mol Microbiol 71:1190-1204.

38. Naujoks J, Tabeling C, Dill BD, Hoffmann C, Brown AS, Kunze M, Kempa S, Peter A, Mollenkopf HJ, Dorhoi A, Kershaw O, Gruber AD, Sander LE, Wittenrath M, Herold S, Nerlich A, Hocke AC, van Driel I, Suttorp N, BedouI S, Hilbi H, Trost M, Opitz B. 2016. IFNs Modify the Proteome of Legionella-Containing Vacuoles and Restrict Infection Via IRG1-Derived Itaconic Acid. PLoS Pathog 12:e1005408.

39. Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, Patel DN, Bauer AJ, Cantley AM, Yang WS, Morrison B, 3rd, Stockwell BR. 2012. Ferroptosis: an iron-dependent form of nonapoptotic cell death. Cell 149:1060-72.

40. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, Schilter HC, Rolph MS, Mackay F, Artis D, Xavier RJ, Teixeira MM, Mackay CR. 2009. Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature 461:1282-6.

41. Speir M, Lawler K, Glaser S, Abraham G, Chow S, Voigrin AJ, Schulze KE, Schuelein R, O’Reilly LA, Mason K, Hartland EL, Lithgow T, Grass A, Lessene G, Huang DC, Vince VE, Naderer T. 2016. Eliminating Legionella by inhibiting BCL-XL to induce macrophage apoptosis. Nature Microbiology 1:15034.

42. Gomez-Valero L, Rusniok C, Carson D, Mondino S, Perez-Cobas AE, Rolando M, Pasricha S, Reuter S, Demirtas J, Crumbach J, Descorps-Declere S, Hartland EL, Jarraud S, Dougan G, Schroeder GN, Frankel G, Buchrieser C. 2019. More than 18,000 effectors in the Legionella genus genome provide multiple, independent combinations for replication in human cells. Proc Natl Acad Sci U S A 116:2265-2273.

43. Blad CC, Tang C, Offermanns S. 2012. G protein-coupled receptors for energy metabolites as new therapeutic targets. Nat Rev Drug Discov 11:603-19.

44. Chen X, Barozzi I, Termanini A, Prosperini E, Recchiuti A, Dalli J, Mietton F, Matteoli G, Hiebert S, Natoli G. 2012. Requirement for the histone deacetylase Hdac3 for the inflammatory gene expression program in macrophages. Proc Natl Acad Sci U S A 109:E2865-74.

45. Geraldy M, Morgen M, Sehr P, Steimbach RR, Moi D, Ridering J, Oehme I, Witt O, Malz M, Nogueira MS, Koch O, Gunkel N, Miller AK. 2019. Selective Inhibition of Histone Deacetylase 10: Hydrogen Bonding to the Gatekeeper Residue is Implicated. J Med Chem 62:4426-4443.

46. Shakespear MR, Iyer A, Cheng CY, Das Gupta K, Singhal A, Fairlie DP, Sweet MJ. 2018. Lysine Deacetylases and Regulated Glycolysis in Macrophages. Trends Immunol 39:473-488.

47. Bradbury CA, Khanim FL, Hayden R, Bunce CM, White DA, Drayson MT, Craddock C, Turner BM. 2005. Histone deacetylases in acute myeloid leukaemia show a distinctive pattern of expression that changes selectively in response to deacetylase inhibitors. Leukemia 19:1751-9.

48. Xiong H, Guo B, Gan Z, Song D, Lu Z, Yi H, Wu Y, Wang Y, Du H. 2016. Butyrate upregulates endogenous host defense peptides to enhance disease resistance in piglets via histone deacetylase inhibition. Sci Rep 6:27070.

49. Tucey TM, Verma J, Harrison PF, Sneglrove SL, Lo TL, Scherer AK, Barugahare AA, Powell DR, Wheeler RT, Hickey MJ, Beilharz TH, Naderer T, Traven A. 2018.
Glucose Homeostasis Is Important for Immune Cell Viability during Candida Challenge and Host Survival of Systemic Fungal Infection. Cell Metab 27:988-1006 e7.

50. Jiang L, Wang P, Song X, Zhang H, Ma S, Wang J, Li W, Lv R, Liu X, Ma S, Yan J, Zhou H, Huang D, Cheng Z, Yang C, Feng L, Wang L. 2021. Salmonella Typhimurium reprograms macrophage metabolism via T3SS effector SopE2 to promote intracellular replication and virulence. Nat Commun 12:879.

51. Escoll P, Song OR, Viana F, Steiner B, Lagache T, Olivo-Marin JC, Impens F, Brodin P, Hilbi H, Buchrieser C. 2017. Legionella pneumophila Modulates Mitochondrial Dynamics to Trigger Metabolic Repurposing of Infected Macrophages. Cell Host Microbe 22:302-316 e7.

52. Fontana MF, Banga S, Barry KC, Shen X, Tan Y, Luo ZQ, Vance RE. 2011. Secreted bacterial effectors that inhibit host protein synthesis are critical for induction of the innate immune response to virulent Legionella pneumophila. PLoS Pathog 7:e1001289.

53. Liu X, Boyer MA, Holmgren AM, Shin S. 2020. Legionella-Infected Macrophages Engage the Alveolar Epithelium to Metabolically Reprogram Myeloid Cells and Promote Antibacterial Inflammation. Cell Host Microbe doi:10.1016/j.chom.2020.07.019.

54. Kajiwara C, Kusaka Y, Kimura S, Yamaguchi T, Nanjo Y, Ishii Y, Udono H, Standiford TJ, Tateda K. 2018. Metformin Mediates Protection against Legionella Pneumonia through Activation of AMPK and Mitochondrial Reactive Oxygen Species. J Immunol 200:623-631.

55. Harada T, Miyake M, Imai Y. 2007. Evasion of Legionella pneumophila from the bactericidal system by reactive oxygen species (ROS) in macrophages. Microbiol Immunol 51:1161-70.

56. Ariffin JK, das Gupta K, Kapetanovic R, Iyer A, Reid RC, Fairlie DP, Sweet MJ. 2015. Histone Deacetylase Inhibitors Promote Mitochondrial Reactive Oxygen Species Production and Bacterial Clearance by Human Macrophages. Antimicrob Agents Chemother 60:1521-9.

57. Svedberg FR, Brown SL, Krauss MZ, Campbell L, Sharpe C, Clausen M, Howell GJ, Clark H, Madsen J, Evans CM, Sutherland TE, Ivens AC, Thornton DJ, Grencis RK, Hussell T, Cunosamy DM, Cook PC, MacDonald AS. 2019. The lung environment controls alveolar macrophage metabolism and responsiveness in type 2 inflammation. Nat Immunol 20:571-580.

58. Cai Y, Yang Q, Liao M, Wang H, Zhang C, Nambi S, Wang W, Zhang M, Wu J, Deng G, Deng Q, Liu H, Zhou B, Jin Q, Feng CG, Sassetti CM, Wang F, Chen X. 2016. xCT increases tuberculosis susceptibility by regulating antimicrobial function and inflammation. Oncotarget 7:31001-13.

59. Zhang Y, Kwon S, Yamaguchi T, Cubizolles F, Rousseaux S, Kneissel M, Cao C, Li N, Cheng HL, Chua K, Lombard D, Mizeracki A, Matthias G, Alt FW, Khochbin S, Matthias P. 2008. Mice lacking histone deacetylase 6 have hyperacetylated tubulin but are viable and develop normally. Mol Cell Biol 28:1688-701.

60. Harrison PF, Powell DR, Clancy JL, Preiss T, Boag PR, Traven A, Seemann T, Beilharz TH. 2015. PAT-seq: a method to study the integration of 3′-UTR dynamics with gene expression in the eukaryotic transcriptome. RNA 21:1502-10.

61. Peterson AL, Siddiqui G, Sloan EK, Creek DJ. 2021. beta-Adrenoceptor regulation of metabolism in U937 derived macrophages. Mol Omics doi:10.1039/d1mo00057h.
Figure 1. Butyrate controls intracellular *L. pneumophila* burdens. a, Colony forming units (CFUs) of *L. pneumophila* ΔflaA after infection of bone-marrow derived macrophages (BMDMs) in the presence of short chain fatty acids (SCFAs) together or individually (1mM each) 6 and 48 hours post infection. Mean and SEM from at least three independent experiments and unpaired two-tailed *t*-test used to determine *p*-values. b, BMDMs infected with GFP-expressing ΔflaA were treated with or without butyrate and imaged over 60 hours with time-lapse microscopy. Top are brightfield images showing GFP signals at 48 hours post-infection. Scale bar, 20µm. Bottom shows quantification of GFP positive BMDMs. Uninfected (UI) BMDMs are include as controls. Mean and SEM are shown from at least three independent experiments. c, CFUs of ΔflaA (48 hours post infection) from BMDMs treated with butyrate during infection (Butyrate), or pre-treated 24 hours prior to infection (Pre-Butyrate) or 24 hours post infections (Post-Butyrate). d, CFUs of ΔflaA in mouse alveolar macrophages 48 hours post infection. Mean from two independent experiments.
Figure 2. Butyrate regulates the expression of nutrient transporters in infected macrophages. 

a, RNAseq of bone marrow-derived macrophages (BMDMs) infected with L. pneumophila ∆flaA in the presence of butyrate 6 hours post infection. Volcano plot indicates fold change expression and false discovery rate (FDR) adjusted p-values from two independent experiments. Selected examples of significantly enriched Gene Ontology (GO) categories among genes up or down-regulated in infected BMDMs treated with butyrate. 

Number of genes and p-values shown. b, Examples of genes involved in inflammation or metabolism that are differentially regulated in uninfected (UI) or ∆flaA infected BMDMs treated with or without butyrate. Heatmap indicates relative expression levels. c, Relative mRNA levels of Slc7a11 normalized to actin mRNA levels in UI and ∆flaA BMDMs with or without butyrate treatment. Mean and SD from two independent experiments. d, Uninfected
(UI) and ΔflaA infected BMDMs treated with or without butyrate were analysed by immunoblotting for Slc7a11 expression. Actin is shown a loading control. e, Colony forming units (CFUs) from ΔflaA infected BMDMs treated with erastin and ferrostatin at 6 and 48 hours post infection. Mean and SEM from at least three independent experiments and unpaired two-tailed t-test used to determine p-values. f, CFUs from ΔflaA infected BMDMs treated with butyrate, glutathione (GSH) or buthionine sulfoximine (BSO) at 6 and 48 hours post infection. Mean and SEM from at least three independent experiments are shown.
Figure 3. Butyrate affects cysteine levels in infected macrophages. a, Targeted metabolite profiling of cysteine using N-ethylmaleimide derivatization (NEM-cysteine) in uninfected (UI) and Δ flaA infected bone-marrow derived macrophages (BMDMs) treated with or without butyrate (1mM). Mean and SEM from 5 biological replicates and unpaired two-tailed t-test was used to determine p-values. b, Colony forming units (CFUs) from Δ flaA infected BMDMs treated with butyrate and cysteine at 6 and 48 hours post infection. Mean and SEM from at least three independent experiments. c, BMDMs infected with GFP-expressing Δ flaA imaged at 18, 20, 26 and 37 hours post infection. Draq7 (blue) indicates dead cells.
Representative of three independent experiments. d, CFUs from ΔflaA infected BMDMs 48 hours post infection. BMDMs were treated with butyrate at the time of infection and cysteine 24 hours post infection (post Cys) or pre-treated with butyrate for 24 hours prior to infection and then supplemented with cysteine at the time of infection (pre-Butyrate+Cys). Mean and SEM from three independent experiments and unpaired two-tailed $t$-test used to determine p-values. e, CFUs from ΔflaA infected BMDMs cultured in cysteine/cystine-free media or supplemented with cysteine. Mean and SEM from at least three independent experiments. f, CFUs from ΔflaA infected BMDMs treated with butyrate and 2-mercaptoethanol. Mean and SEM from at least two independent experiments and p-values are from unpaired two-tailed $t$-test. g, Fold change of untargeted metabolite levels in ΔflaA infected BMDMs treated with butyrate compared to control treatments 6 hours post infection. Amino acids (red), nucleotides (orange), TCA intermediates (blue) and sugar metabolites (green). Mean from 5 biological replicates.
Figure 4. Butyrate affects *L. longbeachae* infections of macrophages. CFUs from ∆flaA *L. pneumophila* strain Lp02 infected BMDMs treated with (a) butyrate, (b) erastin, (c) MG132 or (d) combination of erastin and MG132. e and f, CFUs from ∆flaA *L. pneumophila* 130b infected BMDMs treated with MG132 or bortezomib and cysteine. g and h, CFUs from wild type *L. longbeachae* infected BMDMs treated with butyrate or erastin and cysteine. i, CFUs from wild type *L. longbeachae* infected BMDMs cultured in cysteine/cystine-free media or supplemented with cysteine. Mean and SEM from at least three independent experiments and p-values determined by unpaired two-tailed t-test.
Figure 5. Butyrate restricts intracellular *Legionella* growth by inhibiting HDAC activity. 

**a,** RNAseq of ΔflaA infected BMDMs treated with or without trichostatin A (TSA) at 6 hours post infection. Volcano blot showing fold change expression and false discovery rate (FDR) adjusted p-values from two independent experiments. 

**b,** Colony forming units (CFUs) from ΔflaA infected BMDMs treated with butyrate or TSA at 6 and 48 hours post infection. Mean and SEM from at least three independent experiments and p-values determined by unpaired two-tailed *t*-test. 

**c,** Uninfected (UI) and ΔflaA infected BMDMs treated with butyrate or TSA were analysed for protein levels of histone 3 (H3) and acetylated H3 (H3K9ac) by immunoblotting. 

**d,** e, f, and i, CFUs from ΔflaA infected BMDMs treated with the (d) HDAC3 inhibitor RGF966, (e, f) HDAC6/10 inhibitor Tubastatin A with or without cysteine (1mM), or (i) HDAC4/5 inhibitor LMK235. Mean and SEM from at least three independent experiments and p-values determined by unpaired two-tailed *t*-test. 

**g,** Relative mRNA levels of Slc7a11 normalized to actin mRNA in ΔflaA.
BMDMs with or without Tubastatin treatment. Mean and SD from two independent experiments. h, CFUs from ΔflaA infected WT and HDAC6−/− BMDMs. Mean and SEM from at least three independent experiments.
Figure 6. Butyrate reduces *L. pneumophila* lung burdens. a, C57Bl/6 mice fed control or high fiber diets were intranasally infected with ∆flaA *L. pneumophila* and lung burdens (CFUs/lung) determined 48 hours post infection. b, Mice were intranasally infected with ∆flaA together with the SCFAs acetate, propionate and butyrate. CFUs/lung was determined 48 hours post infections. c, CFUs/lung of mice intranasally infected with ∆flaA together with butyrate (1mM) at 48 hours post infection. Mean, SEM and data from individual mice are shown.
**Extended Data – supplementary figure legends**

**Figure S1. Role of butyrate in controlling *L. pneumophila* growth.** a, ΔflaA *L. pneumophila*, (b) *Salmonella* or (c) *E. coli* were cultured in the presence of increasing concentrations of butyrate and growth measured by optical density at 600nm over time. Mean and SEM from three independent experiments. d, Colony forming units (CFUs) from ΔflaA infected BMDMs cultured at increasing concentration of butyrate at 48 hours post infection. e, f and g, CFUs from ΔflaA infected (e) THP1 differentiated macrophages and (f) immortalized BMDMs treated with or without butyrate or (g) BMDMs differentiated in the presence of butyrate. Mean and SEM from at least three different experiments. h, Uninfected and ΔflaA infected BMDMs were treated with acetate, butyrate or propionate and expression of p62 and the LC3 protein GABARAP determined by immunoblotting. Actin is included as loading control. i, CFUs from ΔflaA infected BMDMs treated with butyrate and bafilomycin A (BafA) at 6 and 48 hours post infection. Mean and SEM from at least three independent experiments except for BafA treatment.
Figure S2. The role of apoptosis in butyrate treated macrophages. a and b, RNAseq analysis of (a) uninfected and ΔflaA *L. pneumophila* infected BMDMs and (b) uninfected BMDMs treated with or without butyrate. Volcano blot showing fold change expression and false discovery rate (FDR) adjusted p-values from two independent experiments. c, Uninfected and ΔflaA infected BMDMs treated with butyrate, propionate or ABT-737 (737) were analysed for MCL-1 (long and short form), BCL-XL and cleaved caspase-3 by
immunoblotting. Tubulin is shown as loading control. Representative of two independent experiments. d, CFUs of ΔflaA infected BMDMs treated with butyrate, QVP or ABT-737 at 6 and 48 hours post infection. Mean and SEM from at least three independent experiments. e, Uninfected and ΔflaA infected BMDMs treated with or without butyrate were analysed for cell death using live-cell imaging. Mean and SEM from three independent experiments.
Figure S3. The metabolism of *Legionella* infected macrophages. a, b, c. Targeted metabolite profiling of N-ethylmaleimide derivatized glutathione (a) and γ-glutamylcysteine (b) or serine (c) in uninfected or Δfla infected BMDMs treated with or without butyrate for 6 hours. Mean from 5 biological replicates. d, Fold change of significantly altered metabolites in ΔflaA infected BMDMs compared to uninfected macrophages. Amino acids (red), nucleotides (orange), TCA intermediates (blue) and sugar metabolites (green). Mean from 5 biological replicates. e, CFUs from ΔflaA infected BMDMs treated with butyrate in the presence of exogenous amino acids at 6 and 48 hours post infection. Mean and SEM from two independent experiments.
**Figure S4. GPCR and HDAC mediated signalling.**

**a,** Colony forming units (CFUs) from \( \Delta \text{flaA} \) infected wild type and GPR deficient BMDMs. Mean from two independent experiments shown.

**b,** Fold change expression of solute carriers in HDAC3 deficient and wild type BMDMs treated with LPS. Data derived from Chen et al (44).
Supplementary Files

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- Table1.xlsx