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

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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 to 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 (Δ flaA). Unlike wild type *L. pneumophila*, which induces rapid macrophage cell death due to the activation of the NLRC4/caspase-1 inflammasome (36), Δ 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 Δ 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 Δ 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, our 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, Pfkfb3) and TCA enzymes (Mdh1, 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).

183

184 To gain insights into how metabolic pathways are regulated by butyrate and whether this
185 affects intracellular *L. pneumophila* replication, we focused on Slc7a11 as this transporter
186 was the most highly upregulated gene in infected BMDMs and its expression was abrogated
187 by butyrate treatment (Fig 2b). We confirmed by qRT-PCR and immunoblot analysis that
188 Slc7a11 is induced in *L. pneumophila* infection but not in the presence of butyrate (Fig 2c
189 and d). The Slc7a11 inhibitor, erastin, reduced intracellular *L. pneumophila* burdens in
190 BMDMs (Fig 2e), suggesting that the effects of butyrate may be connected to Slc7a11
191 downregulation. Inhibition of Slc7a11 via erastin can trigger the programmed cell death
192 pathway of ferroptosis (39). Treatment with the ferroptosis inhibitor, ferrostatin, however,
193 failed to restore *L. pneumophila* burdens during erastin treatment and ferrostatin by itself did
194 not affect *L. pneumophila* infections (Fig 2e). Butyrate treatment can also induce apoptosis
195 (40), which does abrogate *Legionella* replication (41). Consistent with this notion, we noticed
196 that butyrate reduced the expression of the anti-apoptotic BCL-2 member, MCL-1, and
197 induces caspase-3 expression (Fig 2a). Immunoblot analysis confirmed that butyrate, but not
198 propionate, reduced MCL-1 levels in uninfected BMDMs, but not infected BMDMs (Fig
199 S2c). However, butyrate did not cause caspase-3 cleavage, including in *L. pneumophila*
200 infected BMDMs (Fig S2c). Inhibition of caspase activity, using the pan-caspase inhibitor
201 QVD, failed to restore intracellular *L. pneumophila* burdens after butyrate treatment,
202 although QVD restored *L. pneumophila* replication in control treatment with ABT-737 which
203 induced apoptosis (Fig S2d). Finally, butyrate failed to trigger increased cell death in infected
204 macrophages, suggesting that the decreased CFUs are not due to macrophage cell death (Fig
205 S2e). Besides regulating cell death pathways, Slc7a11 promotes glutathione synthesis which
206 depends on the import of cystine in exchange for glutamate and reduction to cysteine.
207 However, butyrate still prevented intracellular survival of *L. pneumophila* in the presence of
208 membrane permeable glutathione and glutathione synthesis inhibitor (BSO) did not affect

intracellular *L. pneumophila* numbers (Fig 2f). Taken together, our data shows butyrate attenuates infection-induced expression of Slc7a11 and limits *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 *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 *L. pneumophila*-infected BMDMs treated with butyrate (Fig 3a). Similarly, there was a trend towards lower levels of the cysteine-related metabolites glutathione and γ -glutamylcysteine in butyrate-treated macrophages, including those infected with *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 *L. pneumophila* intracellular survival, particularly in the presence of butyrate. Cysteine supplementation restored *L. pneumophila* burdens in butyrate-treated BMDMs (Fig 3b). Imaging of GFP-expressing *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 *L. pneumophila* numbers (Fig 3d). Conversely, cysteine supplementation of butyrate pre-treated BMDMs enabled *L. pneumophila* to survive in the absence of the SCFA (Fig 3d). Furthermore, intracellular *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 cysteine-

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 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,

391 unless the culture media is supplemented with cysteine. Cysteine supplementation also
392 enabled intracellular *Legionella* growth after 24 hours of butyrate treatment, suggesting that
393 the decreased cysteine levels in macrophages primarily affect bacterial replication not so
394 much their survival. Commonly used culture media contain cystine as it is more stable than
395 cysteine. Media that contain reducing agents to preserve cysteine and other supplements alter
396 the effects of butyrate on intracellular *Legionella*, highlighting that subtle changes to culture
397 conditions affect experimental outcomes. In addition, Slc7a11 expression is tightly regulated.
398 In contrast to antimicrobial responses, Slc7a11 expression is independent of HDAC3 activity
399 (44) but dependent on tubastatin A. This raises the possibility that butyrate or HDAC specific
400 inhibitors can be harnessed to treat infectious diseases to dampen hyperinflammation and
401 prevent microbial growth. Such an approach has been demonstrated in human macrophages,
402 whereby tubastatin A promoted clearance of intracellular *Salmonella* (56). To further this
403 idea, it will be important to understand the roles of butyrate *in vivo*. In animal models,
404 butyrate prevents inflammation and infections in the gut (24, 31, 33). In addition, increased
405 serum butyrate levels affect other organs and their immune cells and thus prevent detrimental
406 lung inflammation in flu infections (27). Similar approaches using high fibre diets or oral
407 butyrate failed to affect *L. pneumophila* lung burdens, suggesting that alveolar macrophages
408 may have limited access to serum butyrate levels or that serum levels are insufficient to
409 reduce *L. pneumophila* lung burdens. This is in line with recent reports showing that alveolar
410 macrophages have a different metabolic capacity and reduced access to serum-derived
411 metabolites such as glucose (57). Therefore, we have administered butyrate intranasally and
412 show that this is sufficient to reduce *L. pneumophila* lung burdens. Previous studies have
413 further identified an important role of Slc7a11 in alveolar macrophages, as it is upregulated in
414 patients containing active *Mycobacteria tuberculosis* lung infections (58). Slc7a11-deficient
415 mice are protected from *M. tuberculosis* 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 $\Delta flaA$ 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 $\Delta flaA$ is a derivative of *L. pneumophila* Philadelphia-1 that is thymidine auxotroph.

Salmonella enterica subsp. *enterica* serovar Typhimurium SL1344 and *E. coli* DH5 α 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 $\mu\text{g. mL}^{-1}$ 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 anaesthetised by 5 % isoflurane 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 2.5×10^6 *L. pneumophila* ΔflaA 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 CO₂ 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 % CO₂. 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 5×10^5 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 5×10^5 cells/mL in 96-well tissue culture plates and cell death was determined essentially as described previously (41). Cells were stained with 1 μ M 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 μ M 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* Δ flaA (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 $\times$ 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 μ L 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 $^{\circ}$ 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-Time 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	agcaggttcacagcgaagt
Slc7a11 R	tggccagctccgcaaatgaa
Actin F	cattgctgacaggatgcagaagg
Actin R	tgctggaaggtggacagtgagg

RNA Seq

For RNA-seq experiment, 2×10^6 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 cagacgtgtgctcttccgatcttttttttttttttt 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'-ccctacacgacgctcttccg(rA)(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'-aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccg-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=01485e0b3f4264ada6fc6090ecba83a4#/>).

Metabolomics

The BMDMs were plated at cell density of 4×10^6 cells/10 cm dish overnight. Next day, BMDMs were infect the *L. pneumophila* Δ flaA 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.

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Figure 1

Figure 1. Butyrate controls intracellular *L. pneumophila* burdens. a, Colony forming units (CFUs) of *L. pneumophila* Δ flaA 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 Δ flaA 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, 20 μ m. 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 Δ flaA (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 Δ flaA in mouse alveolar macrophages 48 hours post infection. Mean from two independent experiments.

Figure 2

Figure 2. Butyrate regulates the expression of nutrient transporters in infected

macrophages. a, RNAseq of bone marrow-derived macrophages (BMDMs) infected with *L. pneumophila* Δ 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 Δ 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 Δ flaA BMDMs with or without butyrate treatment. Mean and SD from two independent experiments. d, Uninfected

872 (UI) and Δ flaA infected BMDMs treated with or without butyrate were analysed by
873 immunoblotting for Slc7a11 expression. Actin is shown a loading control. e, Colony forming
874 units (CFUs) from Δ flaA infected BMDMs treated with erastin and ferrostatin at 6 and 48
875 hours post infection. Mean and SEM from at least three independent experiments and
876 unpaired two-tailed *t*-test used to determine p-values. f, CFUs from Δ flaA infected BMDMs
877 treated with butyrate, glutathione (GSH) or buthionine sulfoximine (BSO) at 6 and 48 hours
878 post infection. Mean and SEM from at least three independent experiments are shown.
879

Figure 3

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.

890 Representative of three independent experiments. d, CFUs from Δ flaA infected BMDMs 48
891 hours post infection. BMDMs were treated with butyrate at the time of infection and cysteine
892 24 hours post infection (post Cys) or pre-treated with butyrate for 24 hours prior to infection
893 and then supplemented with cysteine at the time of infection (pre-Butyrate+Cys). Mean and
894 SEM from three independent experiments and unpaired two-tailed *t*-test used to determine p-
895 values. e, CFUs from Δ flaA infected BMDMs cultured in cysteine/cystine-free media or
896 supplemented with cysteine. Mean and SEM from at least three independent experiments. f,
897 CFUs from Δ flaA infected BMDMs treated with butyrate and 2-mercaptoethanol. Mean and
898 SEM from at least two independent experiments and p-values are from unpaired two-tailed *t*-
899 test. g, Fold change of untargeted metabolite levels in Δ flaA infected BMDMs treated with
900 butyrate compared to control treatments 6 hours post infection. Amino acids (red),
901 nucleotides (orange), TCA intermediates (blue) and sugar metabolites (green). Mean from 5
902 biological replicates.
903

Figure 4

Figure 4. Butyrate affects *L. longbeachae* infections of macrophages. CFUs from Δ flaA *L. pneumophila* stain Lp02 infected BMDMs treated with (a) butyrate, (b) erastin, (c) MG132 or (d) combination of erastin and MG132. e and f, CFUs from Δ 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

Figure 5. Butyrate restricts intracellular *Legionella* growth by inhibiting HDAC

activity. a, RNAseq of Δ flaA 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 Δ flaA 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 Δ flaA 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 Δ flaA 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 Δ flaA

927 BMDMs with or without Tubastatin treatment. Mean and SD from two independent
928 experiments. h, CFUs from Δ flaA infected WT and HDAC6^{-/-} BMDMs. Mean and SEM from
929 at least three independent experiments.

930

931

Figure 6

932

933 **Figure 6. Butyrate reduces *L. pneumophila* lung burdens.** a, C57Bl/6 mice fed control or
 934 high fiber diets were intranasally infected with Δ flaA *L. pneumophila* and lung burdens
 935 (CFUs/lung) determined 48 hours post infection. b, Mice were intranasally infected with
 936 Δ flaA together with the SCFAs acetate, propionate and butyrate. CFUs/lung was determined
 937 48 hours post infections. c, CFUs/lung of mice intranasally infected with Δ flaA together with
 938 butyrate (1mM) at 48 hours post infection. Mean, SEM and data from individual mice are
 939 shown.

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943 Extended Data – supplementary figure legends

946 **Figure S1. Role of butyrate in controlling *L. pneumophila* growth.** a, Δ flaA *L.*

947 *pneumophila*, (b) *Salmonella* or (c) *E. coli* were cultured in the presence of increasing

948 concentrations of butyrate and growth measured by optical density at 600nm over time. Mean

949 and SEM from three independent experiments. d, Colony forming units (CFUs) from Δ flaA

950 infected BMDMs cultured at increasing concentration of butyrate at 48 hours post infection.

951 e, f and g, CFUs from Δ flaA infected (e) THP1 differentiated macrophages and (f)

952 immortalized BMDMs treated with or without butyrate or (g) BMDMs differentiated in the

953 presence of butyrate. Mean and SEM from at least three different experiments. h, Uninfected

954 and Δ flaA infected BMDMs were treated with acetate, butyrate or propionate and expression

955 of p62 and the LC3 protein GABARAP determined by immunoblotting. Actin is included as

956 loading control. i, CFUs from Δ flaA infected BMDMs treated with butyrate and bafilomycin

957 A (BafA) at 6 and 48 hours post infection. Mean and SEM from at least three independent

958 experiments except for BafA treatment.

961

963

967 immunoblotting. Tubulin is shown as loading control. Representative of two independent
968 experiments. d, CFUs of Δ flaA infected BMDMs treated with butyrate, QVP or ABT-737 at
969 6 and 48 hours post infection. Mean and SEM from at least three independent experiments. e,
970 Uninfected and Δ flaA infected BMDMs treated with or without butyrate were analysed for
971 cell death using live-cell imaging. Mean and SEM from three independent experiments.
972
973

Figure S3

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 Δ fla infected BMDMs treated with or without butyrate for 6 hours. Mean from 5 biological replicates. d, Fold change of significantly altered metabolites in Δ flaA 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 Δ flaA 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

a

b HDAC3 KO vs WT

Figure S4. GPCR and HDAC mediated signalling. a, Colony forming units (CFUs) from Δ 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](#)