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The role of acetate in macrophage`s response against *Streptococcus pneumoniae*

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THE ROLE OF ACETATE IN MACROPHAGE`S RESPONSE AGAINST *STREPTOCOCCUS PNEUMONIAE*

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Abstract

Short chain fatty acids (SCFAs) are metabolites produced mainly by the gut microbiota with a known role in immune regulation. Acetate, the major SCFA, is described to disseminate to distal organs such as the lungs. Moreover, the literature supports that acetate modulates inflammation and improves bacterial clearance. Our group has previously demonstrated that acetate improves *Streptococcus pneumoniae* clearance in the context of a secondary post-viral infection. This protection is mediated by alveolar macrophages, the first line of pulmonary immune defense. Thus, our aim was to evaluate the effect of acetate on the killing ability of alveolar macrophages and to delineate the mechanisms involved in this response. Here we show that acetate supplementation in drinking water modulated the secretion of host defense proteins by murine pulmonary cells and led to reduced *S. pneumoniae* loads in the lungs. To understand the mechanisms of bacterial clearance, alveolar macrophages were used. Transcriptomic analysis (RNAseq) revealed that acetate induced a specific signature of host defense in *S. pneumoniae* conditioned macrophages. This associates with the improved killing ability of acetate treated macrophages mediated by nitric oxide (NO) production. Increased NO concentration triggered by acetate was dependent on augmentation of IL-1β levels. Surprisingly, IL-1β production led by acetate was neither dependent on its cell surface receptor (Free-Fatty Acid Receptor 2), nor on the enzymes responsible for its metabolism (Acetyl-CoA Synthetase 1 and 2). Alternatively, acetate enhanced the glycolytic profile of macrophages resulting in greater HIF-1α activity which culminated in higher transcription of IL-1β. Moreover, the increased secretion of IL-1β triggered by acetate relied on NLRP3 inflammasome activation. In conclusion, we unravel a new mechanism of bacterial killing by acetate-activated macrophages. We show that acetate increased IL-1β production and secretion in a mechanism dependent on the axis

glycolysis/HIF-1α and NLRP3, respectively. Consequently, higher levels of IL-1β resulted in augmented NO production and improved killing ability of alveolar macrophages.

Résume

Les acides gras à chaîne courte (AGCC) sont des métabolites produits principalement par le microbiote intestinal. Ils jouent un rôle important dans la régulation des réponses immunitaires et inflammatoires. L'acétate, le principal AGCC, est décrit pour disséminer dans l'organisme et réguler la fonction d'organes distaux tels que les poumons. Des travaux récents indiquent une fonction dans le contrôle des agents pathogènes, notamment d'origine bactérienne. Notre groupe a précédemment démontré que l'acétate augmente l'élimination de *Streptococcus pneumoniae* dans le cadre d'une infection secondaire post-virale. Cette protection est médiée par les macrophages alvéolaires, la première ligne de défense pulmonaire. Ainsi, notre objectif était d'évaluer l'effet de l'acétate sur l'activité bactéricide des macrophages alvéolaires et d'identifier les mécanismes impliqués dans cette réponse. Nous montrons ici que la supplémentation en acétate dans l'eau de boisson module la sécrétion de protéines de défense par les cellules pulmonaires murines et conduit à une réduction de la charge de *S. pneumoniae*. Nous montrons par analyse transcriptomique (RNAseq) que l'acétate induit une signature spécifique de défense de l'hôte au sein des macrophages alvéolaires conditionnés en présence de *S. pneumoniae*. Cet effet s'accompagne par l'augmentation de l'activité bactéricide des macrophages mediée pour l'oxyde nitrique (NO). L'augmentation de NO induit par acétate dépendait de l'augmentation des niveaux d'IL-1β. De manière surprenante, la production d'IL-1β déclenchée par l'acétate est indépendante de son récepteur de surface (Free-Fatty Acid Receptor 2) et des enzymes responsables de son métabolisme (Acetyl-CoA Synthetases 1/2). En contrepartie, l'acétate a modulé le profil glycolytique des macrophages induisant l'activation de HIF-1α, qui aboutit à la transcription de l'IL-1β. De plus, l'augmentation de la sécrétion de l'IL-1β déclenchée par l'acétate reposait sur l'activation de l'inflammasome NLRP3. En conclusion, nous avons identifié un nouveau mécanisme conduisant à l'élimination des bactéries par les macrophages alvéolaires traité

avec l’acétate. L'acétate augmente la production et la sécrétion d'IL-1β selon un mécanisme dépendant de l'axe glycolyse/HIF-1α et de NLRP3, respectivement. Par conséquent, des niveaux plus élevés d'IL-1β conduit à une augmentation de la production de NO et une meilleure activité bactéricide des macrophages.

Resumo

Ácidos graxos de cadeia curta (AGCC) são metabolitos produzidos principalmente pela microbiota intestinal com um papel conhecido de regulação do sistema imune. Acetato, o mais abundante AGCC, tem a capacidade de disseminar para órgãos distais como os pulmões. Além disso, a literatura endorsa que acetato modula a inflamação e aumenta a eliminação de bactérias. Nosso grupo mostrou previamente que acetato aumenta a eliminação de *Streptococcus pneumoniae* no contexto de infecção secundária. Essa proteção foi mediada por macrófagos alveolares, a primeira linha da resposta imune pulmonar. Portanto, nosso objetivo foi avaliar o efeito de acetato na capacidade bactericida de macrófagos e delinear os mecanismos envolvidos nessa resposta. Aqui, nós mostramos que a suplementação de acetato na água modulou a secreção de proteínas envolvidas na defesa do hospedeiro por células pulmonares murinas e culminou na redução da carga de *S. pneumoniae* nos pulmões. Em seguida, para melhor entender os mecanismos de eliminação bacteriana, macrófagos alveolares foram utilizados. Nós mostramos por análise transcriptômica (RNAseq) que acetato induz uma assinatura de defesa do hospedeiro em macrófagos alveolares condicionados na presença de *S. pneumoniae*. Esse perfil foi associado com o aumento da atividade bactericida de macrófagos mediado pela maior produção de oxido nítrico (NO). O aumento na produção de NO observado em células tratadas com acetato foi dependente dos altos níveis de IL-1β. Surpreendentemente, a produção de IL-1β desencadeada por acetato não foi dependente do seu receptor de superficie (Free-Fatty Acid Receptor 2), nem das enzimas responsáveis por seu metabolismo (Acetyl-CoA Synthetases 1/2). Por outro lado, acetato aumentou o perfil glicolítico de macrófagos, resultando em uma maior atividade de HIF-1α, culminando na transcrição de IL-1β. Além disso, a secreção aumentada de IL-1β foi dependente da ativação do inflamassoma NLRP3. Em conclusão, nós identificamos um novo mecanismo responsável pelo aumento da atividade antimicrobiana de macrófagos tratados com acetato. Nós

mostramos que acetato aumentou a produção e secreção de IL-1β em um mecanismo dependente do eixo glicólise-HIF-1α e NLRP3, respectivamente. Consequentemente, altos níveis de IL-1β resultaram em um aumento da produção de NO e aumento da habilidade bactericida de macrófagos.

List of figures

List of tables

List of abbreviations

2-DG: 2-deoxy-D-glucose

ACOD: Aconitate decarboxylase

ACSS: Acetyl-CoA synthetase

AEC: Alveolar epithelial cell

ALDOA: Fructose-bisphosphate aldolase

AMP: Antimicrobial peptide

AMPK: Adenosine monophosphate-activated protein kinase

AP: Activating Protein

ARG: Arginase

ASC: Apoptosis-associated speck-like protein containing a CARD

ATP: Adenosine 5'-triphosphate

BCA: Bicinchoninic acid

BMDM: Bone marrow derived macrophages

cAMP: Cyclic adenosine monophosphate

CASP: Caspase

CBP: CREB binding protein

CCL: C–C motif chemokine ligand

CD: Cluster of differentiation

CFU: Colony forming units

CRAMP: Cathelicidin-related antimicrobial peptide

CRISPR: Clustered regularly interspaced short palindromic repeats

CXCL: Chemokine (C-X-C motif) ligand

CY-09: 4-[[4-Oxo-2-thioxo-3-[[3-(trifluoromethyl)phenyl]methyl]-5-thiazolidinylidene]methyl]benzoic acid

DAMP: Danger-associated molecular pattern

DC: Dendritic cell

ECAR: Extracellular acidification rate

ELISA: Enzyme-linked immunoassay

ENO: Gamma-enolase

ERK: Extracellular signal-regulated kinase

ETC: Electron transport chain

FADH: Flavin adenine dinucleotide hydrogen

FAO: Fatty acid oxidation

FBS: Fetal bovine serum

FCCP: Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone

FDA: Food and drug administration

FFAR: Free fatty acid receptor

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GLP: Glucagon-like peptide

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GPCR: G protein coupled receptor

GPI: Glucose-6-phosphate isomerase

HDAC: Histone deacetylase

HIF: Hypoxia-inducible factor

HIV: Human immunodeficiency virus

HK: Hexokinase

HβD: Human beta defensin

IAV: Influenza A virus

IFN: Interferon

Ig: Immunoglobulin

IL: Interleukine

ILC: Innate lymphoid cell

iNOS: Inducible nitric oxide synthase

IRF: Interferon regulatory factors

IUPAC: International union of pure and applied chemistry

LDH: Lactate dehydrogenase

L-NMMA: NG-Methyl-L-arginine acetate salt

LPS: lipopolysaccharides

MAC: Membrane attack complex

MARCO: Macrophage receptor with collagenous structure

MCC950: N-[[(1,2,3,5,6,7-Hexahydro-s-indacen-4-yl)amino]carbonyl]-4-(1-hydroxy-1-methylethyl)-2-furansulfonamide sodium salt

MCP: Monocyte chemotactic protein

MCT: Monocarboxylate transporter

MHC: Major histocompatibility complex

MOI: Multiplicity of infection

MPC: Mitochondrial pyruvate carrier

MPI: Max planck institute

MSU: Monosodium Urate

mTOR: Mammalian target of rapamycin

NADH: Nicotinamide adenine dinucleotide hydrogen

NADPH: Nicotinamide adenine dinucleotide phosphate

NF-κB: Nuclear factor kappa light chain enhancer of activated B cells

NLRP: Nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing

NOD: Nucleotide-binding oligomerization domain

OCR: Oxygen consumption rate

OXPHOS: Oxidative phosphorylation

PAFR: Polymeric immunoglobulin receptor

PAMP: Pathogen-associated molecular pattern

PBS: Phosphate-buffered saline

PCV: Pneumococcal conjugate vaccine

PDK: Pyruvate dehydrogenase kinase

PFKFB: 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase

PGK: Phosphoglycerate kinase

PHD: Prolyl Hydroxylase Domain

PKM: Pyruvate kinase M

PLY: Pnemolysin

PPP: Pentose phosphate pathway

PPSV: Pneumococcal polysaccharide vaccine

RIPA: Radio-immunoprecipitation assay buffer

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

RPMI: Roswell park memorial institute

RSV: Respiratory syncytial virus

RT-PCR: real-time polymerase chain reaction

S100A: S100 calcium binding protein A

SCFA: Short chain fatty acid

SLC-A-: Solute carrier family - member -

SLPI: Secretory leukocyte proteinase inhibitor

SMCT: Sodium-coupled monocarboxylate transporter

SP: Surfactant protein

STAT: Signal transducer and activator of transcription

TBP: TATA-Box binding protein

TBS: Tris-buffered saline

TCA: Tricarboxylic acid

TCR: T-cell receptor

TGF-β: Transforming growth factor beta

TLR: Toll like receptor

TNF-α: Tumor necrosis factor alpha

UCP: Uncoupling protein

VEGFA: Vascular endothelial growth factor A

WHO: World health organization

Table of content

Introduction

Short-chain fatty acids

Short chain fatty acids (SCFA) are defined by the International Union of Pure and Applied Chemistry (IUPAC) as carboxylic acids containing aliphatic tails less than 6 carbon atoms. Therefore, SCFAs are formate (C1) acetate (C2), propionate (C3), butyrate (C4) and valerate (C5) being acetate, propionate and butyrate, the major metabolites produced by the human gut microbiota (Figure 1) (Venegas et al. 2019).

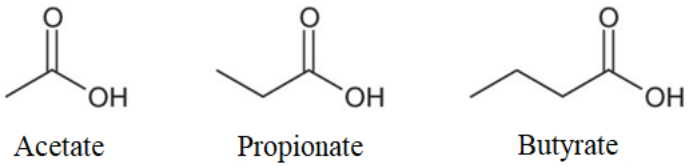


Figure 1: Representation of the two-dimensional structure of acetate, propionate and butyrate.

Production of SCFAs

SCFAs can be derived from the anaerobic fermentation of non-digestible polysaccharides, oligosaccharides, glycoproteins, proteins, and peptides, however the most important substrate for SCFA generation are carbohydrates. These nutrients are mostly originated from resistant starches that escape the digestion and plant cell-wall polysaccharides. The definition of the gut microbiota as the main producer of SCFAs was led by the observation that Germ-free animals had 100-fold less SCFAs than conventional mice. More than 90% of this SCFA encountered in germ-free mice was acetate, which can be produced by the liver. The residual concentrations found for butyrate and propionate are thought to be derived from the diet (Høverstad and Midtvedt 1986).

The production of SCFAs comprises different steps, in which different bacteria are involved. Non-digestible fibers can be processed into short polysaccharides and proteins, which will then be used by other bacteria to form SCFAs. Actinobacteria and Firmicutes are the main phyla to initiate the degradation of non-digestible polysaccharides. They can provide shorter substrates for other bacteria, or they can produce SCFAs by their own (Hee and Wells 2021). Then, the phylum Bacteroidetes continues the breakdown of complex carbohydrates

which will give rise to SCFAs. As shown in Table 1, different species of bacteria are responsible for the production of each SCFA. Acetate is produced by Bacteroidetes, while propionate is mainly produced by Bacteroidetes, and by the Negativicutes class of Firmicutes. The major producers of butyrate belong to the Firmicutes phyla, which are Lachnospiraceae, Ruminococcaceae, Clostridiaceae and Erysipelotrichaceae (Louis and Flint 2017). Of note, the main phyla that composes the gut microbiota of an healthy adult human are Firmicutes and Bacteroidetes, followed by Actinobacteria (Rodríguez et al. 2015). Therefore, producers of SCFA are in high concentrations in the gut.

Table 1: Main producers of acetate, propionate and butyrate, and their physiological effects in the gut.

SCFA	Producer bacteria	Physiological effects
Acetate	*Bifidobacterium adolescentis* *Lactobacillus* spp. *Bacteroides thetaiotaomicron*	Energy Induction of cholesterol biosynthesis
Propionate	*Roseburia insulinovorans* *Veillonella* spp. *Ruminococcus obeum* *Bacteroides* spp. *Dialister* spp. *Phascolarctobacterium* spp.	Energy Gluconeogenesis Inhibition of cholesterol biosynthesis Histone deacetylase (HDAC) inhibition
Butyrate	*Roseburia* spp. *Eubacterium rectale* *Eubacterium hallii* *Faecalibacterium prausnitzii* *Anaerostipes caccae* *Coprococcus eutactus*	Energy, anti-inflammatory Induction of immunosuppressive cytokines and GLP-2 HDAC inhibition Overexpression of detoxifying enzymes Enhancement of mucosal barrier function

Table adapted from Fernádez et al. 2016.

The production of SCFAs is highly variable among individuals because of several factors including the availability of substrates (diet) and the composition of the gut microbiota, which can be affected by ageing, chronic intestinal diseases, antibiotics usage and others. Although the high variety of substrates used for SCFA production, few metabolic steps are involved in the process. While *Bifidobacterium* uses fructose-6-phosphate shunt, the other intestinal bacteria use the glycolytic pathway. Many intestinal bacteria also use the

pentose phosphate pathway to produce metabolites for the glycolytic pathway and then generate SCFAs (Macfarlane and Macfarlane 2003). Those metabolic pathways generate pyruvate and acetyl-CoA, which will be later used for the synthesis of acetate, propionate, and butyrate. Although the gut microbiota is the main producer of SCFAs, acetate can be produced by the liver at low concentrations. Fatty acids can go under β-oxidation to generate free acetate in the liver, and as the rate of acetate consumption in the liver is low, it goes to the bloodstream to be used by other tissues (Yamashita, Kaneyuki, and Tagawa 2001).

The concentrations of SCFAs vary along the intestine, however higher concentrations are found in the proximal large intestine due to the greatest availability of carbohydrates. Therefore, the concentration of SCFAs in the proximal colon ranges from 70-140 mM, whereas in the distal colon it can vary from 20 mM to 70 mM and in the distal ileum from 20-40 mM (Topping and Clifton 2001; Cummings et al. 1987). Among the major SCFAs produced by the gut microbiota, acetate has the higher concentration accounting for 60% of total SCFAs, while propionate and butyrate represent around 20% each (Cummings et al. 1987).

Physiological functions of SCFAs

SCFAs exert many physiological functions in the intestine, (Table 1) (Maslowski et al. 2009; Tan et al. 2014; Thorburn, Macia, and Mackay 2014). By entering some metabolic routes, SCFAs are used as energy source by colonocytes. Butyrate is the preferred substrate for energy generation linked to the respiration and its usage is even higher than glucose and glutamine (Roediger 1980). Also, butyrate and acetate (in a less extent) are used for lipid biosynthesis by colonocytes, whilst propionate is mainly incorporated into gluconeogenesis (Ríos-Covián et al. 2016; Wong et al. 2006). Moreover, SCFAs were shown to increase the proliferation of epithelial cells in the jejunum, ileum, and colon, with butyrate being the main responsible for colonocytes proliferation (Kripke et al. 1989; Sakata and Yajima 1984). In

line, butyrate also maintains and reinforces gut epithelial barrier by inducing the expression of tight junctions and by stabilizing HIF-1α (Venegas et al. 2019). The effect of these metabolites is not restricted to host cells, they can also modulate host microbiota, once they induce a decrease in luminal pH that can be toxic for colonizers and for potential pathogens (Walker et al. 2005).

SCFAs can disseminate through the blood and reach distal organs such as the lungs. To be diffused, SCFAs need to enter in the colonic epithelium via monocarboxylate transporters (MCTs) and sodium-coupled monocarboxylate transporters (SMCTs). Acetate, propionate and butyrate are absorbed by colonocytes at similar rate (Topping and Clifton 2001). The uptake of the anionic form of SCFA happens in the apical membrane of colonocytes and it is coupled to H^+ for MCT1 or to Na^+ for SMCT1 and SMCT2. In addition, the influx of the anionic form of SCFA can be coupled to the bicarbonate efflux, however this transporter is yet unknown. To reach the bloodstream, SCFAs are transported from the colonocytes to the blood by MCT1 and MCT4 present in the basolateral membrane of these cells (Sivaprakasam et al. 2017). The SCFAs that are not degraded by colonocytes metabolism are then transported to the portal circulation. As butyrate is the main source of energy for colonocytes, only a small portion is available to be transported to the blood. The same is true for propionate, which is highly metabolized by the liver and though, less available in the bloodstream (Wong et al. 2006). Therefore, considering the proportion of SCFAs that is metabolized in the gut and liver and the production of acetate by the liver, SCFAs are found in the blood at different ratio from the one described for the intestine. In peripheral blood acetate represents 91% of SCFAs, while propionate represents 5% and butyrate 4% (Cummings et al. 1987). The concentrations of SCFA found in the blood are highly variable, ranging from 10 µM to 500 µM in humans (Verbeke 2017; Müller et al. 2019; Pomare, Branch, and Cummings 1985). On the other hand, in humanized mice the concentration of SCFA in blood can reach until 2 mM (Samuel and Gordon 2006).

Although it is known that SCFAs can disseminate and reach distal organs, there is a lack of evidence regarding their concentrations in each tissue. In the lungs, the concentration of SCFAs can range from undetectable to 3 µM/g of lung tissue in mice and from 30 to 10000 µM in the sputum or in the bronchoalveolar lavage from humans (Ghorbani et al. 2015; Lewis et al. 2019; Trompette et al. 2014; Segal et al. 2017; Mirković et al. 2015). Interestingly, a study raised a hypothesis stating that lung microbiota can also produce SCFAs. Segal and colleagues found that SCFAs were 370 times more concentrated in the lungs than in the blood, thus the explanation that SCFAs comes from the blood was not reasonable. Moreover, they suggest that SCFA production in the lungs is mediated by anaerobic microorganisms that form a complex biofilm to survive (Segal et al. 2017).

Mode of action of SCFAs

To exert those functions, SCFAs display a wide range of mechanism, from direct killing of pathogen to modulation of immune response and enhance of host defense. In the context of infections, SCFAs can act by direct inhibition of the growth of many bacteria and by modulating the virulence of such pathogens (Roe et al. 2002; Prohászka 1980; Roe et al. 1998; Lamas et al. 2019; Sun et al. 2012). The effectiveness of bacterial growth inhibition and bacterial death induction depends on the extracellular environment. Under low pH, SCFA are found undissociated and thus, they diffuse across the cellular membrane. Once in the cytoplasm, SCFAs dissociate and increases the concentrations of ions, disturbing the osmotic balance and the cellular metabolism (Roe et al. 1998). Moreover, SCFAs can reduce the virulence of different *Salmonella* strains by decreasing bacteria motility and reducing biofilm formation (Lamas et al. 2019; Amrutha, Sundar, and Shetty 2017). Alternatively, it was also shown that SCFAs can upregulate some virulence genes of *Borrelia burgdorferi*, facilitating the recognition of the bacterium by the immune system (Lin et al. 2018).

SCFAs can also act by modulating host defense and immune response, via three main mechanisms (Figure 1) (Machado, Sencio, and Trottein 2021). The most described so far is the direct bind on G protein-coupled receptors (GPRs), namely GPR43 (free fatty acid receptor 2, FFAR2), GPR41 (FFAR3) and GPR109A (Ulven 2012). Upon binding, diverse Gα proteins can couple to the receptor, they are $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ (Priyadarshini et al. 2018). Due to the variety of Gα proteins that can be recruited, SCFAs can trigger a vast range of cellular response. The other well established mechanism is the inhibition of histone deacetylase (HDAC), which increases gene expression (Trompette et al. 2018). The third and less described mode of action is on cellular metabolism, where SCFAs can be used as a substrate. The mechanisms and the range of action of those SCFAs are wide. They can either reduce or increase the inflammatory response, according to the model of inflammation and used dose.

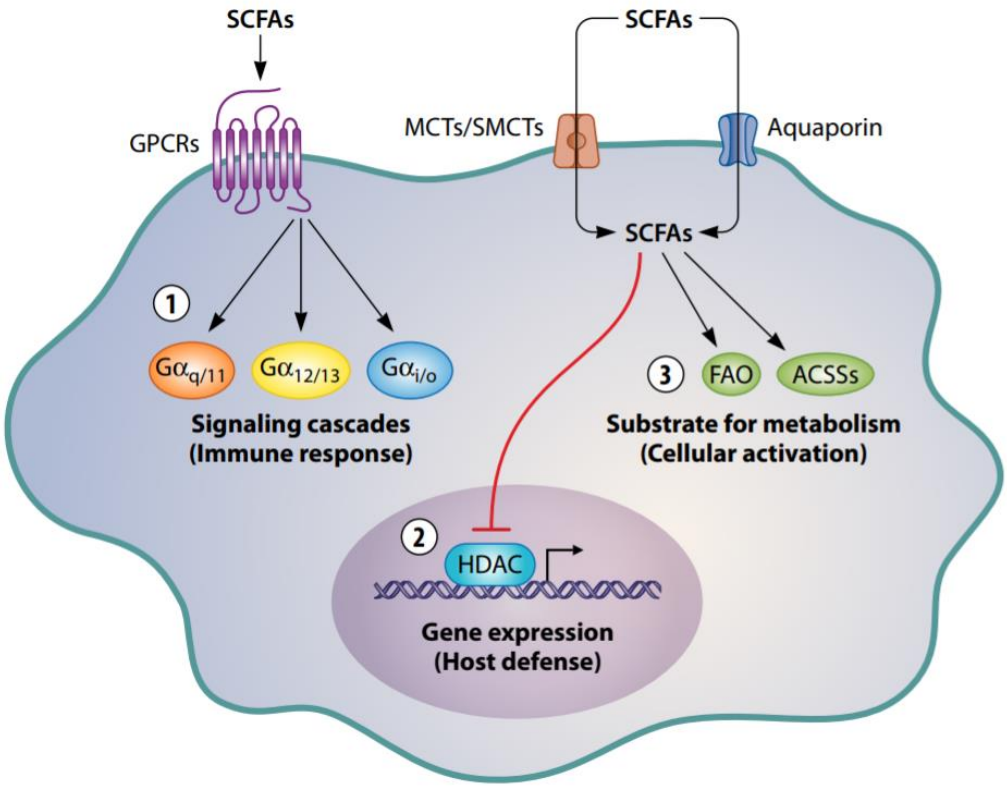


Figure 2: SCFAs have three main mechanisms of action.
(1) SCFAs can act from the extracellular space, activating GPCRs which can signal through Gαq/11, Gα12/13 or Gαi/o resulting in different immune responses. SCFAs can also enter the cell and act from the cytoplasm, where they can (2) inhibit histone deacetylase (HDAC) and induce gene expression, or

(3) be metabolized in the fatty acid oxidation (FAO) cycle or by acetyl-CoA synthetase (ACSSs). Figure from Machado, Sencio and Trottein 2021.

Role of SCFAs in diseases

Besides the physiological role of SCFAs in the intestine, these metabolites are also described to play a role in the context of diseases. As mentioned before, SCFAs can control commensal and pathogenic bacterial outgrowth in the gut, where they are found in higher concentrations. In this context, it is well established that SCFAs protect against enteric infections including shigellosis and salmonellosis (Rabbani et al. 1999; Raqib et al. 2006b; Canani et al. 2011; Raqib et al. 2012; Sunkara, Jiang, and Zhang 2012). The direct effect of SCFAs in bacterial growth can be limited to the gut, however, their impact on bacterial clearance by host defense is broader. Studies demonstrate that SCFAs -especially butyrate- can target gut epithelial cells to promote the production of antimicrobial peptides (AMPs) such as cathelicidins (Steinmann et al. 2009; Sunkara, Jiang, and Zhang 2012; Gallo and Hooper 2012). Besides, SCFAs can also target macrophages and boost their ability to deal with the pathogen. SCFAs were shown to trigger macrophages' pyroptosis resulting in enhanced clearance of *Salmonella* (Tsugawa et al. 2020). Interestingly, this effect was mediated by the activation of the inflammasome, which occurred upon SCFA binding to ASC. Also, butyrate-differentiated macrophages presented an enhanced ability to kill bacteria. This effect was attributed to the inhibition of HDAC, leading to reduced glycolysis, increased AMP production and increased lysosomal acidification (Schulthess et al. 2019). Consistent, SCFAs were also shown to increase the bactericidal activity of macrophages via HDAC, in this setting by increasing the production of lysozymes (J. Zhang et al. 2020).

SCFAs can also modulate sterile inflammation, and diseases such as asthma, and allergy. The anti-inflammatory effects of SCFAs improved inflammatory bowel disease in murine models via NLRP3 inflammasome attenuation, and in humans by lowering NF-κB activation (Harig et al. 1989; Lührs et al. 2002; Maslowski et al. 2009). In a murine model of

gout, acetate was also shown to decrease NF-κB activity via FFAR2 and promote a faster resolution of inflammation (Vieira et al. 2017). Evidence also suggests that SCFAs modulate pulmonary Th2 response via FFAR3 decreasing asthma reactions (Trompette et al. 2014; Cait et al. 2018). SCFAs, especially acetate and butyrate, produced upon digestion of high-fiber diet were shown to enhance oral tolerance against food allergy. This protection was mediated by increased activity of dendritic cells (DCs) and T helper cells, and enhanced IgA production which were mediated by FFAR2 and GPR109A activation (Tan et al. 2016). In the lungs, SCFAs, particularly butyrate, were shown to inhibit innate lymphoid type 2 (ILC2) functions resulting in the control of airway hyperreactivity (Lewis et al. 2019).

The potential function (through supplementation) of SCFAs in respiratory infections has caught the attention of researches (Machado, Sencio, and Trottein 2021). Here, studies with SCFAs, excluding acetate, in the context of pulmonary infection will be briefly presented. SCFAs were shown to ameliorate pulmonary infection caused by *Klebsiella pneumoniae* (Wu et al. 2020). Mice presented decreased inflammation and bacterial loads in the lungs due to the enhanced ability of macrophages to phagocyte and kill the bacteria. This effect was mediated by FFAR2 activation, which triggered a signaling pathway that facilitated the phagosome-lysosome fusion. In the context of viral infection, butyrate was shown to reduce inflammation and damage caused by Influenza A virus (Trompette et al. 2018). Butyrate induced an alternative activation of macrophages via FFAR3, which secreted less CXCL1, recruiting less neutrophils to the site of infection and consequently causing less damage. Additionally, the usage of butyrate as substrate for FAO and its binding to FFAR3 boosted the anti-viral activity of CD8+ T cells. Interestingly, a beneficial effect was also shown in humans. Combined phenyl butyrate and vitamin D3 treatment ameliorated pulmonary tuberculosis in patients, by boosting antibacterial functions of macrophages and increasing the secretion of the antimicrobial peptide LL-37 (Mily et al. 2013; 2015; Rekha et al. 2018).

Those findings are particularly interesting as macrophages and AMPs have a major contribution for the primary response against bacterial infections. Therefore, it is important to better understand how SCFAs improve host defense and whether SCFAs might be used as prophylactics or therapeutics (*e.g.*, in combination with antibiotics or immune stimulators) to combat bacterial infections, especially in the lung compartment.

Acetate

Production of acetate

Although the gut microbiota is the major source of SCFAs, mammalian cells, others than hepatocytes, can also produce and secrete acetate. This phenomenon can happen under stressful conditions, such as infection or limited source of nutrients (Balmer et al. 2016; Yamashita, Kaneyuki, and Tagawa 2001). The first piece of evidence for this phenomenon was in the context of cancer, in which acetate was being used as a source of carbon, however the origin of acetate remained unclear (Comerford et al. 2014; Mashimo et al. 2014). Recently, the pathway by which cells produce acetate *de novo* was elucidated (X. Liu et al. 2018). Despite the possibility to generate acetate by hydrolysis of acetyl CoA or by removal of acetyl groups from histones, the quantity of acetate available would not be enough to make acetate a source of carbon (Inoue and Fujimoto 1969; Knowles et al. 1974). Therefore, acetate was found to be originated from pyruvate through two distinct mechanisms. The first mechanism is a nucleophilic attack of pyruvate by ROS, culminating in release of CO_2 from pyruvate and addition of oxygen, forming acetate. The second mechanism consists of a catalyzation of pyruvate by keto acid dehydrogenases in absence of CoA and NAD^+ generating acetate and acetaldehyde (X. Liu et al. 2018). In 2016 this phenomenon was also observed in the context of infections. Balmer and colleagues observed that upon systemic infection acetate levels increased dramatically in the serum of mice (Balmer et al. 2016). They

also described increased acetate concentrations in peritoneal and subcutaneous infection, and they reported that acetate was released by resident cells.

As eukaryotic cells can produce acetate in some contexts, it is expected to observe a variation in the concentration of acetate available in the blood or in the site of infection or tumor. Upon alcohol consumption, the major portion of ethanol incorporated in the liver is metabolized into acetate, e.g., 10.4 µM/min/liver of ethanol incorporation results in 9.1 µM/min/liver of acetate production (Yamashita, Kaneyuki, and Tagawa 2001). Therefore, an increased concentration of acetate in the bloodstream is observed. Subjects received an injection of 70 mM of ethanol and infusion of 2.7 mM/min of ethanol for acetate quantification in the bloodstream. The concentration of acetate in peripheral blood raised from nearly 0 to 0.8 mM (Lundquist et al. 1962). Another experiment, with a design of alcohol consumption, showed similar results. Subjects who consumed 0.8 g/kg of ethanol in a 15° beverage had 5-fold increase in acetate levels in the peripheral blood (from 0.1 mM to 0.52 mM) (Nuutinen et al. 1985).

Tumor cells were shown to produce acetate in the microenvironment of tumors, like deprivation of glucose or hypoxia. In the glucose deprivation setting, acetate concentration inside the $1x10^4$ cells goes from 0.5 µM to 2.5 µM, whilst in hypoxia acetate increases from 0.5 µM to 1.7 µM (R. Chen et al. 2015). In agreement, different tumor cell lines were shown to secrete acetate under hypoxia condition, however acetate production and secretion seems to happen specifically in cancer cells, as a non-tumor cell line did not increased acetate secretion under hypoxia (Yoshii et al. 2009).

In the context of infections, the concentration of acetate can increase more than 100 times. In mice models, acetate is found at 1-2 mM in the peritoneum of healthy mice, while in mice with peritonitis acetate can range from 2-8 mM. Upon skin infection, acetate can go from 20 mM to 500 mM, whereas in healthy humans control fluid present 1 mM of acetate and during infection abscesses can reach 100 mM of acetate (Balmer et al. 2020).

Mode of action of acetate

Regardless the source, the mechanism of action is unaltered. Whereas SCFAs share similar mode of action to trigger immune response, there are some discrepancies among mechanisms that makes them unique. For example, butyrate, propionate, and acetate can be recognized by two FFAR receptors; propionate is a potent agonist for FFAR2 and FFAR3, meanwhile acetate is more selective for FFAR2 and butyrate for FFAR3 (Poul et al. 2003). In addition, acetate has some particularities regarding its mode of action. To date, acetate has three main modes of action in mammalian cells, (1) it can signal from the extracellular compartment, by binding to the FFAR2 receptor, which can couple with $G\alpha_{i/0}$, $G\alpha_{q/11}$ or $G\alpha_{12/13}$ (Priyadarshini et al. 2018). Therefore, acetate binding on FFAR2 can lead to a broad range of signaling, such as ERK cascade activation, inhibition of cAMP production and increase or decrease intracellular Ca^{2+} mobilization (G. Yang et al. 2018). Alternatively, acetate can also act from the intracellular compartment. It can enter in the cell via MTCs (MCT1, MCT2, MCT4) or aquaporins (Moschen et al. 2012). Then it can be converted in Acetyl-CoA by ACSS1 in the mitochondria or by ACSS2 in the cytoplasm/nucleus. After conversion, (2) acetyl-CoA can enter in the tricarboxylic acid (TCA) cycle serving as metabolic fuel for oxidative phosphorylation (Martínez-Reyes and Chandel 2020; Mashimo et al. 2014). In addition, (3) acetyl-CoA can also be used for acetylation of histones, leading to increased gene expression or for acetylation of proteins/enzymes, modulating their activity (Qiu et al. 2019; R. Chen et al. 2015; Gao et al. 2016; Daïen et al. 2021; J. V. Lee et al. 2018). It is important to note that acetate has also been described to increase histone acetylation via inhibition of HDAC (Bolduc et al. 2017). Although no mechanism has been described, some authors believe that the inhibition of HDAC activity can be a negative feedback due to the excess of free acetyl CoA derived from acetate (Soliman and Rosenberger 2011). Therefore, it is more likely that the inhibition of HDAC is linked to the mechanism (3), in which acetate is converted to acetyl CoA by ACSS2, leading to an increase in histone acetylation (Soliman

and Rosenberger 2011; Qiu et al. 2019). Recently a fourth mode of action has been proposed for acetate. It was shown that acetate can direct bind to glutaminase, increasing its activity (Balmer et al. 2020). Due to the range of mechanisms, acetate can act in many different cell types, from epithelial cells to immune cells and it can even lead to opposite responses, such as attenuation or activation of NLRP3 inflammasome, regarding the cell and the stimulus (Macia et al. 2015; Xu et al. 2019).

As described above, the different modes of action of acetate reflect in a wide range of functions. Under hypoxia, cancer cells increase the uptake rate of acetate, and this exogenous acetate is extensively used for lipogenesis and by the mitochondria. Acetate was shown to increase histone acetylation leading to higher expression of genes responsible for lipogenesis, such as *Fasn* and *Acaca*. In addition to the epigenetic effect, acetate was also used as a substrate for the *de novo* lipid synthesis in cancer cells, promoting cell survival (Gao et al. 2016; Schug et al. 2015). In line with histone acetylation, acetate was also shown to promote the acetylation of proteins such as HIF-2α. In the context of hypoxia or glucose deprivation, acetylation of this protein led to the formation of a CBP/HIF-2α complex, which was required for tumor proliferation, migration, and invasion (R. Chen et al. 2015). Inside the mitochondria, acetate was shown to fuel the TCA cycle for meeting the high bioenergetic demand of tumor growth (Mashimo et al. 2014; Kamphorst et al. 2014; Schug et al. 2015). Although another study also shows that acetate increases mitochondrial respiration and lipid synthesis, they found out that acetate reduced tumor proliferation due to a reduction in glycolysis. Interestingly, these effects were independent of ACSS1 and ACSS2 (Sahuri-Arisoylu et al. 2021).

Role of acetate in the immune response

Apart from the metabolic effects described, acetate can also modulate the immune response in different contexts, such as allergy, cancer, autoimmune disease, inflammation, and infection.

Acetate is shown to reduce anaphylaxis clinical scores and total IgE levels in serum, it also increased $CD103^+$ DCs and T reg cells percentages, as consequence mice were protected from peanut allergy (Tan et al. 2016).

In cancer, acetate also plays an immune-modulatory role. As mentioned before, in the tumor microenvironment the availability of glucose is scarce, and tumors use acetate as an alternative source of carbon. In this context, $CD8^+$ T cells also use acetate to overcome the absence of glucose, however, acetate is not used as a carbon source, but as an epigenetic modulator. Acetate rescues the effector function of exhausted T cells by promoting histone acetylation via ACSS2 and enhancing IFN-γ production (Qiu et al. 2019).

Acetate was also shown to ameliorate autoimmune diseases by different mechanisms. Acetate supplementation to mice with diabetes led to a substantial protection from the disease. This effect was attributed to the reduced frequency of autoreactive T cells, decreased concentration of diabetogenic cytokines and increased gut integrity, mediated, at least in part by FFAR2 (Mariño et al. 2017). A beneficial effect of acetate was also described in the context of arthritis, in which acetate increased the populations of T reg lymphocytes and B reg lymphocytes that secretes IL-10. The observed role of acetate was partially mediated by the conversion of acetate into acetyl CoA that fueled the TCA cycle and it was fully dependent on the induced acetylation of proteins (Daïen et al. 2021).

The modulation of inflammatory response mediated by acetate is also beneficial in different settings. In a murine model of colitis, acetate supplementation, via FFAR2, presented beneficial effects by increasing colon length, and by reducing histology score and cytokine production (Maslowski et al. 2009). In line, acetate was also shown to protect mice

from peritonitis and LPS-induced endotoxemia via FFAR2, yet the protection was mediated by attenuation of NLRP3 inflammasome activation in a Ca^{2+}-dependent manner (Xu et al. 2019). In a murine model of gout induced by monosodium urate (MSU) crystals, acetate was shown to accelerate the resolution of inflammation. In this context, the authors also suggest that FFAR2 mediates the protective effect of acetate, which was associated to decreased NF-κB activity, enhanced production of anti-inflammatory mediators, and increased efferocytosis of apoptotic neutrophils (Vieira et al. 2017). Acetate was also shown to protect mice from acute kidney injury, by reducing the maturation of dendritic cells, and consequently inhibiting their ability to induce $CD8^+$ and $CD4^+$ T cell proliferation. Acetate also increased mitochondrial biogenesis in epithelial cells, ameliorating the effects of hypoxia. Although no mechanism is shown, authors suggests that acetate acts through acetylation of histones to promote this protection (Andrade-Oliveira et al. 2015). Contrary to the presented studies, Daïen and colleagues propose that acetate can promote regulatory B cell differentiation, and thus modulate inflammatory response, independently of FFAR2 and histone acetylation (Daïen et al. 2021). In this setting, promotion of IL-10-producing regulatory B cells by acetate was associated to increased production of acetyl CoA and increase posttranslational lysine acetylation.

As mentioned before, acetate plays an important role in immune modulation, however its effect on infection has just recently caught the attention of researchers. In 2016, Balmer and colleagues demonstrated that acetate-conditioned lymphocytes improved *Listeria monocytogenes* clearance (Balmer et al. 2016). This better effector function of $CD8^+$ T cells cultivated for 3 days in the presence of acetate was attributed to the higher secretion of IFN-γ caused by a shift in the metabolic state of memory T cells, which presented higher glycolytic activity. Increased glycolysis was the result of activation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) via acetylation, initiated by the conversion of acetate to citrate, which enhanced acetyl CoA pool that served as substrate for GAPDH acetylation. As a follow

up of this study, the same group showed that upon long exposure to acetate (7 days) and re-encounter with the pathogen, memory $CD8^+$ T cells reduced the conversion of acetate into citrate and suppressed TCR activation to shut down the effector cell function (Balmer et al. 2020). In this setting, acetate was shown to bind to glutaminase and increase its activity, improving cellular respiration and survival. Therefore, acetate increased $CD8^+$ T cells effector function to deal with the pathogen, then acetate inhibit the TCR-signal to prevent exacerbated inflammation and pathology. In line with the effect of acetate on T lymphocytes described by Balmer, Park and colleagues (2015) showed that acetate could promote T cell differentiation. Here, they describe that acetate regulates mTOR pathway by inhibiting HDAC, which increases the generation of Th17, Th1 and IL-10^+ T cells. These cells induced by acetate ameliorated the histological and inflammatory parameters of mice infected with *Citrobacter rodentium* (J. Park et al. 2015). In 2020, acetate was shown to protect mice against *Clostridium difficile* infection. In this setting, the mechanism depended on neutrophils and ILC3s (Fachi et al. 2020). Acetate, via FFAR2, activated NLRP3 inflammasome in neutrophils causing a higher production of IL-1β. FFAR2 activation by acetate also increased the expression of IL-1R in ILC3, which recognized IL-1β and produced higher concentrations of IL-22. This crosstalk between neutrophils and ILC3s culminated in improvement of epithelial barrier, via IL-22 secretion, and better bacterial clearance.

Role of acetate in respiratory infections

The effect of acetate has also been addressed in pulmonary infections. The group of Vieira has shown that acetate (in drinking water) reduces mice susceptibility to *Klebsiella pneumoniae* (Galvão et al. 2018). The observed protection was linked to reduced neutrophil recruitment, cytokine production and bacterial loads. Also, acetate seemed to increase bacterial killing by alveolar macrophages. In line, acetate was also shown to protect mice against respiratory syncytial virus (RSV) infection (Antunes et al. 2019; Ji et al. 2021). The

treatment activated type I IFN signaling, enhancing IFN-β production via FFAR2. As a result, viral loads and pulmonary inflammation were reduced. Although both research groups observed decreased viral loads associated to increased IFN-β, one describes the pulmonary epithelial cells as the agent for cytokine production while the other states that alveolar macrophages are the responsible for IFN-β production. In 2020, our group showed that, influenza A virus (IAV) infection led to a marked drop in gut microbiota SCFA production, with a major decrease in acetate concentration (Sencio et al. 2020). In addition, those mice presented increased susceptibility to secondary infection caused by *Streptococcus pneumoniae*. Upon supplementation of acetate to mice infected with IAV, and then superinfected with *S. pneumoniae*, bacterial loads were reduced. The protective effect of acetate was mediated by alveolar macrophages, however the mode of action of acetate in these cells remains unknown.

Pneumococcal pneumonia

Epidemiology

A report from WHO published in 2020 presented that lower respiratory tract infections is the fourth cause of death in the world. Although the number of deaths reduced over 19 years, these diseases are still a matter of concern (Figure 2). In addition, among all the infections that commit the population, the one in the lower respiratory tract is responsible for the highest death numbers (World Health Organization 2020).

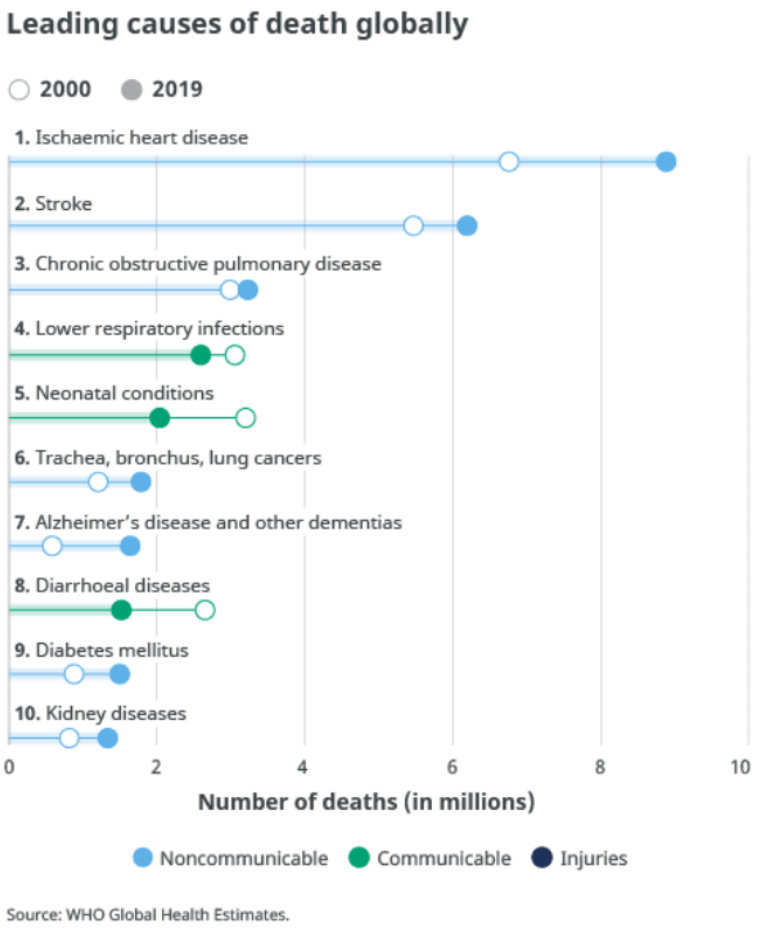


Figure 3: Global leading cause of death in 2000 compared to 2019.
Communicable diseases had decreased number of deaths over 19 years, meanwhile noncommunicable diseases presented an increased number of deaths. Chart from WHO (World Health Organization 2020).

Lower respiratory tract infections include acute bronchitis, bronchiolitis, pneumonia and tracheitis. Among these respiratory sites of infection, the lungs are the most committed, resulting in pneumonia. Pneumonia is characterized by lung inflammation that involves the alveoli and it is considered a severe disease with high risk of death (Filho 2016). In the majority of the cases pneumonia is caused by an infectious agent that triggers an inflammatory response. If the early response is not effective enough, toxic factors (e.g.: protease and toxins) secreted by the pathogen, associated to the substances produced by the host and emanating from the immune response (e.g.: reactive oxygen species, protease, and neutrophil extracellular traps) lead to inflammation of the alveoli. It then causes edema and

compromises the respiratory function of the lungs. This response causes the main symptoms of pneumonia, fever, cough, purulent sputum, and dyspnea.

Children under five years old and elderly people are the most susceptible to pneumonia. Due to vaccination programs the number of children dying because of pneumonia reduced a lot, however this was not true for elderly people (Figure 4). Although the advent of vaccines, pneumonia is still the infection that kills more children in the world, accounting for 16% of death among children under 5 years old ("WHO | Causes of Child Mortality" 2017; "WHO | Pneumonia" 2017). In 2016 it was estimated that 800.000 children died of pneumonia, and it accounted for more deaths than all other causes combined (Troeger et al. 2018). The higher susceptibility of children to pneumonia is linked to several factors, such as exposure to indoor air pollution, low birth weight, incomplete immunization, lack of exclusive breastfeeding, undernutrition, HIV infection and living with more than 7 persons (Jackson et al. 2013).

Individuals over 70 years old present infection rates of pneumonia four times higher than adults, accounting to more than 1 million deaths in 2017 (Dadonaite and Roser 2018) (Figure 4). In addition the risk of hospitalization, the time needed to recover and the death rates caused by the infection are also higher in this population (Stupka et al. 2009). Elderly people may present subtle symptoms, or display atypical symptoms of pneumonia, generally mental confusion, and fever. The higher susceptibility of this population is attributed to factors such as poor nutritional status, chronic comorbidities, smoking and alcohol consumption. Despite this factors, elderly individuals also present physiological alterations, such as reduction in the pulmonary functions (muscle strength and elasticity) and reduced immune function (Fung and Monteagudo-Chu 2010).

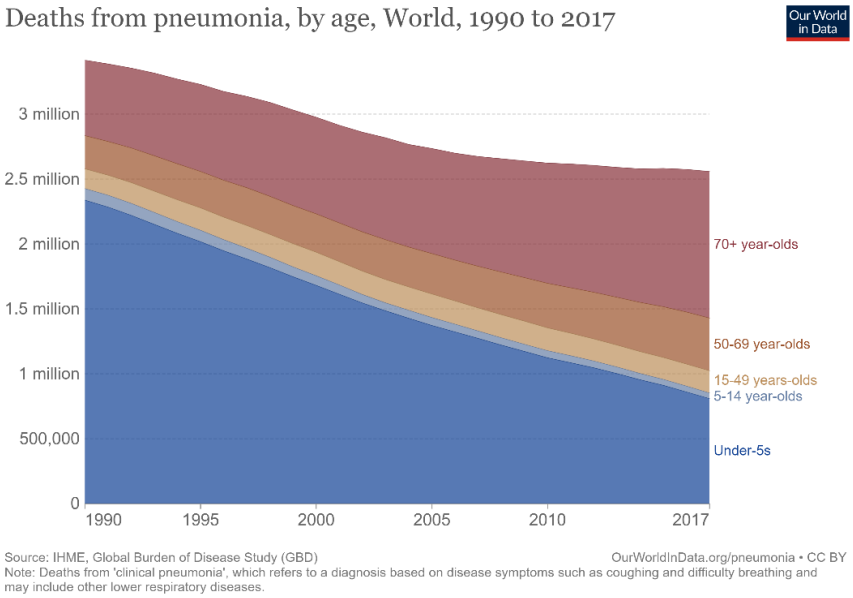


Figure 4: Deaths from pneumonia by age, from 1990 to 2017.
After introduction of vaccination protocols in 2000, death numbers of children reduced considerably, meanwhile no major difference was observed for teenagers and adults. On the other hand, death numbers are higher for elderly people. Chart from Dadonaite and Roser 2019.

Of note, developing countries have higher incidence and mortality due to pneumonia than developed countries (Dadonaite and Roser 2018). This scenario is linked to lower access to adequate healthcare, treatments, and vaccines. In addition, as mentioned before, the nutritional score is critical for pneumonia susceptibility, and it is a major concern in developing countries.

Pneumonia can be caused by fungi, bacteria, and viruses. Among the vast diversity of responsible agents, *Streptococcus pneumoniae* is the main cause of community acquired pneumonia (R. Corrêa, Lundgren, and Silva 2009). Among adults, *S. pneumoniae* is responsible for 36% of pneumonia cases (“CDC | Pinkbook | Pneumococcal” 2015).

Prevention and treatment

Nowadays there are two different types of vaccine in the market to prevent infection caused by *S. pneumoniae,* PCV13 and PPSV23. The nomenclature of the vaccine comprises the type (pneumococcal conjugate vaccine – PCV and pneumococcal polysaccharide vaccine – PPSV) and the number of serotypes covered. The efficacy of PCV13 is 46% against

pneumococcal pneumonia and 75% against invasive pneumococcal disease for covered serotypes, and it is recommended for children younger than 2 years old (Bonten et al. 2015). The efficacy of PPSV23 varies between 60% and 70% against invasive pneumococcal disease caused by covered serotypes and there is no evidence that this vaccine is effective against pneumococcal pneumonia (Wang et al. 2018). In addition, children under 2 years old have a poor antibody response to this vaccine. Therefore, PPSV23 is recommended to people older than 65 years old and to those over 2 years old with specific medical conditions. In June this year PCV20 was approved by the FDA, however it is still not part of vaccination programs (Cber and Fda 2021).

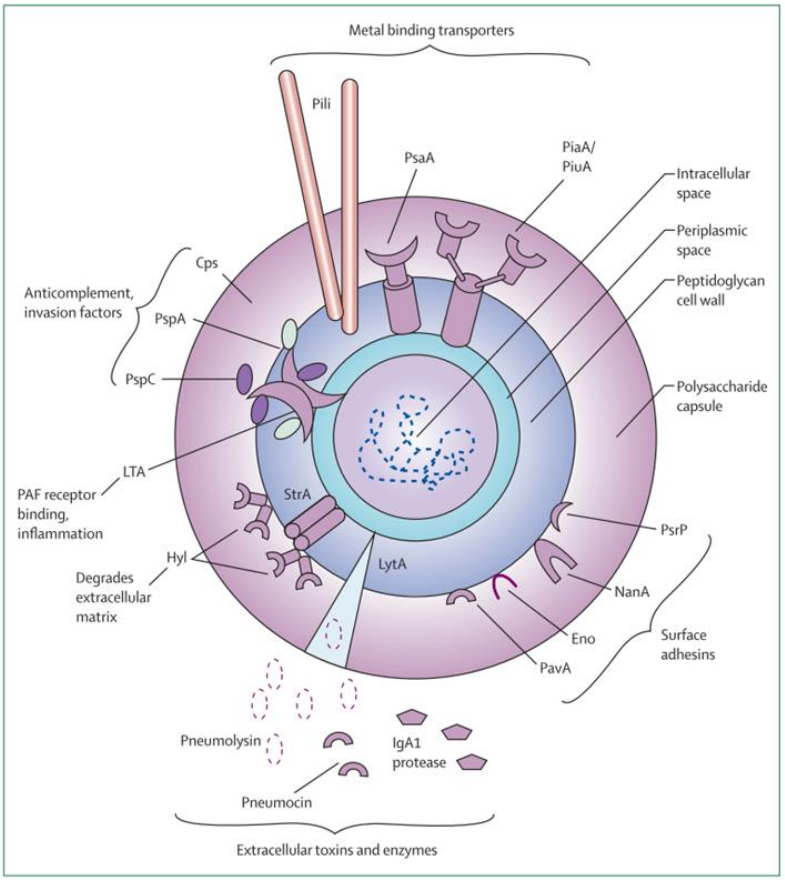
The treatment for pneumococcal pneumonia is based on antibiotics administration. Upon diagnosis of pneumonia, samples are collected for specific diagnosis and antibiogram test, meanwhile an empiric treatment is applied. The empiric treatment usually consists of macrolide and cephalosporin or a fluoroquinolone (Wunderink and Yin 2016). However, resistance against these antibiotics was already reported. Between 20% and 40% of *S. pneumoniae* isolates showed resistance to macrolides, 1-29% were resistant to cephalosporin and a just a small portion, around 1% were resistant to fluoroquinolone (Cherazard et al. 2017).

Due to the low coverage of *S. pneumoniae* serotypes by vaccines and the emerging resistance against antibiotics, it is important to keep studying new therapies against *S. pneumoniae*.

Streptococcus pneumoniae

Streptococcus pneumoniae are coccus Gram-positive, with lancet or oval shape, measuring 0.5 to 1.25 µm of diameter, displayed in pairs or in short chains. It is a facultative anaerobic microorganism, capnophilic (needs a rich atmosphere of CO_2 to grow) and fastidious (needs a very nutritive medium supplemented with blood to grow) (Murray,

Rosenthal, and Pfaller 2014). The classification into serotypes is based in the capsule, and nowadays there are more than 95 serotypes described. Most bacteria are encapsulated and the polysaccharide capsule is a virulence and pathogenesis factor (Murray, Rosenthal, and Pfaller 2014; Kadioglu et al. 2008). *S. pneumoniae* has also a complex cell wall, with heterogeneous composition of peptidoglycan, teichoic acid and lipoteichoic acid (Vollmer, Massidda, and Tomasz 2019). *S. pneumoniae* has several mechanisms to attack and evade the immune system (Figure 5). To establish an infection the bacterium secretes pneumolysine (PLY) and hydrogen peroxide which can impair the mucociliary function, followed by the production of neuraminidase that exposes binding sites for surface adhesins to adhere to the epithelium (van der Poll and Opal 2009; JB et al. 1993). Once attached to the host tissue, the bacterium needs to evade the immune system. To avoid opsonization and killing mediated by complement, *S. pneumoniae* expresses different anti-complement proteins and IgA protease. The mechanisms of action are through inhibition of complement-bacterium binding or via cleavage of complement components or immunoglobulins (Martner et al. 2009). In addition, the presence of capsule can also prevent opsonization and phagocytosis by masking the antigens present in the bacterial surface (Marquart 2021). To deal with cellular response *S. pneumoniae* induces cellular lysis by secreting PLY and hydrogen peroxide (van der Poll and Opal 2009). Once phagocytosed, different genes are expressed (like *sodA*, *nox*, *psaA* and *psaD*) and the produced proteins remove ROS or alter the redox status of the cell, to protect the bacteria from ROS damage (Yesilkaya et al. 2000; LJ et al. 2004). In the lungs, *S. pneumoniae* express pili which contributes to bacterial invasion (Rosch et al. 2008). In line, bacteria can disseminate by expressing pneumococcal surface protein C, which binds to polymeric immunoglobulin receptor (PAFR) to translocate from the luminal side to the apical side of the cell (van der Poll and Opal 2009).



PsaA=pneumococcal surface antigen A.
PiaA/PiuA=pneumococcal iron acquisition and uptake.
PsrP=pneumococcal serine-rich repeat protein.
NanA=neuraminidase.
Eno=enolase.
PavA=pneumococcal adhesion and virulence.
LytA=autolysin.
StrA=sortase A.
Hyl=hyluronate lyase.
LTA=lipoteichoic acid.
PspC=pneumococcal surface protein C.
PspA=pneumococcal surface protein A.
Cps=polysaccharide capsule.
PAF=platelet-activating factor.

Figure 5: Overview of virulence factors of *Streptococcus pneumoniae*.
To avoid being trapped, *S. pneumoniae* secrets toxins, enzymes, and express Hyl. The polysaccharide capsule protects bacteria from recognition and degradation. Surface adhesins are used by the bacteria to attach to the host tissue. LTA allows the bacterium to bind to PAFR and translocate. Anticomplement proteins allow the bacterium to evade opsonization and killing by complement. Metal binding transporters are essential to avoid oxidative damage. Adapted from Poll and Opal 2009.

The respiratory system and its barriers

The respiratory system is divided in upper respiratory tract and lower respiratory tract. The upper respiratory tract consists of nose, pharynges, and associated structures, while the lower respiratory tract comprises larynges, trachea, bronchus, and lungs. The very first step of control from what goes to the lower respiratory tract is in the nose. Inside of the nose there are tick hairs to filter big particles of dust and mucus to trap particles of dusty that will later be expelled. Smaller particles that passed through the nose can arrive in the larynges or even in the trachea. In these sites, mucus secreted by goblet cells traps these particles and the ciliated movements performed by ciliated cells transport the mucus with dust particles to the

pharynges, to then be eliminated. The trachea is divided in two main bronchi, the right main bronchus which goes to the right lung and the left main bronchus which goes to the left lung. Once in the lungs, the bronchi pass through a series of divisions to form smaller units until the terminal bronchioles, and this structure is called bronchial tree. Along these divisions there are many structural changes in the epithelium, however, the epithelium remains ciliated until the smaller bronchioles. Therefore, the elimination of small particles is still mediated by the mucus, which traps the particle, and the ciliated movements that expel this complex. On the other hand, in the terminal bronchioles there is no ciliated cells, thus the elimination of inhaled particles is done by macrophages. The terminal bronchioles give rise to several respiratory units, which are composed by alveolar ducts, alveolar sacs, and the alveolus (Tortora and Derrickson 2016). The alveolus is the anatomic unit of gas exchange in the lungs, and it is composed by type I alveolar epithelial cells (AEC), type II AEC, endothelial cells, alveolar macrophages, interstitial macrophages, lymphocytes, mast cells and fibroblasts. Type I AECs cover more than 90% of the alveoli surface area and are the responsible for gas exchange. Type II AEC are self-renewing cells that secrete surfactant proteins and are progenitors for type I AEC population (Whitsett and Alenghat 2014). Alveolar macrophages phagocyte small particles and microorganisms and are responsible for the surveillance of the tissue, while interstitial macrophages play a role in the inflammatory response together with alveolar macrophages and AECs (Nicod 2005). Mast cells can be activated by antigens, degranulate and lead to increased vascular permeability, causing edema (Krystel-Whittemore, Dileepan, and Wood 2016). Fibroblasts are important for the early development of alveoli and then, to give a structural support for alveolar regeneration (Ushakumary, Riccetti, and Perl 2021).

In the airways, mucus is the main component of the barrier, and it is produced by goblet cells, club cells, alveolar cells, and submucosal glands. Mucus is composed by proteoglycans, large glycoproteins (mucins) and phospholipids and it comprises two phases, the periciliary

liquid and the gel on the surface of the cilium (Whitsett and Alenghat 2014). The periciliary liquid is a physical barrier underlying the epithelial surface which avoids the contact of pathogens with the epithelial surface and allows the ciliary beating (Widdicombe 2002). The gel phase, on the other hand, disrupts bacterial aggregation, traps the pathogens to prevent their adhesion to cell surface and enhance their clearance by ciliary movements (Fahy and Dickey 2010). The physical barrier in the lungs is more complex and involves different factors than mucus and ciliary movements.

Pulmonary surfactant is essential to control the surface tension in the alveolus during the dynamic changes in lung volumes generated by respiration. More than 90% of the surfactant is composed of lipids and less than 10% are surfactant proteins (Whitsett and Alenghat 2014). Surfactant proteins have an important role in the structure, function, and regulation of pulmonary surfactant, despite their intrinsic antimicrobial properties. Although surfactant proteins (SP) A, B, C and D are secreted by type II AEC, they exert different functions. SP-B and SP-C are used to assist the spreading and the stability of surfactant lipids (Whitsett and Alenghat 2014). On the other hand, SP-A and SP-D bind to a wide range of viruses, bacteria, and fungi to neutralize or opsonize these pathogens. Therefore, facilitating their phagocytosis and killing by alveolar macrophages (Takahashi et al. 2006).

Antimicrobial proteins and peptides are also present in the mucus. Lysozyme can be secreted by macrophages, submucosal glands, epithelial cells, and serous cells. It degrades any glycosidic linkage of bacteria, consequently removing bacteria adhered to the epithelium. In addition, lysozyme can also kill Gram-positive bacteria by the hydrolysis of their peptidoglycan wall, culminating in bacterial lysis (Evans et al. 2010). Iron sequestering proteins, such as lactoferrin and lipocalin-2 are produced by epithelial cells, while haptoglobin is also produced by alveolar macrophages. These proteins trigger bacterial killing by reducing the availability of iron, an essential factor for bacterial replication. S100 protein family members are calprotectin produced by airway epithelial cells described to have an

important antimicrobial role against respiratory pathogens (Donato 2003). Their antimicrobial activity with broad-spectrum is due to their ability to sequester the metals Zn_2 and Mn_2. Therefore, S100 proteins have a similar effect on pathogens to the one described for iron sequestering proteins; bacterial growth is impaired due to the lack of nutrients (Zackular, Chazin, and Skaar 2015). As part of antimicrobial proteins, there are the antimicrobial peptides defensins and cathelicidin. Defensins are subdivided into two groups α- and β-defensin. β-defensin is the only group secreted by pulmonary epithelial cells, and only HβD-1 is expressed constitutively. The cathelicidin CAMP is secreted by the pulmonary epithelium and upon cleavage it originates the antimicrobial peptide LL-37 (Evans et al. 2010). To date, the mechanism by which these antimicrobial peptides exert their function is not elucidated. However, the literature suggests two different pathways for the antimicrobial effect of these peptides. One is the induction of transmembrane pore formation in the pathogen, and the other is an alteration in the metabolism of the pathogen, leading to different outcomes, such as inhibition of cell wall, nucleic-acid and protein synthesis (Brogden 2005).

Apart from the classical antimicrobial proteins, other proteins are described to have microbicidal functions. Secretory leukocyte proteinase inhibitor (SLPI) and elafin produced by epithelial cells and macrophages are also present in the airways and are described to have antibacterial effects towards Gram-positive and Gram-negative bacteria. In addition, secretory IgA is secreted by epithelial cells into the alveolar cavity. IgA neutralizes viruses and toxins and blocks the passage of bacteria across the epithelium, additionally it can activate the alternative complement pathway leading to better opsonization of the bacteria (Nicod 2005).

Complement proteins produced by type II AEC, bronchiolar epithelial cells and alveolar macrophages are present in physiological conditions in the lungs (Kulkarni et al. 2018). Complement is an important tool of defense against infections that acts by three mechanisms, opsonization of the pathogen, recruitment of phagocytes to eliminate the pathogen and induction of pathogen's lysis. To exert its function, complement can be activated by three

different pathways (Watford, Ghio, and Wright 2000). The classical pathway involves the formation of immune complexes of IgM or IgG antibodies with the cellular wall of the pathogen, which activates a signaling cascade via complement C1. The lectin pathway, in which a mannose-binding lectin binds to bacterial cell surface and this complex is recognized by mannose-associated serine proteases or IgA, that latter trigger the signaling cascade. The alternative pathway involves a spontaneous hydrolysis of C3 protein. All three pathways converge to the activation of C3 convertase, that can form C3a and C3b. C3b opsonizes the bacteria and C3a is a chemoattractant for phagocytes. The C3 convertase can also trigger the assembly of C5 convertase, which can cleave C5 into C5a and C5b, to attract phagocytes. Or C5 convertase can lead to the formation of membrane attack complex (MAC), composed by C5 – C9. This complex creates pores in the bacterial membrane resulting in bacterial lysis (Pandya and Wilkes 2014).

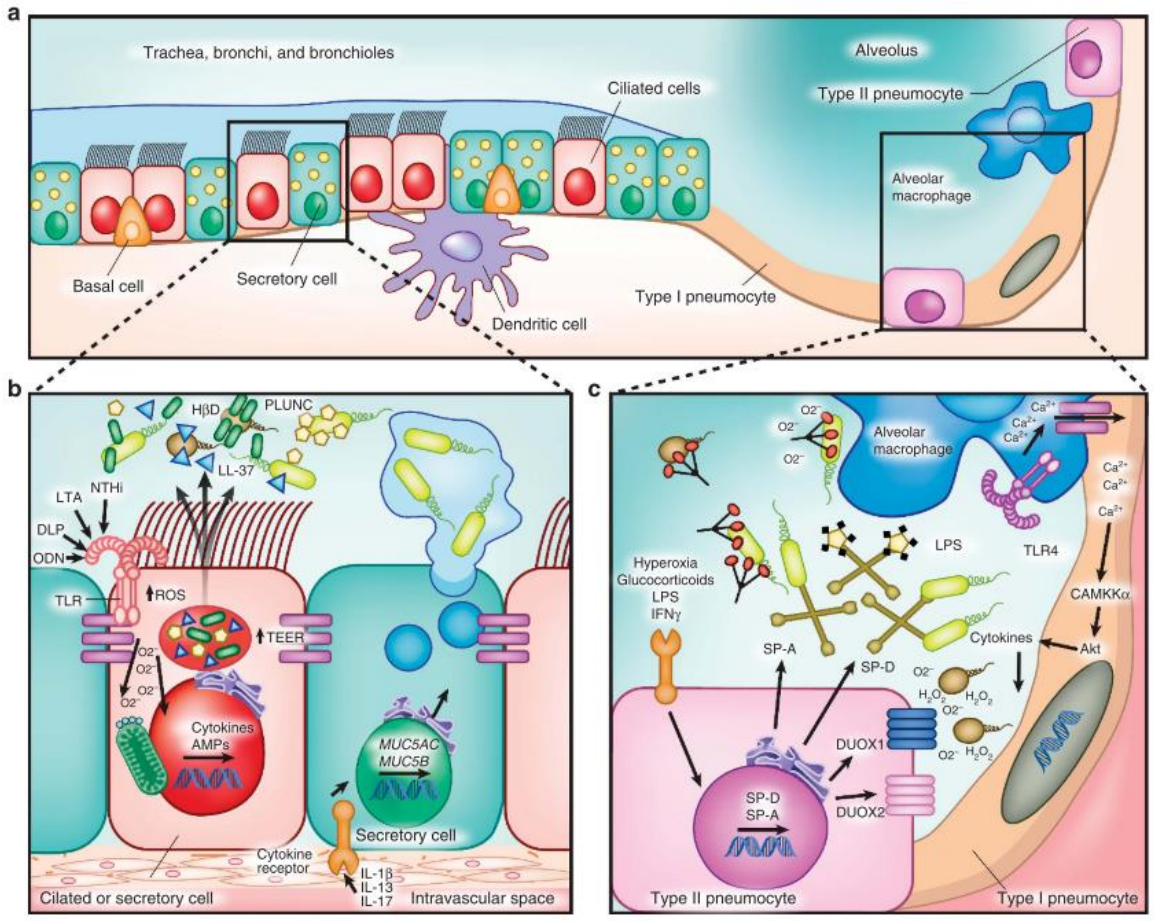


Figure 6: Composition of pulmonary epithelium and its response against intruders.

Panel a represents the epithelium in trachea, bronchi, and bronchioles, which is composed by basal cells, ciliated cells, and secretory cells (goblet and club cells). It also represents alveolar epithelium, composed by AEC type I and II and alveolar macrophages (AM). Panel b shows the airway response upon detection of microorganisms. Ciliated and secretory cells recognize the intruder, signal to the intracellular compartment to produce reactive oxygen species (ROS), cytokines and antimicrobial peptides (AMPs) like LL-37 and HβD. They also reinforce the epithelial barrier (TEER). Secretory cells upon activation produces mucins to trap the pathogens that will be swept by the ciliated movements. Panel c represents the alveolar response upon recognition of intruders. AEC type II produces surfactant proteins (SP-D and SP-A) and ROS (DUOX). In association with AM, AEC type I produces cytokines to recruit and activate immune cells. Figure from Leiva-Juárez, Kolls, and Evans 2017.

Host defense against *Streptococcus pneumoniae*

S. pneumoniae can infect humans by two manners, it can be transmitted among individuals via larger respiratory droplets or smaller aerosols, named exogenous transmission, or it can translocate from the nasopharynges to the lungs, named endogenous dissemination. The endogenous dissemination commits mainly young children, once they present high rates of *S. pneumoniae* colonization in the nasopharynges (Syrjänen et al. 2001).

To establish an infection, the bacteria must be able to overpass the physical barriers, adhere to the epithelium and evade the immune response (Murphy 2011). The bacteria encounter the first mechanism of host defense, the physical barrier, present in the upper and lower respiratory tracts. At this stage, the bacteria is trapped by the mucus and it is eliminated by ciliated movements of epithelial cells (Marquart 2021). If primary barriers are evaded, the bacteria are recognized by alveolar macrophages and AECs, which signal the presence of the invader. Despite the constitutive secretion of proteins, AECs can also upregulate the secretion of surfactant proteins and antimicrobial peptides (AMPs) to contribute to pathogen elimination. In addition, they can release chemokines such as IL-8, CXCL-1, CXCL5, CCL20 or monocyte chemotactic protein-1 (MCP-1) (Yamamoto et al. 2014). They can also secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) to recruit neutrophils and promote macrophage-mediated bacterial killing. In line with GM-CSF function, SP-A

secreted by type II AECs was also shown to promote phagocytosis of *S. pneumoniae* via scavenger receptor (Kuronuma et al. 2004).

Alveolar macrophages (AMs) are the first cells of pulmonary immune defense and, with the help of immunoglobulins and complements, are responsible for phagocytosis and bacterial clearance (Gordon et al. 2000). Although leukocytes and epithelial cells recognize *S. pneumoniae* via pattern recognition receptors (PRRs), alveolar macrophages are the first and most important cell to respond against the infection via activation of signaling cascades, resulting in cytokines and chemokines secretion (Abbas, Lichtman, and Pillai 2015). The main receptors involved in the response against *S. pneumoniae* are the toll like receptors (TLRs) and the NOD like receptors (NLRs) (Figure 6) (Koppe, Suttorp, and Opitz 2012). The recognition of lipoteichoic acid and lipoproteins from bacterial wall by TLR2, triggers the production of cytokines such as TNF-α, IL-6 and IL-1β (G. Tomlinson et al. 2014). For activation of TLR9, the bacteria need to be phagocytosed and their DNA containing CpG need to be exposed. Apart from recognition of bacterial DNA, TLR9 also plays a role in the phagocytic ability of macrophages, however this receptor does not seem to be essential for cytokine secretion (Albiger et al. 2007). The activation of TLR4 by PLY results in production of IL-6, IL-1β and CXCL1, which is responsible for the neutrophil recruitment (Koppe, Suttorp, and Opitz 2012). Similar to TLR9, NOD2 is located inside the cell, and it only recognizes fragments of peptidoglycan after bacterial phagocytosis. As a result of NOD2 activation pro-inflammatory cytokines and CCL2 are produced, leading to recruitment of macrophages to the site of infection (G. Tomlinson et al. 2014). The proteins from the inflammasome complex, NLRP3 and AIM2, binds to PLY and to bacterial DNA, and then they recruit the adaptor protein ASC to activate the complex. Upon activation, caspase 1 is cleaved into its active form (caspase 1 p20), which is responsible for the cleavage of pro-IL-1β into IL-1β, leading to the secretion of this cytokine (Rabes, Suttorp, and Opitz 2016; R. Fang et al. 2011). Despite the well described receptors mentioned so far, there are some

sensors of cytoplasmatic DNA, such as DHX36 and DDX41, which seems to recognize *S. pneumoniae* DNA and induce the production of type I IFN (Koppe et al. 2012; Koppe, Suttorp, and Opitz 2012).

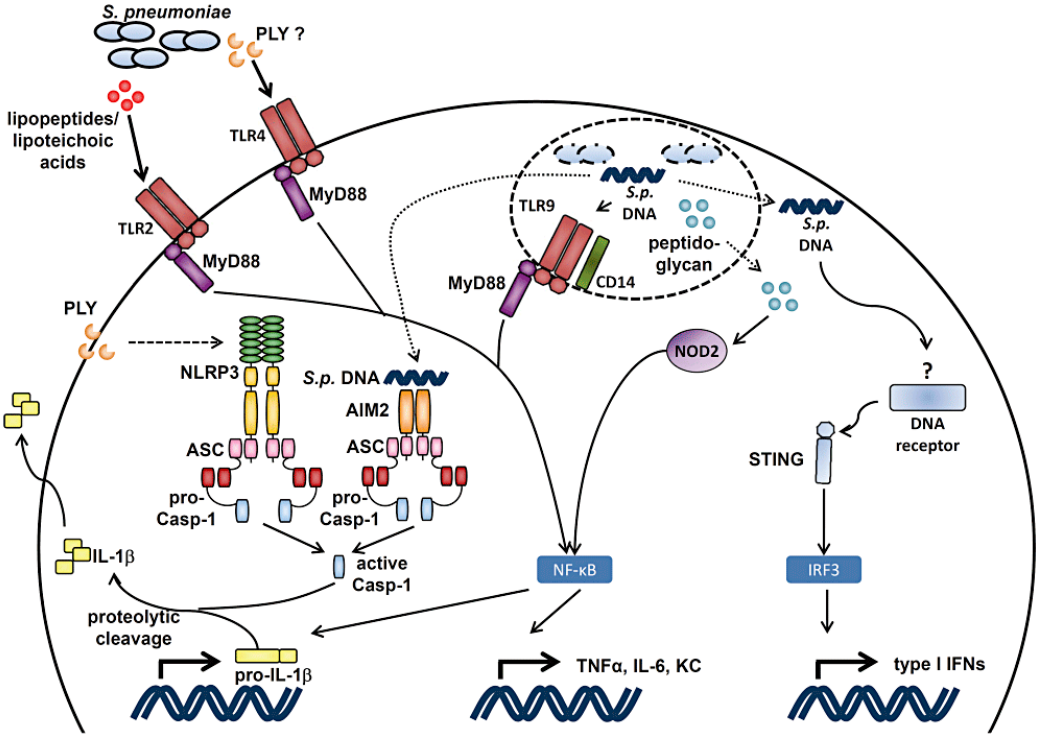


Figure 7: Overview of immune response triggered by *S. pneumoniae*.
Pneumolysin (PLY) and component from bacterial wall are recognized by TLR4 and TLR2. After phagocytosis, bacterial DNA is exposed in the phagolysosome and further recognized by TLR9. These three TLRs recruits the adaptor protein MyD88 which activates NF-κB triggering cytokine production. Peptidoglycan is released from phagolysosome and recognized by NOD2, which also activates NF-κB. PLY and bacterial DNA fragments are recognized by NLRP3 and AIM2, respectively. These two NLRs recruits ASC and form the inflammasome, which cleaves pro-IL-1β into IL-1β. In addition, bacterial DNA also seems to be recognized by DNA sensors that activate STING and IRF3. Figure from Koppe, Suttorp, and Opitz 2011.

The secretion of chemokines recruits neutrophils which also contribute to bacterial elimination via phagocytosis, production of reactive oxygen species (ROS), AMPs, proteases, and neutrophil extracellular traps (NETs). However, if the infection is not controlled at that time point, the recruitment of neutrophils becomes more intense and they can cause injury to the tissue (Koppe, Suttorp, and Opitz 2012). T lymphocytes also migrate to the lungs to try to control the infection, $CD4^+$ T lymphocyte produces microbicidal cytokines such as IFN-γ

(Hirst et al. 2004). Later, T reg lymphocytes are recruited to promote the resolution of the inflammation (Neill et al. 2012). Therefore, the inflammatory response against *S. pneumoniae* is considered acute and driven mainly by the innate immune system.

Alveolar macrophages

Contrary to what was believed during decades, AMs are neither derived from bone marrow monocytes nor replenished from circulating adult monocytes. Instead, AMs are originated from fetal monocytes that go to the lungs before birth and differentiate in response to granulocyte-MF colony-stimulating factor (GM-CSF) (Guilliams et al. 2013). These cells are long-lived self-renewing and under physiological conditions they are the only responsible for the maintenance of their own population (Maus et al. 2006). As mentioned before, AMs are the first line of defense in the lungs, and they were recently shown to be patrolling cells. They can move from one alveolus to another to clean all particulate matter (Neupane et al. 2020). Alveolar macrophages display different markers in their surface when compared to recruited macrophages, such as CD11c, CD200R, CD206 and SIGLEC-F (Hussell and Bell 2014). Macrophages can have different profiles, and although they are grouped into M1 and M2 when differentiated *in vitro*, these cells can also display intermediate states. The pro-inflammatory macrophage, M1, induced by LPS and IFN-γ is linked to antimicrobial response through production of NO, and they are described to secrete pro-inflammatory Th1 cytokines like TNF-α, IL-6, IL-1β, and IL-12. The alternatively activated macrophage, M2, is induced by IL-13 or IL-4 produced by immune cells, such as Th2 lymphocytes, mast cells and basophils. These cells secrete high levels of IL-10 and are linked to anti-inflammatory and pro-resolutive roles (Aberdein et al. 2013). Interestingly, a study found that most human alveolar macrophages express M1 and M2 surface markers at the same time in a steady state condition ($CD86^{hi}CD206^{hi}$) (Mitsi et al. 2018). The expression of these two markers might be linked to the need of a dual response by these cells. Alveolar macrophages have to phagocyte

cellular debris and small particles without triggering an inflammatory response, meanwhile, the recognition and phagocytosis of pathogens need to trigger an inflammatory response to clear the infection (Hussell and Bell 2014).

Studies demonstrating the polarization of alveolar macrophages are scarce and contradictory. A study published in 2012 showed that the profile of alveolar macrophages in acute pulmonary inflammation by LPS or chronic inflammatory lung disease was distinguished by CD11c and Mac-1 expression rather than classical M1 or M2 markers (Duan et al. 2012). In line, a study with human alveolar macrophages showed the difficulty to classify alveolar macrophages in M1 or M2 in the context of chronic obstructive pulmonary disease (COPD). Authors classified 35% of alveolar macrophages as double positive for M1 and M2 markers, and 25% as negative for M1 and M2 markers. Interestingly, the 25% of macrophages that were negative for both markers had a pro-inflammatory gene signature (Takiguchi et al. 2021). In accordance, the profiling of alveolar macrophages in patients with asthma is rather contradictory. One study showed that alveolar macrophages from patients with asthma presented an increase in M2 profile, and the severity of the disease was correlated to the higher expression of CD206 and MHCII (Girodet et al. 2016). On the other hand, another study also described an increase in M1 population characterized by $IRF5^+$ in asthmatic patients together with an increase in $CD206^+$ M2 profile (Draijer et al. 2017). Hence, it is hard to subdivide alveolar macrophages into M1 and M2 *in vivo*. This difficulty can be linked to the complexity of the pulmonary environment and to the fact that alveolar macrophages at steady state already present surface markers of M1 and M2, meanwhile monocytes are differentiated into macrophages with a “neutral” profile, M0. In addition, to enhance the complexity of analysis each study uses a different set of markers to evaluate M1 and M2 profiles. While it is complex to phenotype macrophages *in vivo*, stimulation *in vitro* can polarize alveolar macrophages into M1 or M2 profile as summarized in Table 2 (S. Chen et al. 2016; H. Liu et al. 2019; G. S. Tomlinson et al. 2012). Although the profiling of

macrophages into M1 and M2 is useful in many conditions, studies have shown that macrophages can have several different profiles, even *in vitro*, that are not comprised among the axis M1 – M0 – M2. In addition, macrophages have remarkable plasticity, which allows them to respond to environmental changes and switch phenotypes (Mosser and Edwards 2008; Xue et al. 2014).

S. pneumoniae **killing by alveolar macrophages:**

AMs have many different methods to kill bacteria (Figure 8). They can produce and secrete antimicrobial peptides (AMP) such as β-defensin and cathelicidin (LL-37 in humans and CRAMP in mouse). Generally, AMPs exert its function by inducing membrane permeabilization of microorganisms (Beisswenger and Bals 2005). Also, in the presence of bacteria or particles such as zymosan, AMs from rats secrete higher concentrations of lysozyme which is shown to effectively kill bacteria (Biggar and Sturgess 1977). To date, the effectiveness of AMPs and lysozyme against *S. pneumoniae* is not clear, apparently a good percentage of bacteria can evade these mechanisms regardless their serotypes (Habets, Rozen, and Brockhurst 2012; Assoni et al. 2020). Additionally, AM can also produce and secrete complement components, which can opsonize and kill bacteria (Lubbers et al. 2017; Cole et al. 1980). Apart from the extracellular killing, macrophages can also promote intracellular killing of pathogens. For this, bacterial need to be internalized (phagocytosed). Phagocytosis is initiated through the recognition of the particle/bacteria by a vast range of surface receptors. Particle/bacteria binding to receptors triggers a deformation in the membrane, a formation of pseudopod and later the particle is engulfed by membrane contraction (Niedergang and Grinstein 2018). The recognition can be direct, i.e., scavenger receptors (like MARCO) expressed by AMs bind to the surface of the particle/bacteria. It can also be indirect, in this case, receptors (like Fcγ and complement receptor-CR) bind to opsonins (immunoglobulins and complements) which are bound to the particle/bacteria (Groves et al. 2008; Lafuente,

Niedergang, and Rosales 2020). In the case of *Streptococcus pneumoniae*, opsonization is extremely important for effective phagocytosis (Gordon et al. 2000). Once phagocytosed, the bacteria can be killed by oxygen-independent or -dependent mechanisms. Inside the mature phagolysosome, which has an acid pH, AMPs, lysozymes and proteases directly promote bacterial killing and the absence of Fe^{2+}, Zn^{2+} and Mn^{2+} (which are extruded from the phagolysosome) limits bacterial growth (Flannagan, Cosío, and Grinstein 2009; Uribe-Quero and Rosales 2017). The oxygen-dependent mechanism relies on the production of ROS and RNS. Although ROS is mainly produced by neutrophils, macrophages can also produce it inside the phagosome, via NADPH oxidase system. On the other hand, nitric oxide (NO) is highly produced by macrophages in the cytoplasmic side of phagosomes. NO can easily spread and diffuse across the membrane and once in the luminal side it is converted into a range of RNS (Flannagan, Cosío, and Grinstein 2009). ROS and RNS can interact with proteins, lipids and nucleic acids from bacteria leading to death (F. C. Fang 1997). Despite the high toxicity of ROS, *S. pneumoniae* presents mechanisms to counteract these species, thus it does not play an important role on its clearance (Marriott et al. 2007; Aberdein et al. 2013). Contrary, NO is described as the main responsible to mediate the killing of *S. pneumoniae* by alveolar macrophages (Kerr et al. 2004; Marriott et al. 2006; 2007).

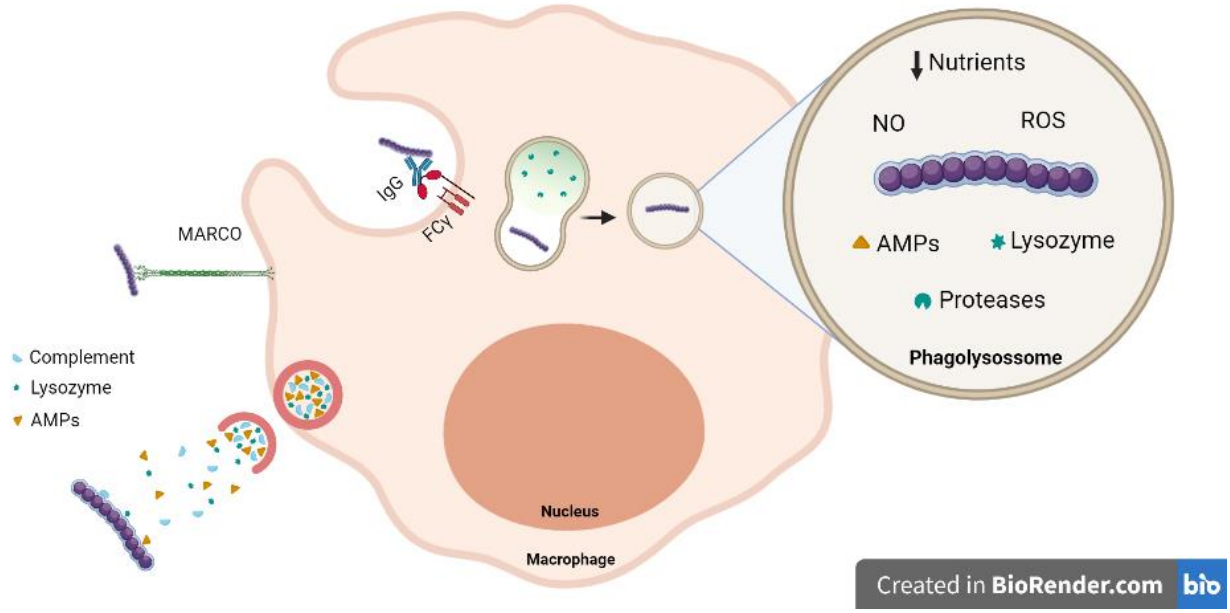


Figure 8: Mechanisms of S. pneumoniae killing promoted by macrophage.
Extracellular *S. pneumoniae* killing has low efficiency and it is mediated by the secretion of lysozyme, complement and antimicrobial peptides (AMPs). *S. pneumoniae* can be recognized by scavenger receptors such as MARCO, although the effectiveness of phagocytosis led by this recognition is low. The most effective mechanism is the phagocytosis of opsonized bacteria (by immunoglobulins -IgG- or complement). Once opsonized, Fc receptors or complement receptors bind to this complex and triggers the phagocytosis. Then, the phagosome is fused with lysosome where the pH is lower, and there is an efflux of nutrients and a high concentration of proteases, AMPs, lysozyme, reactive oxygen species (ROS) and nitric oxide (NO). The combination of those factors, especially the NO presence, promotes bacterial killing.

Immunometabolism of macrophages

In the early 50's the metabolism of immune cells started to caught attention from researchers. The first publications were describing the metabolism of glycogen and oxygen consumption by phagocytes during *in vitro* phagocytosis (Puchkov NV 1955; De Gregorio 1956; Bazin, Delaunay, and Avice 1953). At this period, correlations between oxygen consumption, glycolysis and phagocytosis started to raise (Stähelin, Suter, and Karnovsky 1956). These researchers observed that after adding bacteria to macrophages, there was an increase in oxygen consumption, but no changes were observed in the lactic acid production. Besides these early descriptions, immunometabolism has just recently became a trend. Nowadays, with the advances in technology, there are several methods to evaluate the

metabolism of macrophages. The main metabolic pathways correlated with immune response are the glycolysis, tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OXPHOS), pentose phosphate pathway (PPP) and fatty acid metabolism (Figure 9).

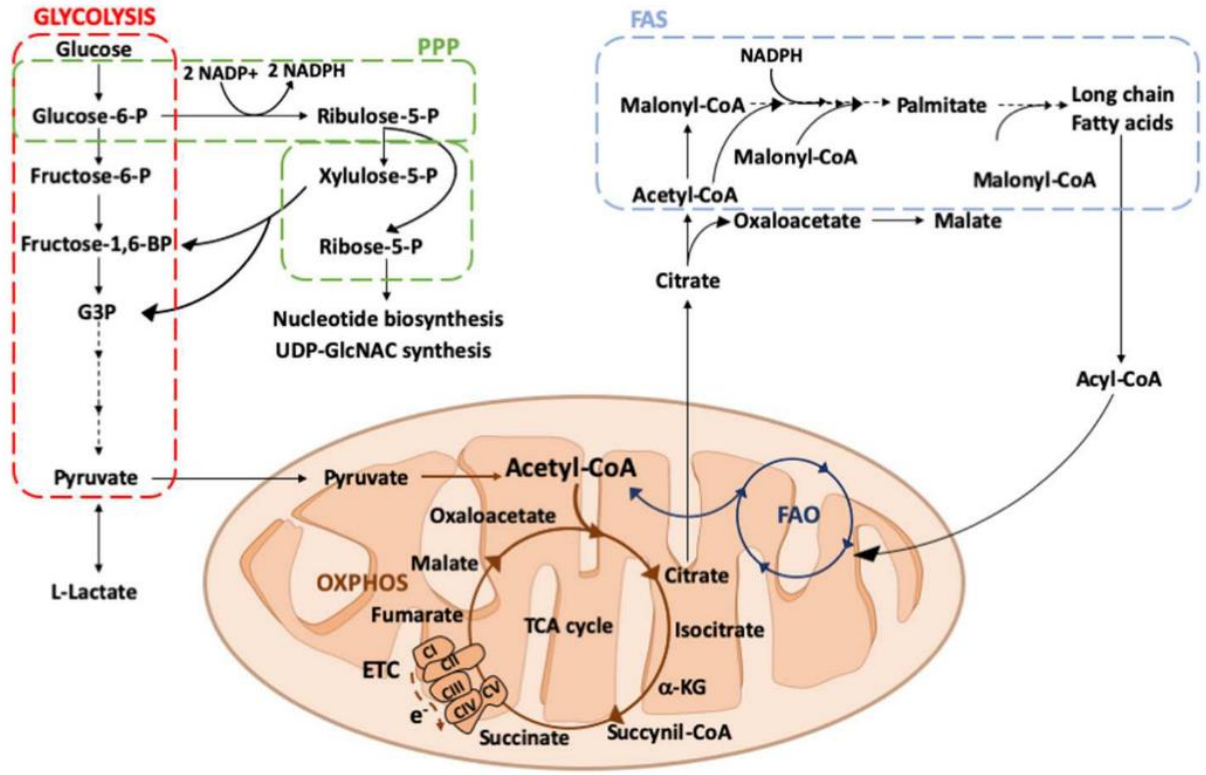


Figure 9: Overview of major metabolic pathways involved in macrophage's function.
Glucose is converted into pyruvate by the glycolytic pathway (red square). Glucose 6-phosphate, a metabolite of glycolysis can be used in the PPP (green square) to generate NADPH and ribose 5-phosphate, which is used for glycosylation of proteins. Pyruvate originated from glycolysis goes to the mitochondria and is converted into acetyl CoA to enter the TCA cycle (red circle). TCA cycle produces NADH and $FADH_2$ to fuel the electron transport chain (ETC) and generate ATP. Citrate, a metabolite of TCA cycle, goes to the cytoplasm where it is converted into acetyl CoA. Acetyl CoA condensates malonyl CoA to form fatty acids in the fatty acid synthesis (FAS). Generated fatty acids are then activated with acetyl CoA to go to the mitochondria and be oxidized in the fatty acid oxidation (FAO) pathway. Image from Viola et. al 2019.

To recapitulate, glycolysis starts with the entrance of glucose in the cell via solute carrier family 2 members (SLC2A1, SLC2A2, SLC2A3, SLC2A4), being SLC2A1 the most expressed by macrophages (Freemerman et al. 2014). Glycolysis happens in the cytoplasm and can be divided in three stages. At stage 1, glucose is modified to be trapped in the cell and then transformed in a compound that can be readily cleaved into three-carbon units. In this step, two molecules of ATP are consumed. At stage, 2 fructose 1,6-biphosphate is cleaved

into two three-carbon fragments. At stage 3, three-carbon fragments are oxidized to pyruvate and generate 4 molecules of ATP and one of $NADH^{+}$. Therefore, the end-products of glycolysis are two molecules of ATP and two of pyruvates. Pyruvate can have different fates; in the presence of oxygen, it is converted to Acetyl CoA to enter in the TCA cycle, while in the absence of oxygen, it is converted to lactic acid by lactate dehydrogenase (LDH) (Berg and Tymoczko, John L and Stryer 2002).

The glycolytic profile of macrophage has been the focus of several studies. As described above, glycolysis does not generate high amounts of energy (ATP), however, activated macrophages with a pro-inflammatory profile have increased glycolytic activity and increased secretion of lactate (Kelly and O'Neill 2015; Galván-Peña and O'Neill 2014). This phenomenon is described as Warburg effect (Warburg, Wind, and Negelein 1927). The Warburg effect happens under normoxic conditions, in which the cell shifts the metabolism from respiration toward glycolysis, and pyruvate is converted to lactate instead of entering in the TCA cycle. This seems to be controversial, once activated cells need more energy to respond to stimulus, and glycolysis alone generates only 2 molecules of ATP, meanwhile mitochondrial respiration generates 36 molecules of ATP. Nevertheless, aerobic glycolysis results in higher rates of ATP than mitochondrial respiration due to the ability to over-activate the reaction (Vazquez et al. 2010). Activated cells overexpress glucose transporters and glycolytic enzymes, frequently led by the transcription factor HIF-1α, which results in higher glycolytic activity. On the other hand, the cells cannot increase the concentration of mitochondria to match the increased glucose uptake, therefore, pyruvate is converted to lactate, via LDH enzymes, which are also upregulated in the aerobic glycolysis.

The TCA cycle is essential to maintain a series of other metabolic pathways. It uses acetyl CoA to harvest high-energy electrons and it is a source of precursors for nucleotides, amino acids, cholesterol, and porphyrin. Briefly, oxaloacetate condenses with acetyl CoA (originated from pyruvate) to form citrate. Then, an isomer of citrate is decarboxylated to

form α-ketoglutarate, which will be decarboxylated to form succinate. Later, succinate is used to regenerate oxaloacetate. In the overall reaction, two molecules of carbon dioxide, three of NADH and one $FADH_2$ are generated. As TCA cycle generates NADH and $FADH_2$, it is directly linked to respiration, *i.e.*, oxidative phosphorylation (OXPHOS). The OXPHOS is the pathway that generates the highest concentration of ATP in aerobic cells. Briefly, NADH and $FADH_2$ are oxidized to release electrons, which will flow through the electron-transport chain and reduce oxygen to water. The flux of electrons generates a proton gradient across the membrane, which then flow through ATP synthase to generate ATP (Berg and Tymoczko, John L and Stryer 2002). As mentioned before, pro-inflammatory macrophages rely on glycolysis rather than respiration to generate energy. In this setting, the TCA cycle is broken in two points allowing succinate and citrate to escape the mitochondria and exert modulatory functions (towards inflammation), such as blockage of prolyl hydroxylase (PHD), enzyme that degrades HIF-1α (Jha et al. 2015; Tannahill et al. 2013). On the other hand, anti-inflammatory/pro-resolving macrophages rely on respiration. They display intact TCA cycle and OXPHOS, which are responsible for ATP production necessary for the glycosylation of lectin and mannose receptors expressed by these macrophages (Viola et al. 2019).

The pentose phosphate pathway (PPP) is the source of nicotinamide adenine dinucleotide phosphate (NADPH) for different pathways, such as fatty acid biosynthesis and detoxification by reduction of oxidized glutathione. The PPP is divided in two steps: The generation of NADPH (oxidative) and the interconversion of sugars (nonoxidative). In the first step, glucose 6-phophate is oxidized into ribulose 5-phosphate, generating NADPH. The organism needs higher concentrations of NADPH than ribulose 5-phophate, thus the excess of ribulose 5-phosphate is used in the second step. To optimize the usage of carbons, ribulose 5-phosphate is converted into different 5-carbon sugar (as ribose 5-phosphate), which then are converted into intermediates of the glycolytic pathway (Berg and Tymoczko, John L and Stryer 2002). The PPP is crucial for macrophage polarization and function. The oxidative step

is fundamental for pro-inflammatory macrophages, and it is generally upregulated in these cells. NADPH generated in PPP is used for antioxidant defense mechanisms, macrophage's killing activity (via ROS production by NADPH oxidase) and fatty acid biosynthesis (Kelly and O'Neill 2015; Galván-Peña and O'Neill 2014). On the other hand, anti-inflammatory/pro-resolving macrophages depends on the nonoxidative production of ribose 5-phosphate, which is required for N-glycosylation of surface proteins expressed by these cells (Jha et al. 2015).

Fatty acids have essential physiological roles which are: building blocks for biological membranes, attach to proteins to localize them in the membrane, be a source/stock of energy and serve as intracellular messenger and hormones. The fatty acid synthesis takes place in the cytosol and starts with an activated acyl group (*e.g.*, an acetyl unit) and a malonyl unit. The acetyl is condensed with malonyl to form a four-carbon unit, which is submitted to reduction, dehydration, and another reduction to form butyryl CoA. Then, to extend the fatty acid chain, another malonyl unit is added to the butyryl unit, and this process is repeated several times to form a C16 fatty acid. The fatty acid oxidation (FAO) takes place in the mitochondria and proceeds the activation of the fatty acid with acetyl CoA units. The activated fatty acid is transported by the mitochondria to be degraded by a series of oxidation, hydration, and oxidation, followed by the cleavage by coenzyme A to yield a 2 carbon shorter fatty acid, acetyl CoA $FADH_2$ and NADH (Berg and Tymoczko, John L and Stryer 2002). Macrophages with a pro-inflammatory profile have an increased synthesis of fatty acid, that is essential for the phagocytic activity, NLRP3 inflammasome activation, and production of pro-inflammatory cytokines and ROS (Diskin and Pålsson-McDermott 2018). Consistent, FAO is enhanced in anti-inflammatory/pro-resolving macrophages, however it is not essential for macrophage polarization and function. Degradation of fatty acid fuels mitochondrial OXPHOS increasing ROS production, which activates NLRP3 inflammasome (Diskin and Pålsson-McDermott 2018). Activation of NLRP3 inflammasome and ROS production show

that opposite metabolic processes can have the same outcome, even in different profiles of macrophages.

Although some overlaps are observed in the metabolism, gene expression and surface markers of pro-inflammatory (M1) and anti-inflammatory/pro-resolutive (M2) macrophages, it is possible to distinguish them using a combination of markers/functional analysis described in Table 2.

Table 2: Summary of polarized macrophages' profile. Adapted from Viola et al. 2019.

Polarization	**M1**	**M2**
Transcription factors	NNF-κB (p65), STAT1, STAT3, IRF-4, HIF-1α, AP1	STAT6, NF-κB (p50), IRF4
Metabolic enzymes	iNOS, PFKFB3, PKM2, ACOD1	ARG1
Metabolic profile	Glycolysis, pentose phosphate	Oxidative phosphorylation, fatty acid oxidation
Surface markers	CD80, CD86, MHC-II	CD206, CD36, CD163
Produced cytokines	TNF-α, IL-1β, IL-6, IL-12	IL-10, TGF-β
Function	Pro-inflammatory, pathogen elimination	Anti-inflammatory and pro-resolving

Objective

Rationale

It is well established that SCFAs have multiple beneficial effects in the gut, such as increased AMPs production and strength of intestinal barrier (Raqib et al. 2006b; Fachi et al. 2019). However, less is known about the effect of SCFAs on pulmonary cells. Additionally, SCFAs, specially butyrate, were shown to boost the ability of macrophages to kill bacteria, by facilitating the fusion of lysosome and phagosome, and enhancing the production of AMPs, ROS and lysozyme (Schulthess et al. 2019; J. Zhang et al. 2020; Wu et al. 2020).

Based on these emerging studies, acetate seems to be an interesting immune regulatory candidate to focus on. Acetate can modulate the inflammatory response, boost host defense, and reduce viral and bacterial loads in the lungs. The observed effects in the lungs are described to be mediated by pulmonary epithelial cells and alveolar macrophages. It was shown that acetate can improve the secretion of IFN-β by alveolar epithelial cells and macrophages, improving RSV viral clearance in the lungs (Antunes et al. 2019; Ji et al. 2021). In the context of bacterial infection, acetate was shown to improve phagocytosis and clearance of *Klebsiella pneumoniae* by alveolar macrophages (Galvão et al. 2018). Our group also showed that in the context of prior influenza infection, acetate reduces *S. pneumoniae* loads in the lungs via alveolar macrophages. Thus, acetate might exert protective effects on pneumococcal pneumonia. Although acetate seems to impact alveolar macrophage's response, the mechanism by which this SCFA acts remains elusive.

Despite the existence of treatments and vaccines for pneumococcal pneumonia, this disease still causes high mortality worldwide. This can be attributed to the high number of serotypes associated to lower efficacy of vaccines, emerging resistance against antibiotics, and exacerbated inflammation triggered upon infection. Therefore, it is of extreme importance to study novel interventions for this disease and elucidate the mechanisms by which *S. pneumoniae* clearance might be enhanced.

Objectives

The main objective of this study was to decipher the role and the mode of action of acetate during *S. pneumoniae* infection, with focus on alveolar macrophages. To reach this aim, we investigated:

1- The global effect of acetate supplementation in drinking water on protein production by pulmonary cells and acetate's effect on bacterial loads of *S. pneumoniae* infected mice

2- The global impact of acetate pre-treatment in alveolar macrophages stimulated with *S. pneumoniae*

3- The impact of acetate in the function, metabolism, and immune response of macrophages

4- The mechanism by which acetate modulates macrophage's profile

Materials and methods

Mice and ethics statement

Specific pathogen-free C57BL/6J mice (7-week-old, male) were purchased from Janvier (Le Genest-St-Isle, France) and *Ffar2*$^{-/-}$ mice (>10 backcrosses) were produced as previously described(Maslowski et al. 2009). Mice were maintained in a biosafety level 2 facility in the Animal Resource Centre at the Institut Pasteur de Lille for at least two weeks prior to usage to allow appropriate acclimatation. Mice were fed a standard rodent chow (SAFE A04, SAFE, Augy, France) and had access to water *ad libitium*. All experiments complied with current national and institutional regulations and ethical guidelines (Institut Pasteur de Lille/B59-350009). The protocols were approved by the institutional ethical committee 'Comité d'Ethique en Experimentation Animale' (CEEA) 75. Nord Pas-de-Calais. All experiments were approved by the "Education, Research and Innovation Ministry", France under registration number APAFIS22304-201910011647335v3.

Reagents

Sodium acetate, lipopolysaccharides (LPS) from Escherichia coli O111:B4, nigericin sodium salt, potassium chloride (KCl), 4-[[4-Oxo-2-thioxo-3-[[3-(trifluoromethyl)phenyl]methyl]-5-thiazolidinylidene]methyl]benzoic acid (CY-09), N-[[(1,2,3,5,6,7-Hexahydro-s-indacen-4-yl)amino]carbonyl]-4-(1-hydroxy-1-methylethyl)-2-furansulfonamide sodium salt (MCC-950), 2-deoxy-D-glucose (2DG), and NG-Methyl-L-arginine acetate salt (L-NMMA) were purchased from Sigma (Saint Louis, MO). IL-1β recombinant protein was purchased from Invitrogen (Waltham, MA). Anti-IL-1β neutralizing monoclonal antibody was purchased from Thermo Fisher Scientifics (Waltham, MA). Rabbit monoclonal antibody anti-ACSS2 (D19C6) was purchased from Cell Signaling (Danvers, MA), mouse monoclonal antibody anti-caspase-1 (Casper-1) was purchased from Adipogen (San Diego, CA). Rabbit polyclonal antibody anti-IL-1β was a kind gift from Proteintech (Rosemont, IL). Mouse IgG kappa binding protein conjugated to horseradish peroxidase

(HRP) and mouse anti-rabbit IgG-HRP were purchased from Santa Cruz Biotechnology (Dallas, TX).

S. pneumoniae culture and preparation

S. pneumoniae was plated into tryptic soy blood agar until reach logarithmic phase grow (18 h) at 37 °C and 5% CO_2. Then colonies were transferred to liquid Todd Hewitt 0,5% Yeast extract (THY) and incubated at 37 °C and 5% CO_2 until reach logarithmic phase grow and optical density of 0.6 at 600 nm. Bacteria were washed 2 times and resuspended in THY 25% sterile glycerol, aliquoted and stored at -80 °C. Later, three random aliquots were plated to determine the concentration of the stock. For mice infection, bacteria were washed two times in sterile PBS and then diluted to the final concentration of $2x10^7$ CFU/mL. For *in vitro* experiments, bacteria were washed, resuspended in RPMI medium without antibiotics and heat killed at 65 °C for 15 min. For killing assay, bacteria were washed, opsonised with a mixture of 50% RPMI without antibiotics and 50% fetal bovine serum (FBS) with complement at 37 °C for 30 min. Then, bacteria were washed and resuspended in RPMI without antibiotics.

Infection, treatment, and assessment of bacterial loads

Mice received acetate 200mM in drinking water for 5 days prior to infection. Then, mice were anesthetized subcutaneously with 80 mg/Kg ketamine and 15 mg/Kg and intranasally infected with 50 µl of sterile PBS containing $1x10^6$ colony forming units (c.f.u.) of *S. pneumoniae* serotype 1 (clinical isolate E1586). Mice were euthanized with a lethal dose of pentobarbital sodium (460mg/Kg) after 30 h of infection for collection of organs. Lungs and spleen were homogenized in 1 mL of sterile PBS. Tissue homogenates were submitted to 10-fold serial dilutions in PBS and 20 µl of each dilution were added in triplicate onto blood agar plates. Then, plates were incubated overnight at 37 °C 5% CO_2 for further CFU quantification.

Proteomics

C57BL/6J mice were supplemented or not with acetate 200mM in drinking water for 5 days and were euthanized with a lethal dose of pentobarbital sodium (460mg/Kg). Mouse trachea was exposed and an 18G cannula was inserted and broncho-alveolar cavity was flushed 3x with 1mL of ice-cold PBS and this procedure was repeated once more. Then, BAL was centrifuged at 1800rpm for 8min at 4°C for removal of cells and debris. The supernatant was collected and submitted to protein concentration protocol with TCA and acetone. Then, samples were resuspended in Laemmli buffer 1x in order to have the same concentration of proteins in all samples. Later samples were sent to the proteomics platform from Institut Pasteur de Lille for protein identification and relative quantification by mass spectrometry.

Alveolar macrophages expansion

C57BL/6J and *Ffar2*$^{-/-}$ mice were euthanized with a lethal dose of pentobarbital sodium (460 mg/Kg) and the trachea was exposed to insert an 18 G cannula. Bronchoalveolar lavage (BAL) was performed with 1 mL of warm PBS 0.5% (FBS) and 2 mM EDTA, this procedure was repeated 9 times, and the total 10mL of lavage were added to 3mL of RPMI Glutamax 10% FBS. Recovered BAL was centrifuged at 350 g for 7 min, supernatant was discarded, the pellet was resuspended in RPMI Glutamax 10% FBS and cells were counted. One million cells were plated in a non-treated 100 mm petri-dish with 10mL of RPMI Glutamax, 10% FBS, 1% Pen/Strep, 1% pyruvate, and 5 ng/mL GM-CSF and incubated overnight at 37 °C 5% CO_2. Then, culture medium was replaced by fresh medium and cells were incubated for 2 days. Later, the media was removed and centrifuged at 350 g for 7 min to keep non-adherent cells. The pellet was resuspended in fresh media and added back to attached cells, which were incubated for additional 3 days (Busch et al. 2019). After 6 days in culture, cells were removed from the plate, centrifuged, counted, and added to 96 well plates at $1.5x10^6$ cells/mL, where they were stimulated with *S. pneumoniae* in the presence or not of acetate.

Cell culture and *in vitro* experiments

Max Plank Institute (MPI) cells are self-renewing and non-transformed cells originated from fetal liver of C57BL/6J mouse. MPI cells were used as a model of alveolar macrophages, due to their closer profile (Fejer et al. 2013). Cells were cultivated in RPMI Glutamax with 10% FBS, 1% Penicillin/streptomycin (Gibco) and 30 ng/mL GMCSF (Peprotech) at 37°C 5% CO_2. MPI cells were used from passage 6 until passage 30, as described by Fejer, until passage 90 no changes were observed in cells response and phenotype (Fejer et al. 2013). Cells were tested for mycoplasma contamination with MycoAlert^{TM} Mycoplasma Detection Kit (Lonza - Basel, Switzerland). *In vitro* experiments were done with the seeding density of $1.5x10^6$ cells/mL. All treatments were diluted in culture medium at indicated concentrations and added to the cells one hour before stimulation with *S. pneumoniae*. Sodium acetate 30 mM, KCl 90 mM, CY-09, MCC950, 2-DG, L-NMMA 500 µM, IL-1β recombinant protein 800 pg/mL and anti-IL-1β monoclonal antibody at 1.25 µM. Except for the killing assay, in which live bacteria was used, all *in vitro* experiments were done with heat-killed *S. pneumoniae* MOI of 30.

Nitrite quantification

Supernatant from MPI cells was collected at indicated time points for nitrite quantification using the Griess Reagent Kit following the protocol provided by from Life Technologies (Carlsbad, CA). In brief, equal volumes of reagent A and B were mixed, then 14 µl was added to 86 µl of water and the 100 µl were loaded on each well. Later, with a multichannel pipette 100 µl of samples or standard were added to the plate. The absorbance was measured in 548 nm. Samples values were interpolated with the standard curve for the calculation of nitrite concentration.

ELISA

Cytokine production was measured from the supernatant of cells, accordingly to protocol's manufactures for IL-1β, IL-6, IL-12p40 (Invitrogen - Waltham, MA) and TNF-α (R&D Systems - Minneapolis, MN).

Killing assay

MPI cells were previously incubated with heat-killed *S. pneumoniae* MOI 30 in the presence or not of acetate for 24 h, a time point in which only acetate treated cells produce significant concentrations of nitrite. In certain experiments 2-DG 10 mM and CY-09 30 µM were also added to the cells at the same moment as acetate. Later, killing assay was performed as described (Machado et al. 2020; Sencio et al. 2020). Briefly live *S. pneumoniae* MOI 10 was added to the cells in the presence or not of acetate, L-NMMA 500 µM (NO inhibitor), 2-DG 10 mM and CY-09 30 µM, incubated for 1 h at 4 °C, for bacterial attachment and then at 2 h 30 min at 37 °C for internalization. At this point cells were incubated with Penicillin/Streptomycin 40 U/mL for 30 min to eliminate extracellular bacteria. Then, cells were incubated for 2 h to allow bacterial killing. To quantify intracellular viable bacteria left (CFU), cells were lysed, diluted, and plated in blood agar plate. Due to the variation of infection, we set the mean of CFU from vehicle group at 100% of bacteria left for each experiment. Then, we calculated the % of bacterial left for other groups over the vehicle group.

RNA extraction, cDNA synthesis and RT-PCR

Total RNA from cellular lysate was extracted using the NucleoSpin® RNA kit (Macherey-Nagel, Hoerdt, Germany). RNA was used to generate cDNA with High-Capacity cDNA Archive Kit (Life Technologies, USA). Later, the cDNA was mixed with SYBRGreen (Thermo Fisher Scientific, Waltham, MA) based real-time PCR and amplified for detection on QuantStudio 12K Flex Real-Time PCR Systems (Applied Biosystems, USA) according to

manufacturer's protocol. Specific primers were generated using Primer Blast and ordered at Eurofins Scientifics. The expression of all genes was normalized with the housekeeping gene TATA-Box Binding Protein (*Tbp)* (ΔCT) and the fold increase was calculated over the control group ($2^{-\Delta\Delta CT}$).

Table 3: Primer sequences used for PCR

Tbp F - GGCGGTTTGGCTAGGTTTCT *Tbp* R - TGCCGTAAGGCATCATTGGA	*Pfkfb3* F - CCAGAGCCGGGTACAGAAGA *Pfkfb3* R - GAGGCCACAACAGTAGGGTC
Slc2a4 F - CAGATCGGCTCTGACGATGG *Slc2a4* R - GCCACGTTGCATTGTAGCTC	*Pgk1* F - CGAGCCTCACTGTCCAAACT *Pgk1* R - TCTGTGGCAGATTCACACCC
Serpine1 F - GTCGTGGAACTGCCCTACC *Serpine1* R - GCGTCTCTTCCCACTGTCAA	*Aldoa* F - CGCTCCTTAGTCCTTTCGCC *Aldoa* R - AATGCAGGGATTCACACGGT
Pdk1 F - CCACTGAGGAAGATCGACAGAC *Pdk1* R - AGAGGCGTGATATGGGCAATCC	*Vegfa* F - GCAGCTTGAGTTAAACGAACG *Vegfa* R - GGTTCCCGAAACCCTGAG
Ucp3 F - ACCCGATACATGAACGCTCC *Ucp3* R - TCATCACGTTCCAAGCTCCC	*Inos* F - CAGCTGGGCTGTACAAACCTT *Inos* R - CATTGGAAGTGAAGCGTTTCG
Il-1b F - TCGTGCTGTCGGACCCATA *Il-1b* R - GTCGTTGCTTGGTTCTCCTTGT	*Casp1* F - ACAAGGCACGGGACCTATG *Casp1* R - TCCCAGTCAGTCCTGGAAATG
Nlrp3 F - ATTACCCGCCCGAGAAAGG *Nlrp3* R - TCGCAGCAAAGATCCACACAG	*Asc* F - CTTGTCAGGGGATGAACTCAAAA *Asc* R - GCCATACGACTCCAGATAGTAGC
Mct1 F - TGTGTGGAAAACCTACCGGG *Mct1* R - TGCCAACCACTCCCTACCTA	*Mct4* F - GGCGGTAACAGGTGAAAGCA *Mct4* R - ATAGGGCGACGCTTGTTGAA
Acss1 F - GTTTGGGACACTCCTTACCATAC *Acss1* R - AGGCAGTTGACAGACACATTC	*Acss2* F - TGCCACCATAAGTCAACCCC *Acss2* R - ACAGGGCATTCAGAAGGGTG
Ffar2 F - TTAATCTGACCCTGGCGGAC *Ffar2* R - AGC GCGCACACGATCTTT	*Hif1a* F - ACCTTCATCGGAAACTCCAAA *Hif1a* R - ACTGTTAGGCTCAGGTGAACT

RNA-Seq and enrichment analysis

To assess gene expression profile by RNA-sequencing, extracted RNA was send to Nice-Sophia-Antipolis Functional Genomics Platform. Next generation sequencing was performed on Illumina NextSeq500. The obtained libraries of sequences (reads) were aligned with STAR on the mm10 genome version during the primary analysis. Secondary analysis was done with STAR aligner RNA-seq pipeline. In total, 14501 genes were included in the analysis, with at least 20 reads each. The data was normalized over total gene expression and

the log2 fold change was calculated over *S. pneumoniae* stimulated cells without acetate treatment.

Enrichment analysis was done in the Metascape platform, for GO biological process. It was included all genes with adjusted p-value < 0.05, and fold change > 1.5. The pathway enrichment had a p-value cutoff of 0.01 and minimum enrichment of 1.5. The raw data was analyzed and pathways that were redundant or related to other cellular type were excluded. Then, two graphics were done, one containing all enriched process by acetate, and another with 10 pathways associated to immunological and metabolic processes.

Western Blot

Protein extracts were obtained from the lysis of $1x10^6$ cells using RIPA buffer. Then protein extraction was centrifuged at 10000 g for 10 min and supernatant was collected for quantification of protein with BCA assay. For each sample 30 µg of protein was added to Laemmeli buffer (EcoTech Biotechnology - Istanbul, Turkey) to a final concentration of 1x and boiled at 95 °C. For caspase 1 assessment, supernatant was concentrated as described (Fernandes-Alnemri et al. 2009). Briefly, 900 µl of supernatant was collected and precipitated with 900 µl of methanol and 225 µl of chloroform. Then it was centrifuged at 20000 g for 10 min, the first phase was discarded, 500 µl of methanol was added and centrifuged again. Later, the supernatant was removed, the pellet dried, resuspended in Laemmli buffer 1x and boiled at 95 °C for 5 min. Protein samples were loaded into SDS page stain-free 4-12% acrylamide gel (BIO-Rad). After electrophoresis the gel was activated by UV to allow quantification of total protein. Then, proteins were transferred to a PVDF membrane, which was later blocked with TBS-T 5% of powder milk. Primary antibodies were diluted 1:1000 in TBS-T 5% BSA incubated ON at 4 ºC and secondary antibodies were diluted 1:3000 in TBS-T 5% BSA incubated for 1 h at RT in a rocking platform. Protein expression was normalized with total protein, and the fold change was calculated over the control.

Knockdown with siRNA

ON-TARGETplus siRNA (SMARTpool) for HIF-1α 20 µM (Dharmacon) or scramble siRNA 20 µM (Eurofins) were mixed with PBS and lipofectamine RNAiMAX (Thermo) for 30 min in the center of the well from a 6 well plate. MPI cells were counted and added to each well at the concentration of $2.5x10^5$ cells/mL. Cells were immediately incubated at 37 ºC and 5% CO_2 for 24 h. Later, medium was changed, and cells were incubated again during 3h. After incubation, medium was removed and cells were pre-treated with acetate 30 mM for 1h, followed by *S. pneumoniae* stimulation during 18h. Supernatant was collected for ELISA and cells were collected for RT-PCR.

Knockout with CRISPR Cas9

Knockout cells generation was performed following the protocol from Ann Ran and colleagues (Ran et al. 2013). Briefly, ACSS1 and ACSS2 guide RNAs were designed using the data base Gecko2 Mouse library. Six target sequences were designed for each gene, to further allow the selection of the best knockout. Annealed oligonucleotide pairs containing a guide RNA-coding sequence were inserted in the BsmBI sites of Lenti CRISPR v2 (Addgene #52961) or Lenti CRISPR v2-blast (Addgene #83480) plasmids containing a resistance gene for puromycin or blasticidin, respectively. Lentiviral particles were produced by transient cotransfection of 293TT cells with a guide RNA-carrying lentiCRISPRv2 or lentiCRISPRv2-blast plasmid, a packaging vector (psPAX2, Addgene #12260), and a vector expressing the vesicular stomatitis virus glycoprotein (VSV-G), using Turbofect as a transfection reagent according to the manufacturer's protocol (Thermo Fischer). Control lentiviruses were generated using lentiCRISPRv2 or lentiCRISPRv2-blast plasmid with no guide RNA coding sequence inserted. Transfected cells were incubated for 3 days at 33° C. Cell culture supernatants containing the lentiviral particles were collected, passed through 0.45-µm filters and stored at -80° C. Later, lentiviruses were added to MPI cells at 30% of confluence for 24

h. After two weeks of antibiotics selection (puromycin 7 µg/mL or blasticidin 1 µg/mL), cells were expanded to have the KO gene assessed by RT-PCR and Western Blot. As ACSS1 is low expressed, we could not detect it by western blot. KO cells with a reduction higher than 90% of mRNA expression were kept in culture for further experiments.

Table 4: Guide RNA sequences

Acss1.1 5'-CACCGAAGAGACATGGAGTGCACCG-3' 5'-AAACCGGTGCACTCCATGTCTCTTC-3'	Acss2.1 5'-CACCGAAACATCTGCTACAACGTGC-3' 5'-AAACGCACGTTGTAGCAGATGTTTC-3'
Acss1.2 5'-CACCGGCTCCTACCTTGTGCGTCA-3' 5'-AAACTGACGCACAAGGTAGGAGCC-3'	Acss2.2 5'-CACCGTACTGGAAAACCGCATGCCC-3' 5'-AAACGGGCATGCGGTTTTCCAGTAC-3'
Acss1.3 5'-CACCGGCTCACAGGACGGACACCA-3' 5'-AAACTGGTGTCCGTCCTGTGAGCC-3'	Acss2.3 5'-CACCGACCACAAGTTCCAAGATCAT-3' 5'-AAACATGATCTTGGAACTTGTGGTC-3'
Acss1.4 5'-CACCGTCTGGAGACCACATGCCGCC-3' 5'-AAACGGCGGCATGTGGTCTCCAGAC-3'	Acss2.4 5'-CACCGGTCACCTGTAGTGATGAGC-3' 5'-AAACGCTCATCACTACAGGTGACC-3'
Acss1.5 5'-CACCGTATGCCGCCATGACGCACA-3' 5'-AAACTGTGCGTCATGGCGGCATAC-3'	Acss2.5 5'-CACCGATCACATACCGTGAACTCC-3' 5'-AAACGGAGTTCACGGTATGTGATC-3'
Acss1.6 5'-CACCGTCTGGATATCCCCCTTGAAC-3' 5'-AAACGTTCAAGGGGGATATCCAGAC-3'	Acss2.6 5'-CACCGCAGCAATGTTCTCCGTAAAC-3' 5'-AAACGTTTACGGAGAACATTGCTGC-3'

Metabolic analysis with Seahorse

MPI cells ($1x10^6$ cells/mL) were seeded in a seahorse 96 wells plate with complete RPMI for 3 h and then pre-treated with acetate 30 mM for 1 h followed by *S. pneumoniae* stimulation. After 24 h, cells were washed with Seahorse RPMI medium (Agilent - Santa Clara, CA) with 2 mM glutamine in the presence or not of 10 mM glucose and incubated for 1h at 37° C without CO_2. The cartridge was hydrated with sterile water overnight at 37° C without CO_2 and then loaded into the calibrant solution for 1 h at 37° C without CO_2. Then, different drugs were added to the ports and the cartridge was loaded into Agilent Seahorse XFe96 Analyzers for calibration. In this meantime, supernatant was removed from cells and 180 µl of Seahorse RPMI medium with 2 mM glutamine in the presence or not of 10 mM

glucose. After completion of calibration, the plate containing the cells was loaded in the equipment and oxygen consumption and acidification were measured for at least 1 h 30 min. Different drugs combination was used to assess each of the metabolic parameters. For mito stress analysis: Port A Oligomycin - Oligo 1 µM (Cayman - Ann Arbor, MI), Port B: Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone - FCCP 1.5 µM (Sigma), Port C: Rotenone and Antimycin – R/A 1 µM (Sigma).

Calculations for OCR:

- Non-mitochondrial oxygen consumption: The lower measurement after rotenone and antimycin injection
- Basal respiration: Subtraction of the lower measurement after rotenone and antimycin injection from the last measurement of basal respiration.
- ATP linked production: Subtraction of the lower measurement after rotenone and antimycin injection, and the lowest measurement after oligomycin injection from last measurement of basal respiration.
- Proton leak: Subtraction of the lower measurement after rotenone and antimycin injection from the lowest measurement after oligomycin injection.
- Maximal respiration: Subtraction of the lower measurement after rotenone and antimycin injection from the maximal measurement after FCCP injection.
- Spare capacity: Subtraction of the lower measurement after rotenone and antimycin injection, and the last measurement of basal respiration from the maximal measurement after FCCP injection.
- Coupling efficiency: Value obtained for ATP production divided by the value obtained for basal respiration multiplied by 100.

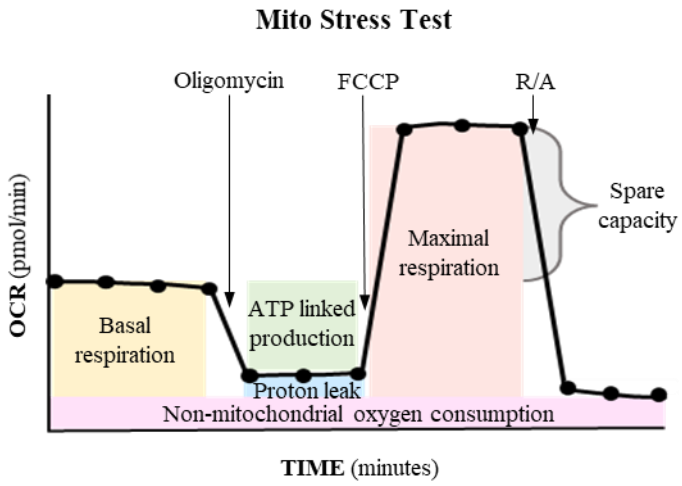


Figure 10: Representative image of Mito Stress Test.
Oxygen consumption rate (OCR) is measured under basal conditions for four cycles, then oligomycin is injected to block complex V (ATP synthesis dependent on mitochondrial respiration) and other three measures are done. Next FCCP, an uncoupling agent, is added to collapse mitochondrial inner membrane gradient, allowing the mitochondria to reach the maximal oxygen consumption. Lastly, rotenone and antimycin A (R/A) are injected to inhibit complex III and I, blocking the whole mitochondrial respiration.

For glycolysis analysis: Port A: Glucose - Gluc 10 mM (Agilent), Port B: Oligomycin 1 µM (Cayman), Port C: 2-Deoxy-D-Glucose (2-DG) 50 mM (Sigma).

Calculations for ECAR:

- Non-glycolytic acidification: The last measurement of basal acidification (before glucose injection).
- Glycolysis: Subtraction of the last measurement of basal acidification (before glucose injection) from the maximal measurement after glucose injection.
- Glycolytic capacity: Subtraction of the last measurement of basal acidification (before glucose injection) from the maximal measurement after oligomycin injection.
- Glycolytic reverse: Subtraction of the last measurement of basal acidification (before glucose injection), and the maximal measurement after glucose injection from the maximal measurement after oligomycin injection.

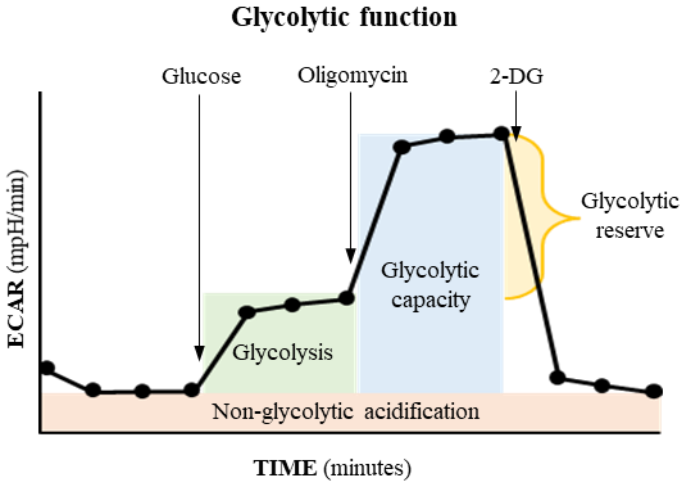


Figure 11: Representative image of Glycolytic function test.
First, basal extracellular acidification rate is assessed during four cycles. Then, glucose is injected to assess the extracellular acidification led by secretion of lactate originated from glycolysis. Next, oligomycin is injected to impair mitochondrial function and drive glycolysis to the maximum. Lastly 2-DG is injected to block glycolysis.

Statistical analysis

Results are expressed as the mean with standard error of the mean (SEM) unless otherwise stated. The statistical analyses were done using R software (RNA-Seq) or GraphPad Prism v8 software. For each analysis, at least three independent experiments were performed. Due to the variation led by the infection, we show here a representative data from the three independent experiments. All experiments were done in triplicates unless otherwise stated. Normality test was done for all results to determine the usage of parametric or non-parametric tests. Data containing two groups with normal distribution were analyzed with Unpaired Student's t-test, and the nonparametric ones were analyzed with Mann-Whitney U test. When more than two factors were present in the analysis Two-way ANOVA test followed by Sidak's multiple comparisons test was performed. Statistical details of experiments can be found in the figure legends.

For RNA-Seq, normalization and differential analysis were performed using the DESeq2 package in R. Log2 fold change was calculated over *S. pneumoniae* stimulated cells without acetate treatment. Volcano plot represents genes with log2 fold change > 0.6 and

adjusted p-value < 0.05 were considered as up-regulated and genes with log2 fold change < -0.6 and adjusted p-value < 0.05 were considered as down-regulated.

Enrichment analysis was done in the Metascape platform, for Gene Ontology (GO) biological process. It was included all genes with adjusted p-value < 0.05, and fold change > 1.5. The pathway enrichment had a p-value cutoff of 0.01 and minimum enrichment of 1.5. The raw data was analyzed and pathways that were redundant or related to other cellular type were excluded. Then, two graphics were done, one containing all enriched process by acetate, and another with 10 pathways associated to immunological and metabolic processes.

Results

Acetate supplementation modulates protein secretion in alveolar compartment and improves bacterial clearance

Most studies addressing acetate effect in pulmonary infection uses a protocol of acetate supplementation in drinking water, which is generally performed five days before infection (Antunes et al. 2019; Sencio et al. 2020; Galvão et al. 2018; Ji et al. 2021). In two studies, acetate was shown to directly impact the immune response of pulmonary cells to infection. Ji and colleagues demonstrated that acetate induced IFN-β production by AMs in mice infected with RSV (Ji et al. 2021). Interesting, Antunes and colleagues also showed that acetate increased IFN-β production in mice infected with RSV, however in this study, the cytokine production was attributed to pulmonary epithelial cells (Antunes et al. 2019). Although some studies already demonstrated the effect of acetate in the lungs, so far, no study has addressed whether acetate impacts pulmonary physiology before the infection. As mentioned before, the pulmonary cavity is composed by several different proteins that play an important role in physical barrier and in immune response. Aiming to address if acetate treatment could alter the basal secretion of proteins in the lungs, we performed mass spectrometry to identify all proteins present in the alveolar space of naïve mice treated or not with acetate. We observed that acetate supplementation in drinking water modulated the expression of 84 proteins (Figure 12A and Annex I). Among these different expressed proteins, we observed proteins implicated in signaling cascades, metabolic pathways, cytoskeleton composition and others. Despite the random modulation in protein expression, we observed the upregulation of proteins secreted by alveolar macrophages and epithelial cells involved in host defense. Mice treated with acetate presented higher concentrations of complement, haptoglobin, antimicrobial peptides and surfactant proteins (Figure 12B). To better understand the importance of these proteins, a summary of their functions was written below. Complement C8 gamma can be secreted by AECs and mononuclear cells, and it is described to enhance bacterial killing by playing a role in the MAC complex (Parker and Sodetz 2002; Kulkarni et

al. 2018). Haptoglobin can also be secreted by AECs and alveolar macrophages and it has an antimicrobial function, due to its capacity to sequester iron (Parrow, Fleming, and Minnick 2013; F. Yang et al. 1995; 2000). Similar to haptoglobin and complement C8, the antimicrobial peptide S100A7 can be produced by AECs and alveolar macrophages (Andresen et al. 2011). S100A7 is described to kill *E. coli* and enhance the expression of tight junction proteins in the skin (Gläser et al. 2004; Andresen et al. 2011; F et al. 2014). Lysozyme can also be produced by both AEC and alveolar macrophages, and its production is described to enhance bacterial clearance (Gibson and Phadke 1994; Akinbi et al. 2000; J. Zhang et al. 2020). Lysozyme has a broad range of action against microorganisms due to its ability to hydrolase the polysaccharide portion of bacterial cell wall (Kalfa and Brogden 1999). Pulmonary surfactant proteins can be secreted by AECs and they can modulate inflammatory response and enhance phagocytosis of microorganisms by macrophages (Takahashi et al. 2006).

Although we observed a reduction in the expression of 34 proteins, their functions were diverse and only three of them had a potential link to immune response (Figure 12C). These proteins can be secreted by both AECs and alveolar macrophages and they are: Leukotriene A-4 hydrolase, which converts leukotriene A-4 in the pro-inflammatory mediator leukotriene B4 (MB, M, and JZ 1997). Sulfhydryl oxidase 1 that catalyzes the formation of hydrogen peroxide (I et al. 2016). Plastin 2, by similarity, is described to play a role in the activation of T-cells (GH et al. 2007). Therefore, acetate seemed to modify the pulmonary environment, and this could contribute to a more effective response against *S. pneumoniae*.

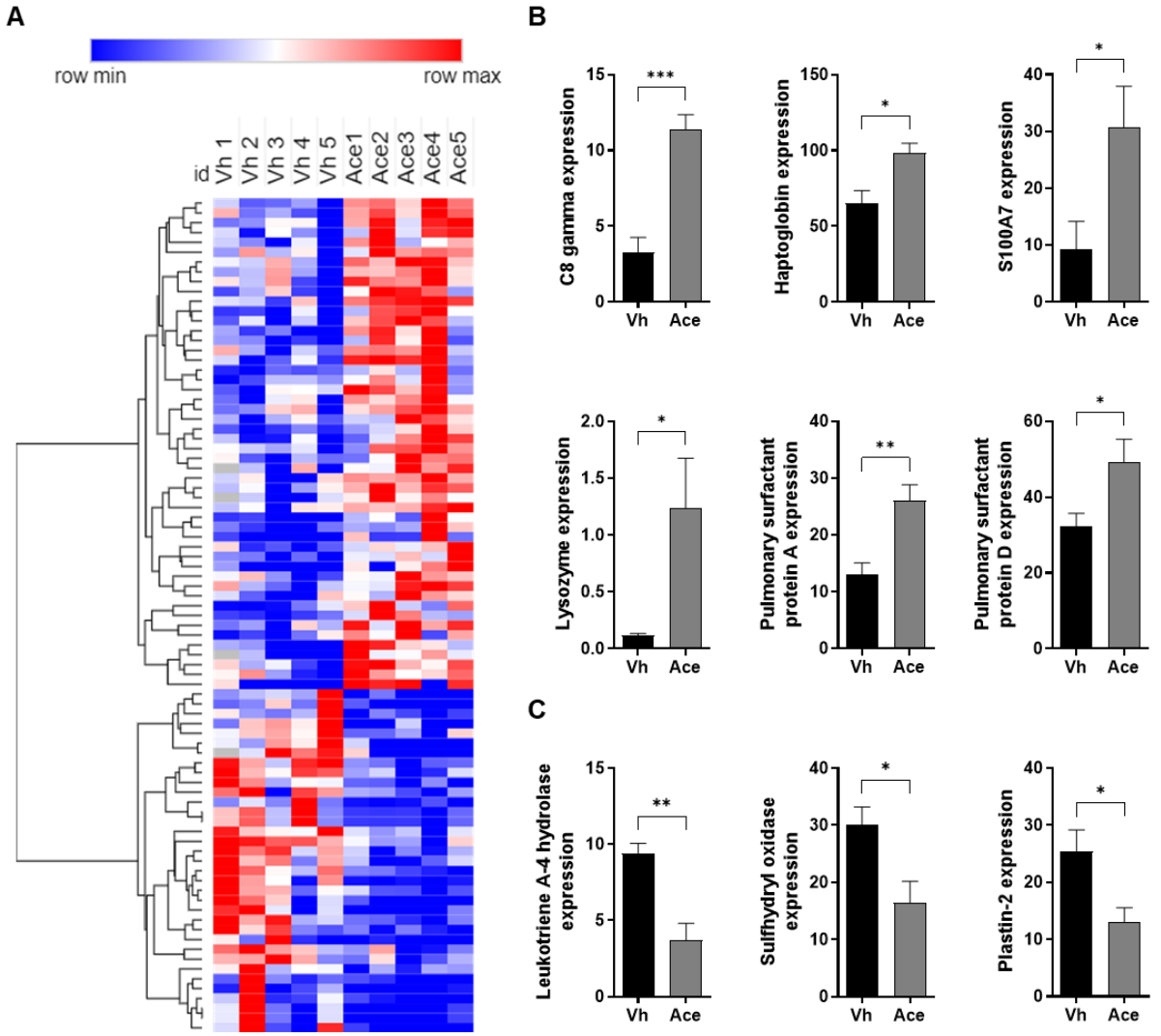


Figure 12: Acetate supplementation modulates secretion of proteins in the alveolar compartment.
(**A**) Heat map of all differentially expressed proteins (p-value < 0.05) in the BALF of naïve mice treated or not during 5 days with acetate 200 mM in drinking water. Small rectangles indicate protein expression of each sample, being blue low expressed and red high expressed. (**B**) Proteins involved in host defense and antibacterial response highly expressed in acetate treated mice. (**C**) Proteins potentially involved in immune response reduced in acetate treated mice. (**B** and **C**) Bars show the mean and errors show the SEM of n=5. Statistical analysis was done using Unpaired Student's t-test or Mann-Whitney U test (*p < 0.05, **p < 0.01, ***p < 0.001). Comparison was made among indicated groups.

Based in the protective effect of acetate described by the literature and in our promising results of proteomics, we addressed the role of acetate supplementation in host defense against *Streptococcus pneumoniae*. For this, bacterial loads were assessed in mice treated or not with acetate in drinking water and then infected with *S. pneumoniae*. We observed that prophylactic treatment with acetate significantly reduced bacterial loads in the lungs (Figure

13 *left panel*) and in the spleen, reflecting reduced bacterial dissemination (Figure 13 *right panel*).

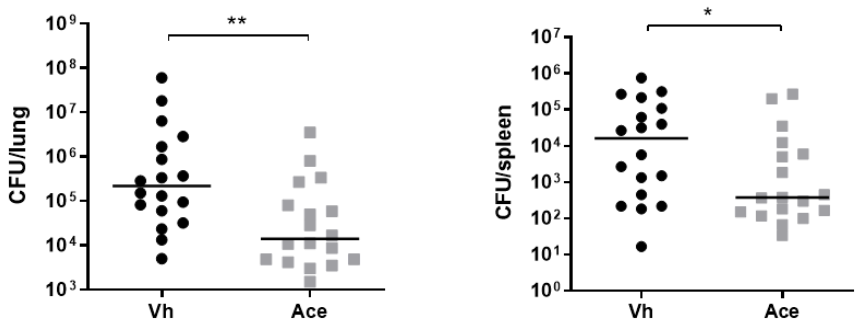


Figure 13: Acetate supplementation reduced bacterial loads in the lungs and spleen of *S. pneumoniae* infected mice.
Mice received 200 mM acetate treatment in drinking water for 5 days, followed by intranasal infection with *S. pneumoniae* ($1x10^6$CFU). Result shows the median from the pool of 3 independent experiments. Statistical analysis was done using Mann-Whitney U test (*p < 0.05, **p < 0.01).

Acetate modulates the response of alveolar macrophage like cells against *S. pneumoniae*

Alveolar macrophages are the first line of pulmonary immune defense and they are known to play a crucial role in the resistance to *S. pneumoniae* (Knapp et al. 2003). The killing capacity of those cells resulted in reduction of bacterial loads in the lungs and consequently decreased inflammatory response. In addition, our group has previously shown that alveolar macrophages were responsible to mediated acetate's protection from secondary pneumococcal infection (Sencio et al. 2020). Thus, we investigated the effect of acetate on alveolar macrophages profile and function.

Bone marrow derived macrophages (BMDM) are the most used macrophages to study immune response. Indeed, this is the best model to study infiltrating/recruited macrophages, however, for resident macrophages this model is not the best due to their different profile. To address the effect of acetate on alveolar macrophages, we used Max Planck Institute (MPI) cells that resembles this population. MPI cells are self-renewing, non-transformed macrophages originated from murine fetal liver, such as alveolar macrophages, and they share

a similar immune response (Fejer et al. 2013). To validate the usage of this cell line for the study of acetate, we evaluated the expression of genes involved in transport, recognition, and metabolism of acetate. We could detect mRNAs for all assessed genes, with variation in expression level, being *Ffar2* the highest expressed gene, followed by *Mct1* and *Acss2* (Figure 14A). *Mct4* presented a lower expression and *Acss1* was the lowest expressed gene. After validating that MPI can sense acetate, we investigated the role of acetate in macrophages' response against *S. pneumoniae*. For this, MPI cells were pre-treated or not with acetate, stimulated with *S. pneumoniae* and after 18 h total mRNA was collected and sent for RNA sequencing. Despite acetate's ability to induce upregulation of gene expression, we observed a similar number of up- and down-regulated genes (Figure 14B). To evaluate the impact of acetate in the profile of *S. pneumoniae*-conditioned macrophages an enrichment analysis was performed. Acetate favored a wide range of processes, from localization and metabolism to immune system (Figure 14C).

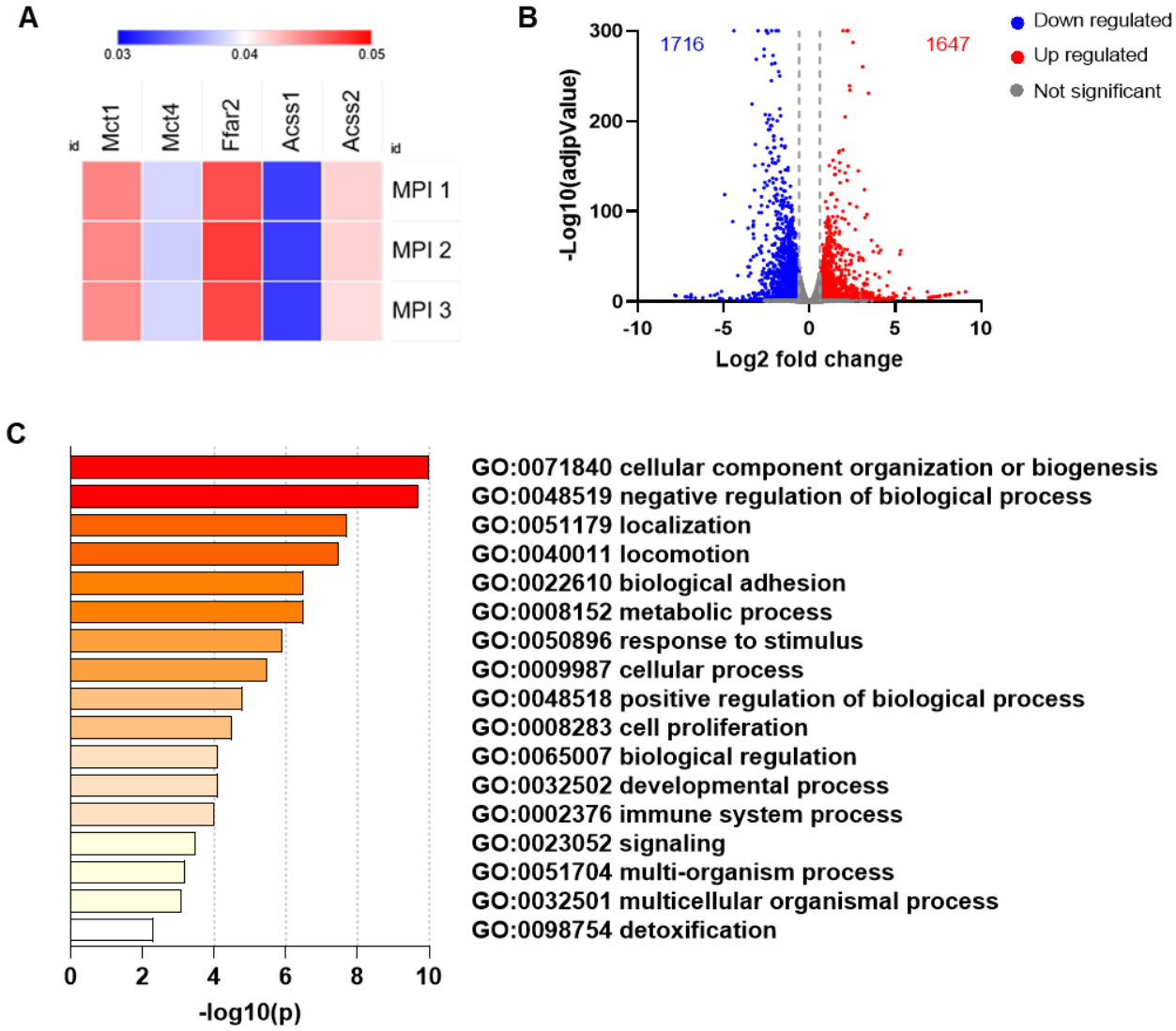


Figure 14: Acetate modulates the response of MPI cells against *S. pneumoniae.*
(**A**) Expression levels of monocarboxylate transporters (*Mct1* and *Mct4*), Free Fatty Acid Receptor 2 (*Ffr2*), Acetyl CoA synthetase (*Acss1* and *Acss2*) of MPI naïve cells. (**B** and **C**) Data obtained from RNA-Seq experiment of MPI cells pre-treated or not with acetate and stimulated with *S. pneumoniae* for 18h. (**B**) Volcano plot representing all genes expressed by MPI cells, up-regulated genes, in red, were set as $Log_2FC > 0.6$ and adjusted p-value < 0.05, down-regulated genes, in blue, had $Log_2FC < -0.6$ and adjusted p-value < 0.05 and genes without variation, in grey had $-0.6 > Log_2FC < 0.6$ and adjusted p-value > 0.05. (**C**) Biological processes enriched by acetate using genes that had $Log_2FC > 0.6$ and adjusted p-value < 0.05.

Acetate improves the killing ability of macrophages via NO

As acetate enriched a wide range of biological processes, we selected some pathways from the immune system and the metabolic process. In this analysis we observed that acetate impacted the metabolism of lipids and fatty acids, probably because it is a substrate for these

metabolic processes (Figure 15A). Acetate also enriched the processes of inflammatory response, defense response, and macrophage activation. Of note nitric oxide (NO) mediated signal transduction was also upregulated, and it might indicate an increase in this reactive species, which has a critical role in the killing of *S. pneumoniae* by macrophages (Marriott et al. 2007; 2008).

To address if this profile of macrophages was coherent with their function, MPI cells were submitted to the same treatment-protocol from RNA-Seq, and a killing assay was performed. MPI cells previously treated with acetate presented less viable intracellular bacteria than the vehicle group (Figure 15B). It is important to mention that despite the ability of SCFAs to directly induce bacterial death, our group showed that acetate alone had no effect on *S. pneumoniae* growth and viability (Sencio et al. 2020). Thus, acetate was enhancing the ability of macrophages to kill *S. pneumoniae*. To investigate whether NO had a role in acetate-mediated *S. pneumoniae* killing, we quantified nitrite production (an indirect measure of NO) from macrophages pre-treated or not with acetate and stimulated or not with *S. pneumoniae*. Acetate alone had no impact in nitrite concentration, on the other hand, stimulation by *S. pneumoniae* increased nitrite production (Figure 15C). Interestingly, acetate boosted the production of nitrite induced by *S. pneumoniae*. Thus, to assess the effect of NO in the killing promoted by acetate a nitric oxide synthase inhibitor (L-NMMA) was used. We observed that the blockage of NO production reduced the effectiveness of acetate (Figure 15D). Thus, acetate boosted the killing ability of macrophages by increasing NO production.

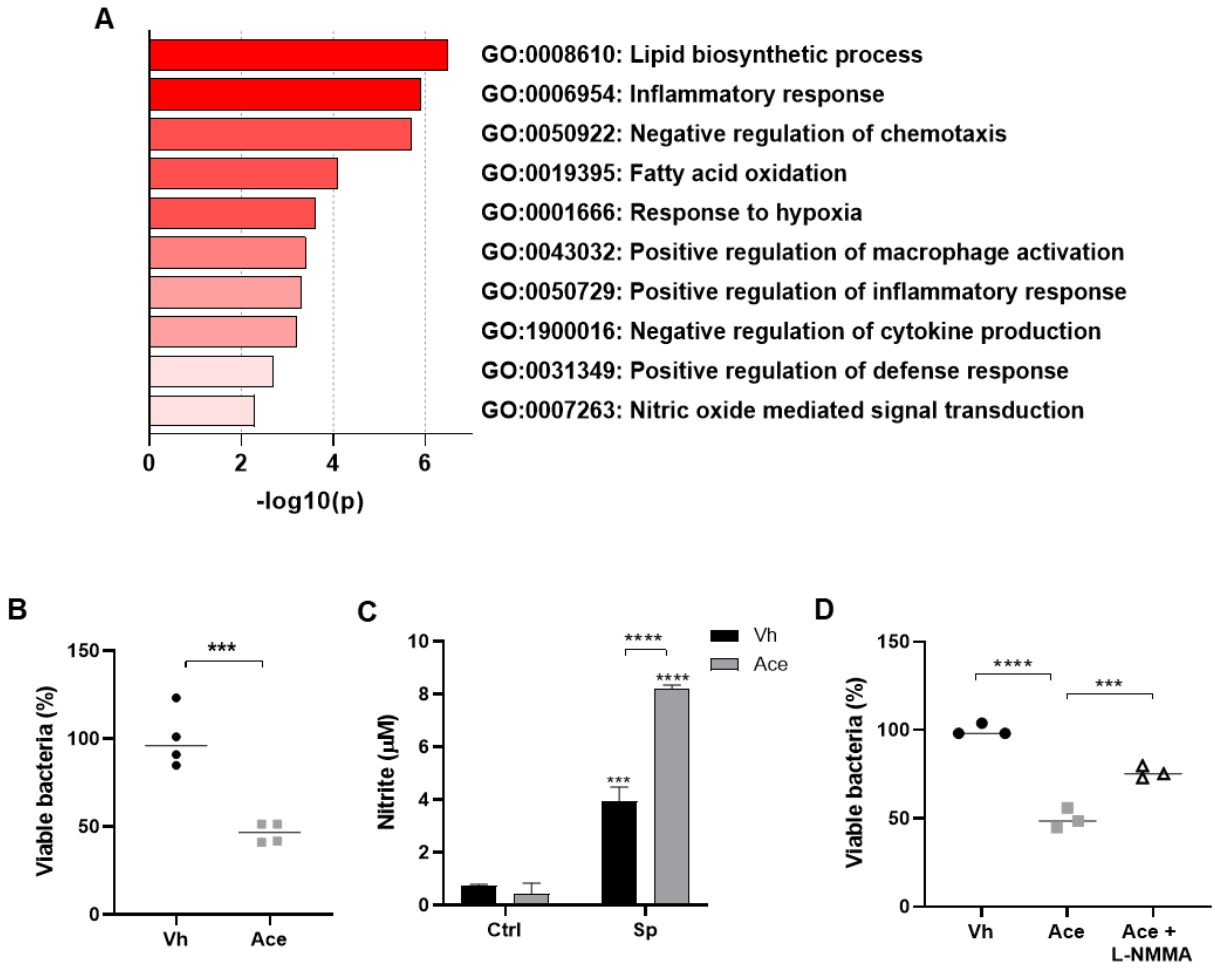


Figure 15: Acetate improves the killing ability of macrophages via NO.
(**A**) Immune and metabolic pathways enriched by acetate using genes that had Log2FC > 0.6 and adjusted p-value < 0.05 Data obtained from RNA-Seq experiment from lysate of MPI cells pre-treated or not with acetate and stimulated with *S. pneumoniae* for 18h. (**B** and **D**) Killing assay of macrophages previously activated with *S. pneumoniae* in the presence or absence of acetate for 24h. Then incubated with live *S. pneumoniae* (**D**) in the presence of NO inhibitor, L-NMMA. Later cells were lysed, and viable intracellular bacteria were assessed. (**C**) Nitrite levels assessed by Griess assay in the supernatant of MPI cells treated or not with acetate and stimulated or not with *S. pneumoniae* for 48h. (**B** and **D**) Data showing the median and (**C**) bars showing the mean and error showing the SEM of triplicates/quadruplicates. Results are representative of three independent experiments. Statistical analysis was done using (**B**) Unpaired Student's t-test, (**C**) Two-way ANOVA corrected with Sidak's multiple comparisons test and (**D**) One-Way ANOVA (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Comparison was made among respective control group or as indicated.

Acetate increases nitric oxide production via IL-1β

Due to the important role of NO in bacterial killing induced by acetate, we investigated how acetate was enhancing NO production. As NO can be induced by secreted proteins, especially cytokines (Rafa et al. 2013; Soufli et al. 2016), we addressed whether acetate had a

direct effect on NO or if the effect was mediated by a secreted factor. For this, filtered supernatant from cells stimulated during 24 h with *S. pneumoniae* with or without acetate were used. Naïve cells were incubated with the supernatants and stimulated with *S. pneumoniae* during 24 h for nitrite assessment. Interestingly, the conditioned medium derived from acetate treated cells induced higher production of nitrite when compared to conditioned medium from non-treated cells (Figure 16A). Therefore, a soluble factor produced and secreted upon acetate treatment was responsible for the augmentation in NO concentration.

As mentioned before some pro-inflammatory cytokines can improve nitric oxide production. The most and best described one is IFN-γ, nevertheless there are other cytokines that can stimulate or improve NO production according to the context (*i.e.*: cellular type and stimulus). IL-17A, IL-6, TNF-α and IL-1β were shown to increase nitric oxide levels in PBMC from healthy patients (Rafa et al. 2013), in addition, IL-1β and TNF-α were also described to induce iNOS activity in cardiac myocytes (Ungureanu-Longrois et al. 1995). However in hepatocytes, only IL-1β was able to induce NO production (Kitade et al. 1996). Therefore, we quantified pro-inflammatory cytokines secreted by bacteria-conditioned MPI cells pre-treated or not with acetate. We detected high production of all tested pro-inflammatory cytokines (IL-12p40, TNF-α, IL-6 and IL-1β) upon *S. pneumoniae* stimulation (Figure 16B). Interestingly, acetate alone increased only the production of IL-1β protein (Figure 16B *first panel*). In line, in the presence of *S. pneumoniae*, acetate markedly boosted IL-1β levels. On the other hand, acetate treatment of bacteria-stimulated macrophages decreased the production of TNF-α and IL-12p40, meanwhile no difference in IL-6 production was observed. To address if acetate effect on cytokine production was transposable for primary cells, alveolar macrophages obtained from C57BL6 mice were pre-treated with acetate and then stimulated with *S. pneumoniae* for 24 h. Although the response from alveolar macrophages was weaker than the one from MPI cells, *S. pneumoniae* induced the secretion of all cytokines (Figure 16C). In accordance with the data obtained from MPI

cells, in the presence of *S. pneumoniae*, acetate increased the secretion of IL-1β, when compared to vehicle treated group (Figure 16C *first panel*). On the other hand, acetate decreased the production of TNF-α, IL-12p40, and IL-6 triggered by *S. pneumoniae* stimulation (Figure 16C). Of note, alveolar macrophages displayed a similar response to the one observed in MPI cells, with a marked increase in IL-1β production upon acetate pre-treatment.

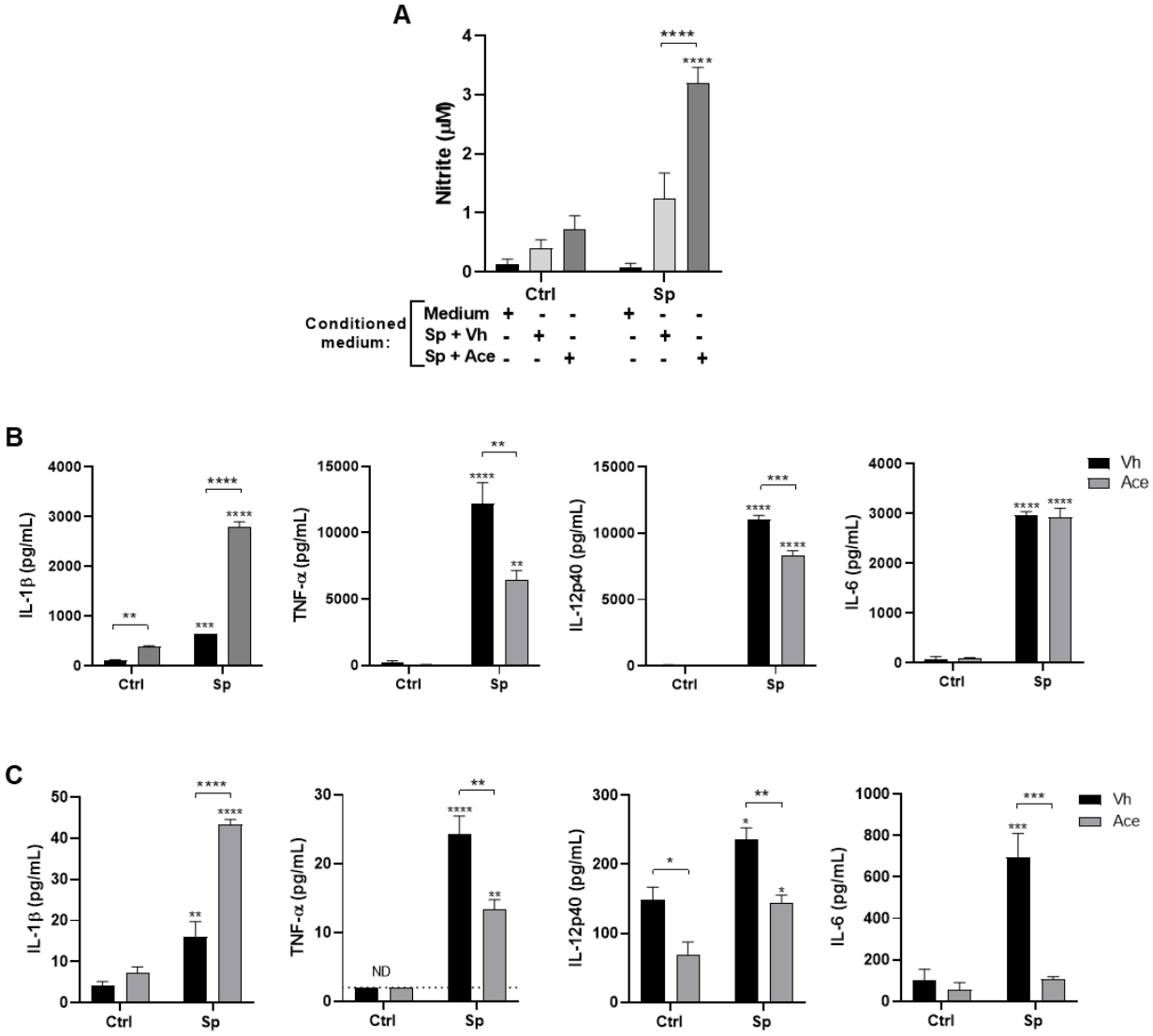


Figure 16: Acetate induces NO in an indirect manner and increases IL-1β production in MPI and alveolar macrophages.
(**A**) Nitrite production assessed by Griess assay from supernatant of MPI cells incubated for 1 h with conditioned medium (supernatant from cells pre-treated or not with acetate and stimulated with *S. pneumoniae* for 24 h), and then stimulated or not with *S. pneumoniae* for 24 h. IL-1β, TNF-α, IL-12p40, and IL-6 levels assessed by ELISA from supernatant of (**B**) MPI cells or (**C**) alveolar macrophages pre-treated or not with acetate and then stimulated or not with *S. pneumoniae* for 24 h. Bars show the mean and errors show the SEM of triplicates/quadruplicates. Results are representative

of three independent experiments. Statistical analysis was done using Two-way ANOVA corrected with Sidak's multiple comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Comparison was made among respective control group or as indicated.

To address if the sole cytokine increased by acetate could increase NO production, we evaluated the time course of *Il1b* gene expression and IL-1β protein production together with *Inos* expression and nitrite production. We observed that *S. pneumoniae* stimulation increased the expression of *Il1b* gene only at 12 h post stimulation, while protein levels were increased at 12 h post stimulation and remained at the same levels in the time points after 12 h (Figure 17A). In bacteria-conditioned macrophages, acetate induced an increase in *Il1b* gene expression at 12 h and 24 h post stimulation, and the peak of expression was at 12 h. Consistently, the increase in IL-1β protein levels induced by acetate started 12 h post stimulation and peaked at 36 h. In addition, acetate increased *Il1b* gene expression and protein secretion in all time points after 12 h of stimulation when compared to the vehicle group. *Inos* gene expression started to increase after 12 h of stimulation in both groups, and the expression levels was enhanced in acetate pre-treated macrophages when compared to vehicle group (Figure 17B *left panel*). In contrast, nitrite levels started to raise only 24 h post stimulation in the acetate group and 36 h post stimulation in the vehicle group (Figure 17B *right panel*). In keeping with *Inos* gene expression, acetate increased nitrite levels when compared to vehicle group from 24 h until 48 h. Of note, the effect of acetate on nitrite production had a delay when compared to its effect on IL-1β production.

As IL-1β can induce NO production in some cells and have no effect in others(Kitade et al. 1996), we first addressed IL-1β effect on MPI cells. We observed that IL-1β recombinant protein alone had no effect in nitrite production, however in the presence of *S. pneumoniae*, it enhanced the production of nitrite (Figure 17C *left panel*). To confirm that IL-1β was indeed the responsible for higher levels of nitrite driven by acetate, we blocked it. The neutralization of IL-1β abrogated the effect of acetate on nitrite production (Figure 17C *right panel*). Hence,

acetate increased IL-1β production, which acted in an autocrine manner to enhance NO production.

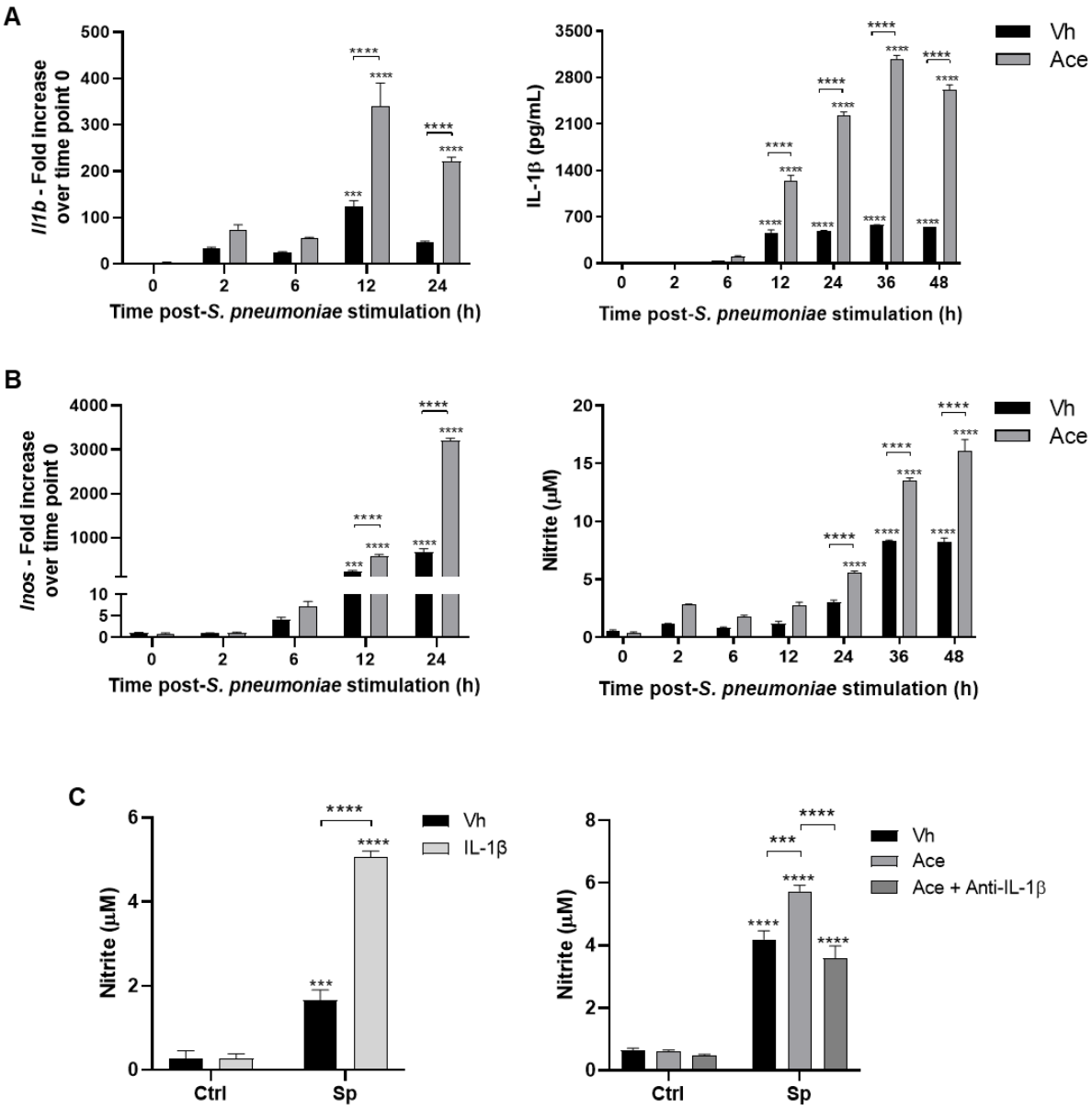


Figure 17: Acetate enhances NO production via IL-1β.
Kinetics of (**A *left panel***) *Il1b* and (**B *left panel***) *Inos* gene expression assessed by RT-PCR and (**A *right panel***) IL-1β levels assessed by ELISA and (**B *right panel***) nitrite production assessed by Griess assay from lysate or supernatant of MPI cells pre-treated or not with acetate and stimulated with *S. pneumoniae*. (**C *left panel***) Nitrite production assessed by Griess assay from supernatant of MPI cells pre-treated or not with recombinant IL-1β protein and stimulated or not with *S. pneumoniae* for 24 h. (**C *right panel***) Nitrite production assessed by Griess assay from supernatant of MPI cells pre-treated or not with acetate and/or anti-IL-1β neutralizing antibody stimulated or not with *S. pneumoniae* for 48 h. Bars show the mean and errors show the SEM of triplicates/quadruplicates. Results are representative of three independent experiments. Statistical analysis was done using Two-way ANOVA corrected with Sidak's multiple comparisons test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). Comparison was made among respective control group or as indicated.

Acetate-induced IL-1β is independent of FFAR2, ACSS1 and ACSS2

To better understand the mode of action of acetate to improve the defense of macrophages we decided to investigate the main mechanisms by which acetate could act. We showed that killing ability of macrophages was enhanced by NO production and this was not directly impacted by acetate, on the contrary it was mediated by IL-1β. Due to the important role of IL-1β in the immune defense of macrophages we sought to investigate the mechanism by which acetate was enhancing its production.

As mentioned before, acetate has three main mechanisms. It can signal from the extracellular compartment by binding to FFAR2 receptor (1), and it can enter the cells via MCTs or aquaporins to then be converted in acetyl CoA by ACSS1 in the mitochondria to enter the TCA cycle (2) or by ACSS2 in the cytoplasm/nucleus to acetylate proteins (3) (Martínez-Reyes and Chandel 2020; Qiu et al. 2019; R. Chen et al. 2015; Poul et al. 2003). Although the wide description of three modes of action for acetate, FFAR2 activation is the main one linked to the modulation of IL-1β production by acetate. Acetate was shown to reduce IL-1β production in BMDM stimulated with LPS and nigericin by binding to FFAR2 and attenuating NLRP3 inflammasome activation (Xu et al. 2019). On the other hand, another study showed that acetate increased IL-1β production by neutrophils stimulated with LPS and nigericin via activation of FFAR2 and NLRP3 inflammasome (Fachi et al. 2020). Therefore, the cellular type seems to be crucial for the response generated upon activation of FFAR2 by acetate.

Due to the importance of FFAR2 described in the literature, we first evaluated the production of IL-1β by WT and *Ffar2*$^{-/-}$ alveolar macrophages pre-treated or not with acetate and stimulated or not with *S. pneumoniae*. WT alveolar macrophages pre-treated with acetate presented a trend in increasing IL-1β levels, while in *Ffar2*$^{-/-}$ cells this trend was not observed (Figure 18A). On the other hand, the stimulation with *S. pneumoniae* increased IL-1β production in both genotypes. Surprisingly, acetate, in the presence of *S. pneumoniae*, boosted

IL-1β production in a similar fashion in WT and *Ffar2*$^{-/-}$ cells. Hence, acetate alone might depend on FFAR2, while in the presence of *S. pneumoniae*, acetate increases IL-1β production even in the absence of FFAR2.

To date, there is no study showing that acetate can induce IL-1β production via ACSS1 or ACSS2. However, since these two mechanisms are broadly described by acetate and they could be hypothetically linked to IL-1β production, we decided to investigate them. To first address if acetate could impact these mechanisms, we evaluated the gene expression of *Mct*s and *Acss*s upon acetate treatment and *S. pneumoniae* stimulation. We observed that acetate alone increased the expression of *Mct4*, *Acss1* and *Acss2* genes (Figure 18B). In addition, *S. pneumoniae* stimulation upregulated only the expression of *Mct4*, while in the presence of acetate the expression of all genes was boosted. Therefore, we raised two hypothesis that could link IL-1β production mediated by acetate to ACSS1 or ACSS2. We first thought that acetate could be converted in acetyl-CoA by ACSS1 in the mitochondria and enter the tricarboxylic acid cycle (TCA cycle). The higher production and entrance of acetyl CoA could unbalance the TCA cycle and culminate in the accumulation of metabolites, such as succinate, which is described to increase IL-1β production (Tannahill et al. 2013). We also raised a second hypothesis, in which acetate could be converted into acetyl CoA by ACSS2 in the nucleus, leading to histone acetylation and further opening the chromatin to increase *Il1b* gene expression. To investigate these hypotheses and evaluate the contribution of these two enzymes in IL-1β production, we generated CRISPR Cas9 KO cells for ACSS1, ACSS2 and a double knockout to discard compensatory mechanisms (Figure 18C-E). Although the depletion of these enzymes, macrophages were still able to produce IL-1β upon acetate pre-treatment in the presence of *S. pneumoniae* (Figure 18F). In line, we observed that all three knockout cells presented the same production of IL-1β by all groups when compared to their respective controls. Hence, acetate induction of IL-1β did not depend on FFAR2, ACSS1 or ACSS2.

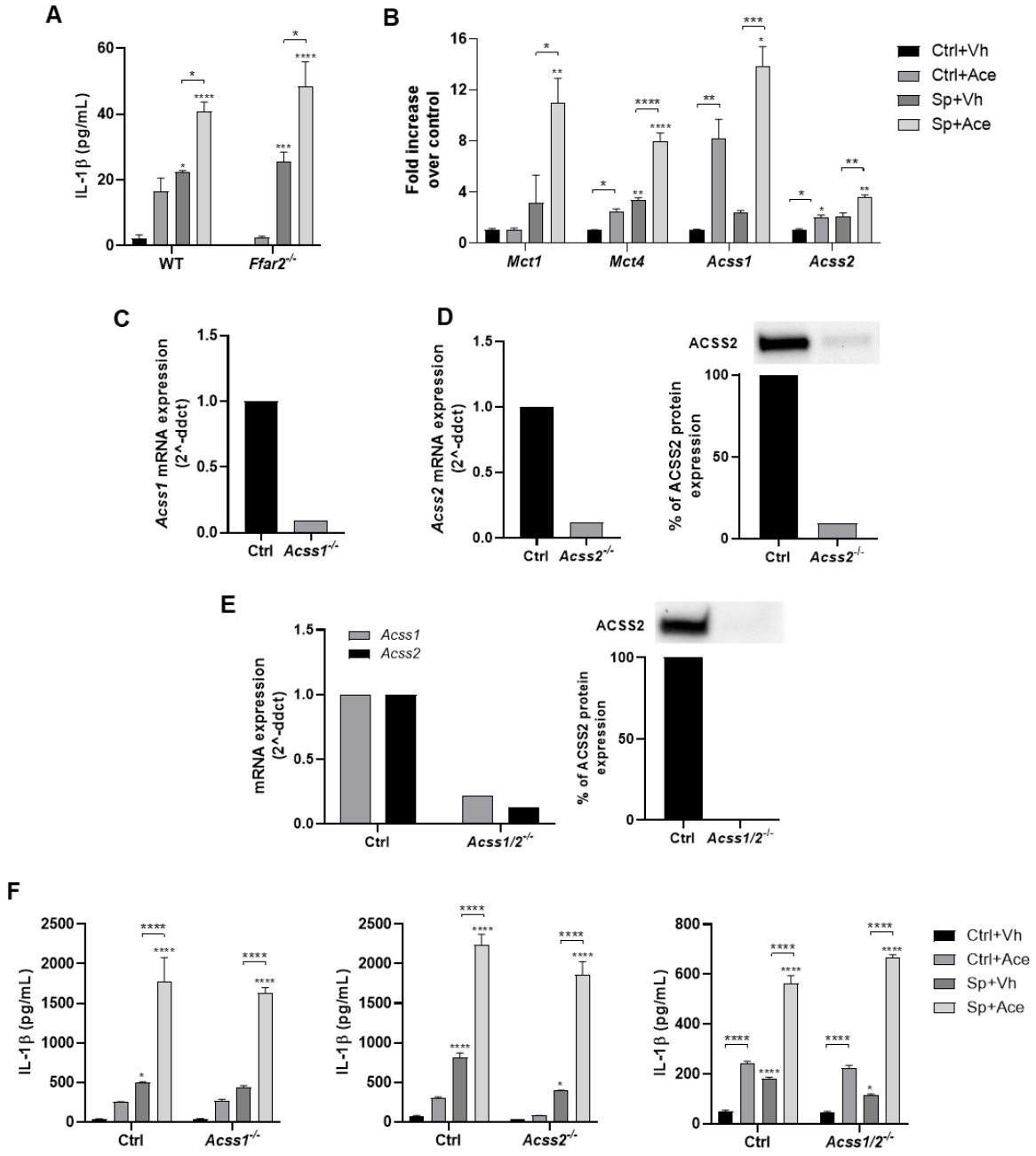


Figure 18: Production of IL-1β induced by acetate is independent of FFAR2, ACSS1 and ACSS2.

(**A**) IL-1β levels assessed by ELISA from supernatant of alveolar macrophages of C57Bl6 WT mice and *Ffar2*$^{-/-}$ mice pre-treated or not with acetate and stimulated or not with *S. pneumoniae* for 24 h. (**B**) Mct1, Mct4, Acss1 and Acss2 expression levels assessed by RT-PCR from lysates of MPI cells pre-treated or not with acetate and stimulated or not with *S. pneumoniae*. Fold increase was calculated over control (Ctrl+Vh). (**C**-**E**) *Acss1* and *Acss2* expression levels assessed by RT-PCR and ACSS2 protein levels assessed by Western Blot from lysate of control, *Acss1*$^{-/-}$, *Acss2*$^{-/-}$ and *Acss1/2*$^{-/-}$ MPI cells. Fold increase was calculated over each control (Ctrl). (**F**) IL-1β levels assessed by ELISA from supernatant of Ctrl, *Acss1*$^{-/-}$ *Acss2*$^{-/-}$, and *Acss1/2*$^{-/-}$ MPI cells pre-treated or not with acetate and stimulated or not with *S. pneumoniae* for 24 h. (**A**, **B** and **F**) Bars show the mean and errors show the SEM of triplicates/quadruplicates. Results are representative of three independent experiments. Statistical analysis was done using Two-way ANOVA Sidak's multiple comparisons test (*p < 0.05, ***p < 0.001, ****p < 0.0001). Comparison was made among respective control group or as indicated.

Increased IL-1β concentration induced by acetate is mediated by NLRP3 inflammasome

Since we did not figure out the mechanism by which acetate was acting, we decided to go back to the literature of IL-1β to understand and investigate which mechanisms of production and processing could be modulates by acetate.

IL-1β requires two signals to be produced, processed, and secreted by macrophages. The first step is to promote the synthesis and cytoplasmatic accumulation of pro-IL-1β (inactive precursor of IL-1β). The best described signals for this step are the PAMPs and DAMPs acting through PRRs (Garlanda and Jaillon 2016). However, hypoxia induction can also be a first signal and induce pro-IL-1β (Corcoran and O'Neill 2016). The second step is to activate the inflammasome pathway which will ultimately activate caspase 1 and/or caspase 8, which cleave pro-IL-1β in IL-1β (Lopez-Castejon and Brough 2011; Pyrillou, Burzynski, and Clarke 2020). Usually, the activation of this step is triggered by PAMPs and DAMPs, however other substances can also activate it. For example, NLRP3 inflammasome can be activated by K^+ efflux, or Ca^{2+} influx, triggered by other substances rather than DAMPs and PAMPs (He, Hara, and Núñez 2016).

According to the literature, acetate seems to play a role in NLPR3 inflammasome. In a model of colitis acetate was shown to increase NLRP3 inflammasome activation in intestinal epithelial cells by increasing Ca^{2+} mobilization (Macia et al. 2015). In contrast, acetate was also shown to impair NLRP3 inflammasome activation in BMDM by reducing Ca^{2+} mobilization and increasing ubiquitination and degradation of NLRP3 (Xu et al. 2019).

As NLPR3 was the only inflammasome described to be modulate by acetate, we investigated its involvement in our context. For this, we first performed RT-qPCR for the genes of the inflammasome from cells pre-treated or not with acetate and stimulated or not with *S. pneumoniae*. We could observe that acetate alone did not impact inflammasome genes expression, while *S. pneumoniae* alone only increased *Casp* gene expression (Figure 19A).

Contrarily, acetate pre-treatment together with *S. pneumoniae* stimulus enhanced the expression of *Nlrp3* and *Asc* (Figure 19A *left* and *central panel*). Since increased gene expression does not necessarily means activation of the inflammasome complex, we checked for caspase 1 activation (caspase-1 p20) and pro-IL-1β concentrations. We could see from the western blot, that supernatant from cells pre-treated or not with acetate and stimulated or not with *S. pneumoniae* had intact caspase 1 (Figure 19B). Nevertheless, acetate group only presented a tendency to increase caspase-1 p20, while *S. pneumoniae* stimulated group presented enhanced levels of caspase active form. In addition, the combination of acetate and *S. pneumoniae* increased even more the concentration of caspase-1 p20. In accordance, intracellular levels of pro-IL-1β were increased upon acetate or *S. pneumoniae* addition, and again, the combination of acetate and *S. pneumoniae* boosted pro-IL-1β production (Figure 19C). Therefore, acetate might act through NLPR3 inflammasome, once it increased *Nlrp3* and *Asc* genes expression, caspase 1 activation and pro-IL-1β production.

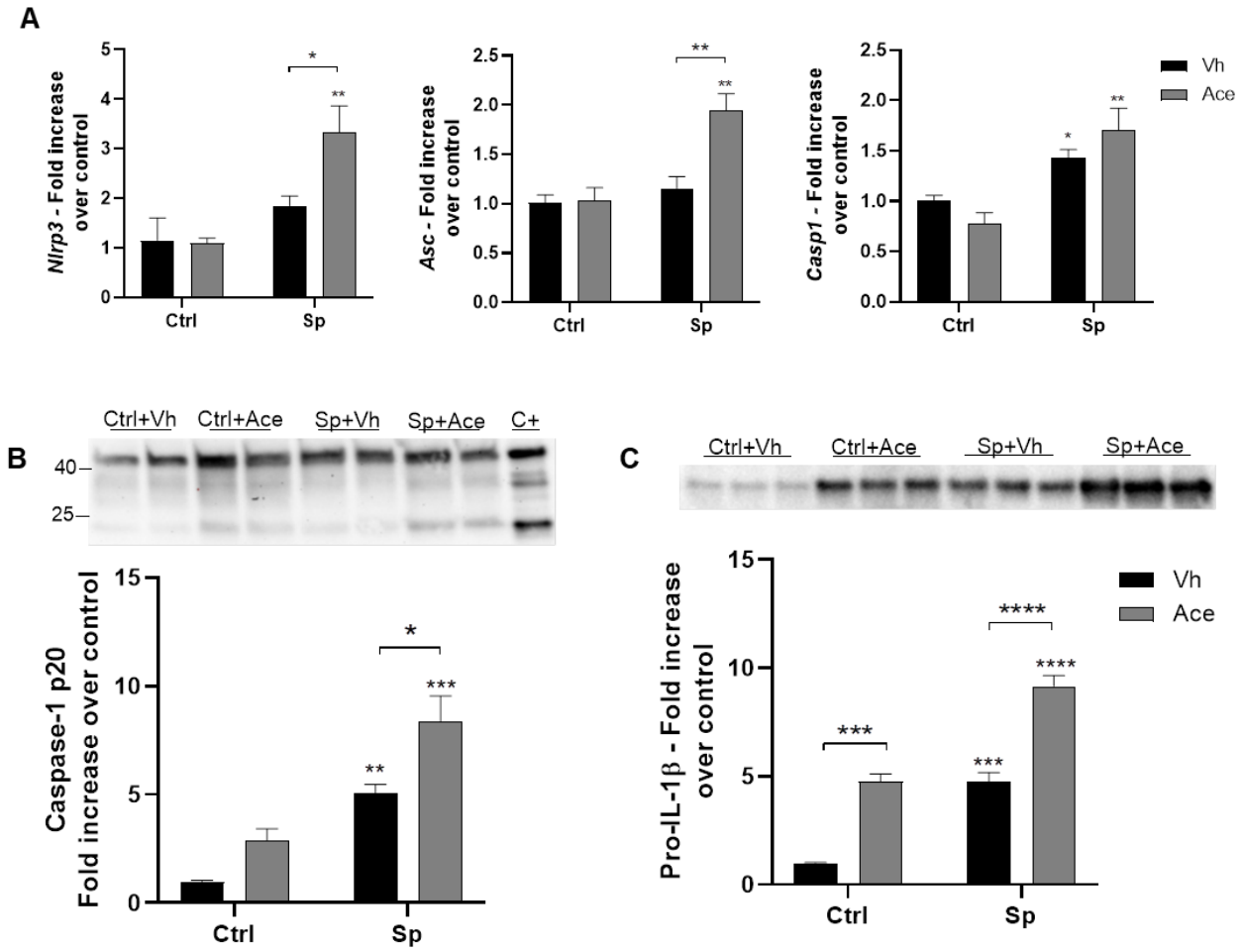


Figure 19: Acetate increased inflammasome gene expression and activation upon stimulation with *S. pneumoniae*.
(**A**) *Nlrp3*, *Asc* and *Casp1* expression assessed by RT-PCR from the lysate of MPI cells pre-treated or not with acetate and stimulated or not with *S. pneumoniae* for 18h. (**B**) Caspase 1 intact and cleaved assessed by Western Blot from supernatant of MPI cells pre-treated with acetate and stimulated with *S. pneumoniae* for 24h. A positive control was done adding 300 ng/mL of LPS for 4 h, followed by 20 µM of nigericin for 30 min. This experiment was repeated three times in duplicates/triplicate. Graphic shows a pool from the mean of three independent experiments. (**C**) Pro-IL-1β protein assessed by Western Blot from lysate of MPI cells pre-treated with acetate and stimulated with *S. pneumoniae* for 24h. For all experiments, fold increase was calculated over control (Ctrl+Vh). Bars show the mean and errors show the SEM of triplicates. (**A** and **C**) Results are representative of three independent experiments. Statistical analysis was done using Two-way ANOVA corrected with Sidak's multiple comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Comparison was made among respective control group or as indicated.

The results obtained so far do not clarify which mechanism is involved in IL-1β production, since the increased expression of those genes doesn't necessarily mean an increase in the protein production, neither an assembly of those proteins in complexes. However, it gives us a clue that NLRP3 inflammasomes could be activated. Aiming to decipher the participation of NLRP3 inflammasome in IL-1β production, a non-specific

inhibitor of NLRP3, KCl, was used. One of the mechanisms that activate NLRP3 is the K^+ efflux. Hence, high concentrations of extracellular K^+ lead by KCl administration blocks K^+ efflux and abrogate NLRP3 activation (Kelley et al. 2019). Therefore, alveolar macrophages were pre-treated or not with acetate in the presence or not of KCl, and then, stimulated or not with *S. pneumoniae*. Pre-treatment with KCl did not affect IL-1β production by *S. pneumoniae* stimulation, however IL-1β was dramatically decreased in *S. pneumoniae* stimulated macrophages pre-treated with acetate (Figure 20A). This result suggests that NLRP3 inflammasome pathway was the responsible for IL-1β production in macrophages pre-treated with acetate and stimulated with *S. pneumoniae*. Nevertheless, it was necessary to use specific inhibitors for NLRP3, since high concentrations of KCl may also inhibit AIM2 inflammasome (Fernandes-Alnemri et al. 2010). Thus, MPI cells were pre-treated or not with acetate in the presence or absence of MCC950 or CY-09 and then stimulated or not with *S. pneumoniae* for IL-1β quantification. In the group stimulated with *S. pneumoniae*, there was a trend in reducing IL-1β levels, while in the acetate pre-treated and *S. pneumoniae* stimulated group all concentrations of MCC950 reduced IL-1β production in similar proportions (Figure 20B). Interestingly, the effect observed upon CY-09 treatment was more expressive than the one with MCC950. All concentrations of CY-09 reduced *S. pneumoniae* induced IL-1β (Figure 20C). Remarkably, we observed a dose dependence reduction of IL-1β from acetate pre-treated macrophages stimulated with *S. pneumoniae*. Therefore, NLRP3 inflammasome was, at least in part, responsible for the secretion of IL-1β induced by acetate. To evaluate whether the contribution of NLRP3 for IL-1β production impacts the effector function of macrophages, we blocked NLRP3 inflammasome in acetate treated cells and assessed their killing ability. We observed that CY-09 treated cells presented the same percentage of viable bacteria as the vehicle group, thus it abolished the effect of acetate (Figure 20D). Hence, the axis NLRP3- IL-1β induced by acetate is important for the ability of macrophages to kill

bacteria.

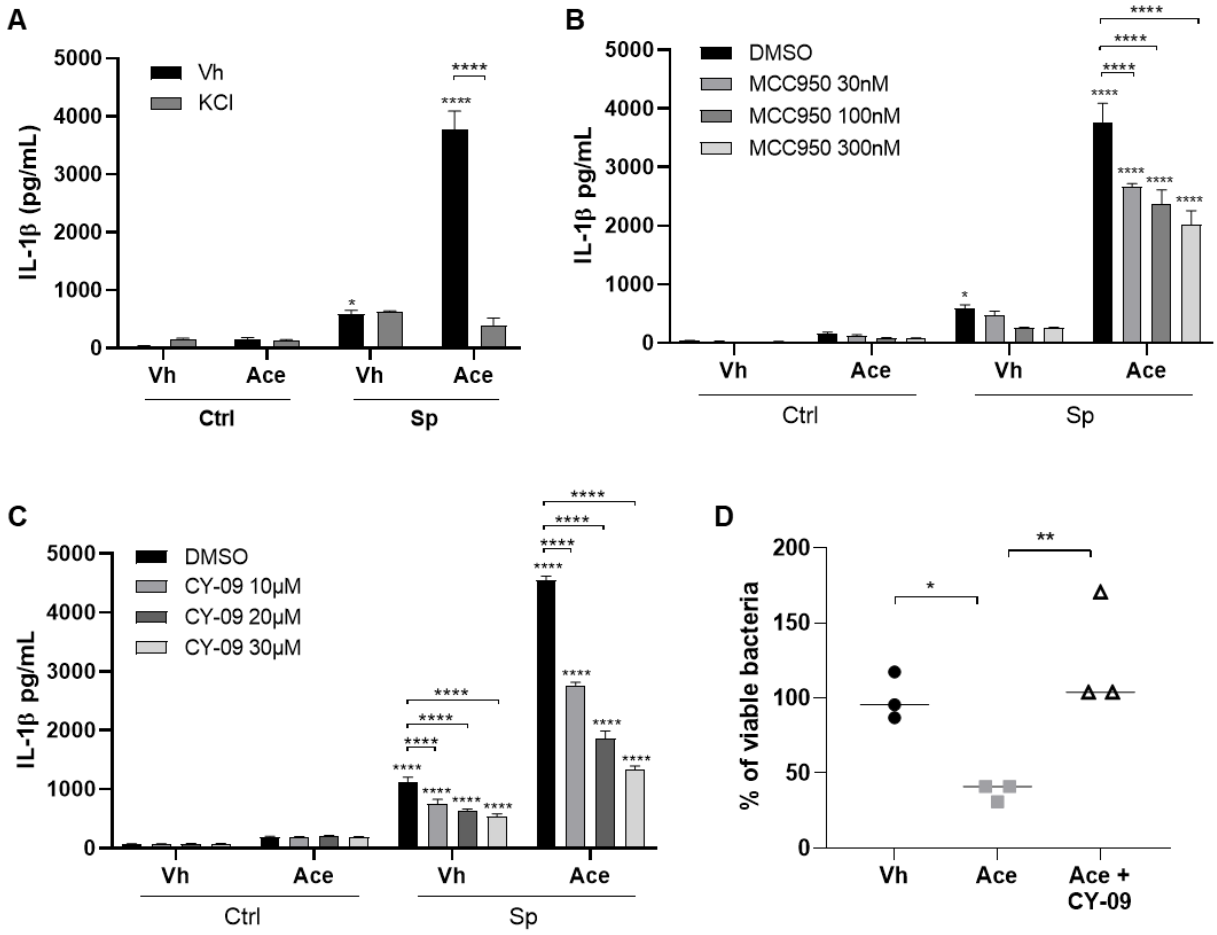


Figure 20: IL-1β secretion increased by acetate depends on NLRP3 inflammasome.
IL-1β levels assessed by ELISA from supernatant of MPI cells pre-treated with acetate, (**A**) KCl 90 mM, (**B**) MCC950 or (**C**) CY-09 at indicated concentrations and then stimulated with *S. pneumoniae* for 24 h. (**D**) Killing assay of macrophages previously activated with *S. pneumoniae* in the presence or absence of acetate and CY-09 30 μM for 24 h. Then incubated with live *S. pneumoniae*. Later cells were lysed, and viable intracellular bacteria were assessed. (**A-C**) Bars showing the mean and error showing the SEM, and (**D**) data showing the median of triplicates/quadruplicates. Results are representative of three independent experiments. Statistical analysis was done using (**A-C**) Two-way ANOVA corrected with Sidak's multiple comparisons test and (**D**) One-Way ANOVA (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Comparison was made among respective control group or as indicated.

Acetate modulates cellular metabolism and increases IL-1β production via glycolysis

Although the vast majority of publications about inflammation and infection associate the beneficial effect of acetate to FFAR2 receptor, there are few publications showing acetate's effect mediated by metabolism. Acetate was described to enhance glycolysis,

leading to a more effective T cell response, and it was also shown to increase respiration resulting in improved T memory cell response (Balmer et al. 2016; 2020; Buck, O'Sullivan, and Pearce 2015). Despite no link to immune response, acetate is described to induce a quick modulation in oxygen consumption and extracellular acidification (Sahuri-Arisoylu et al. 2021; Balmer et al. 2016; 2020). Therefore, to evaluate the acute effect of acetate in our context, we performed a seahorse analysis upon injection of acetate and 1h later we injected *S. pneumoniae*, in accordance with our protocol. We observed that acetate injection immediately increased the consumption of oxygen, while *S. pneumoniae* injection did not alter oxygen consumption in the absence or in the presence of acetate (Figure 21A). Interestingly, acetate also had an acute effect on extracellular acidification rate (ECAR), decreasing this parameter (Figure 21B). On the other side, *S. pneumoniae* injection alone seemed to sustain the extracellular acidification, while *S. pneumoniae* addition post-acetate did not present any alteration in ECAR. These results show that acetate, upon injection, increased cellular respiration, assessed by OCR, and decreased glycolytic rates, assessed by ECAR.

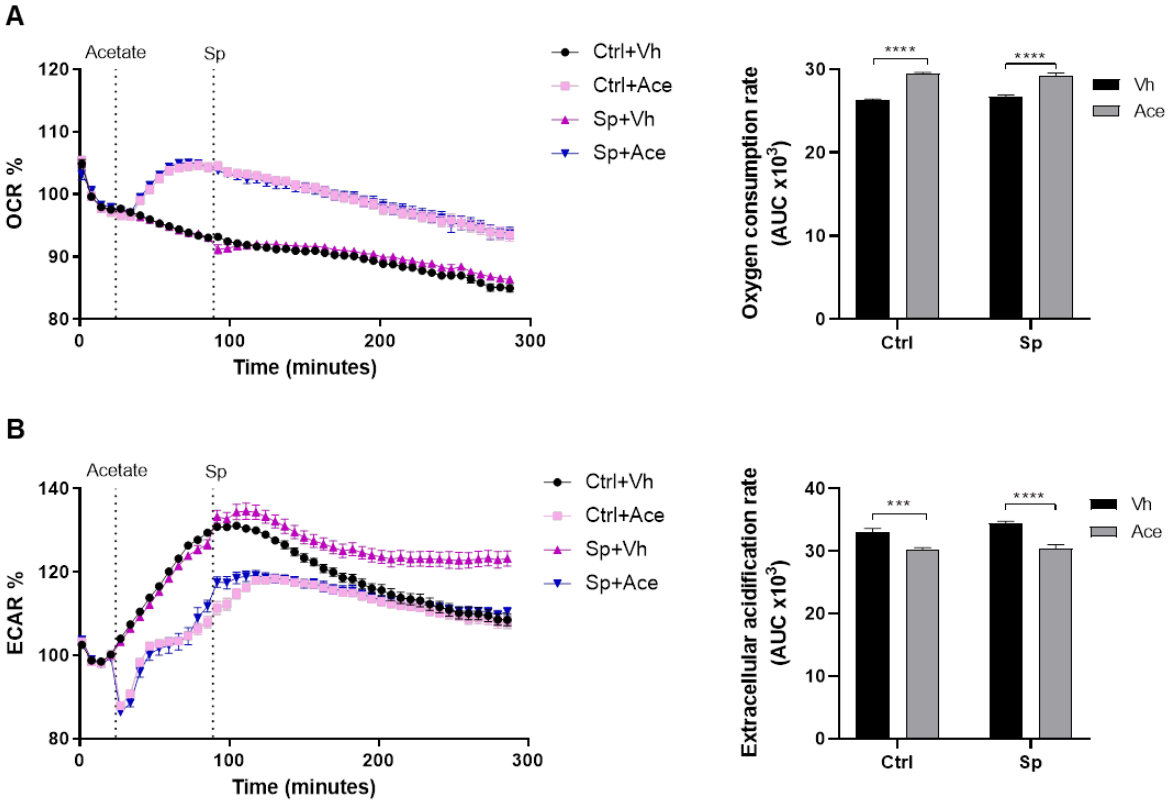


Figure 21: Acute injection of acetate modulates mitochondrial respiration and glycolysis. (**A**) Oxygen consumption rate (OCR) from MPI cells upon injection of acetate and 1 h later injection of *S. pneumoniae*. (**A *left panel***) Percentage of OCR considering the second measurement as 100% for each group and (**A *right panel***) area under the curve from A left panel. (**B**) Extracellular acidification rate (ECAR) from MPI cells upon injection of acetate and 1 h later injection of *S. pneumoniae*. (**B *left panel***) Percentage of ECAR considering the second measurement as 100% for each group and (**B *right panel***) area under the curve from B left panel. Bars and lines showing the mean and error showing the SEM of sextuplicates. Results are representative of three independent experiments. Statistical analysis was done using Two-way ANOVA corrected with Sidak's multiple comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Comparison was made among respective control group or as indicated.

Once we observed an acute effect of acetate, we wondered if this effect would be maintained in a long-term stimulation. In addition, as pro-inflammatory stimuli are known to induce metabolic changes upon long exposure, we also wanted to evaluate the effect of acetate upon *S. pneumoniae* stimulus. To address these questions, we pre-treated cells with acetate, stimulated with *S. pneumoniae* and after 24 h seahorse analysis was performed. We first assessed the mitochondrial stress via oxygen consumption rate (OCR), and we calculated the contribution of some steps for mitochondrial function.

In order to facilitate the comprehension, a brief description of each parameter analyzed will be given. Non-mitochondrial respiration is the oxygen consumption by a subset of cellular enzymes that are not linked to mitochondrial respiratory complex. The basal respiration shows the consumption of oxygen under baseline conditions. ATP production represents the participation of mitochondrial respiration in the generation of energy. Proton leak represents the basal respiration which is not coupled to ATP production. Maximal respiration mimics energy demand or stress, by triggering the maximal consumption of oxygen by the cell, mediated by the rapid oxidation of substrates such as fats, sugar, and amino acids. Spare respiratory capacity shows the capability of the cell to respond to stress/ energetic demand, and how close it is to the maximal respiration. Coupling efficiency shows the proportion of oxygen consumed for ATP synthesis compared to proton leak.

As expected, *S. pneumoniae* stimulation reduced almost all parameters assessed, indicating impairment in the mitochondrial function (Figure 22A-D and F-H). Different from our observation upon acute injection, after 24h of exposure acetate did not impact the basal respiration (Figure 22C). On the other hand, maximal respiration and spare respiratory capacity were increased in acetate treated cells in the presence or absence of *S. pneumoniae* (Figure 22D and G). The increase in these two parameters might indicate an increase in substrate availability, an increase in mitochondrial mass or good electron transport chain (ETC) integrity (Hill et al. 2012). Interestingly, acetate increased non-mitochondrial respiration and coupling efficiency from macrophages stimulated with *S. pneumoniae* (Figure 22B and H). The increase in non-mitochondrial respiration might indicate an increase in the activity of enzymes that depends on oxygen, (*e.g.*: NADPH oxidase and lipoxygenases), as well as increased ROS and RNS (Chacko et al. 2014; Dranka, Hill, and Darley-Usmar 2010). The observed increase in coupling efficiency indicates that in acetate pre-treated macrophages stimulated with *S. pneumoniae* a higher proportion of consumed oxygen is directed to ATP generation (Amo et al. 2008). Acetate, in the presence of *S. pneumoniae* also reduced proton

leak (Figure 22E), which can be interpreted as good mitochondrial membrane and ETC integrity or decreased uncoupling protein activity (Hill et al. 2012).

Thus, we could see that *S. pneumoniae* reduced oxygen consumption, and although acetate had no impact in basal respiration, it enhanced the capacity of the cells to respond to increased energy demand. In addition, acetate treatment seemed to improve ETC integrity.

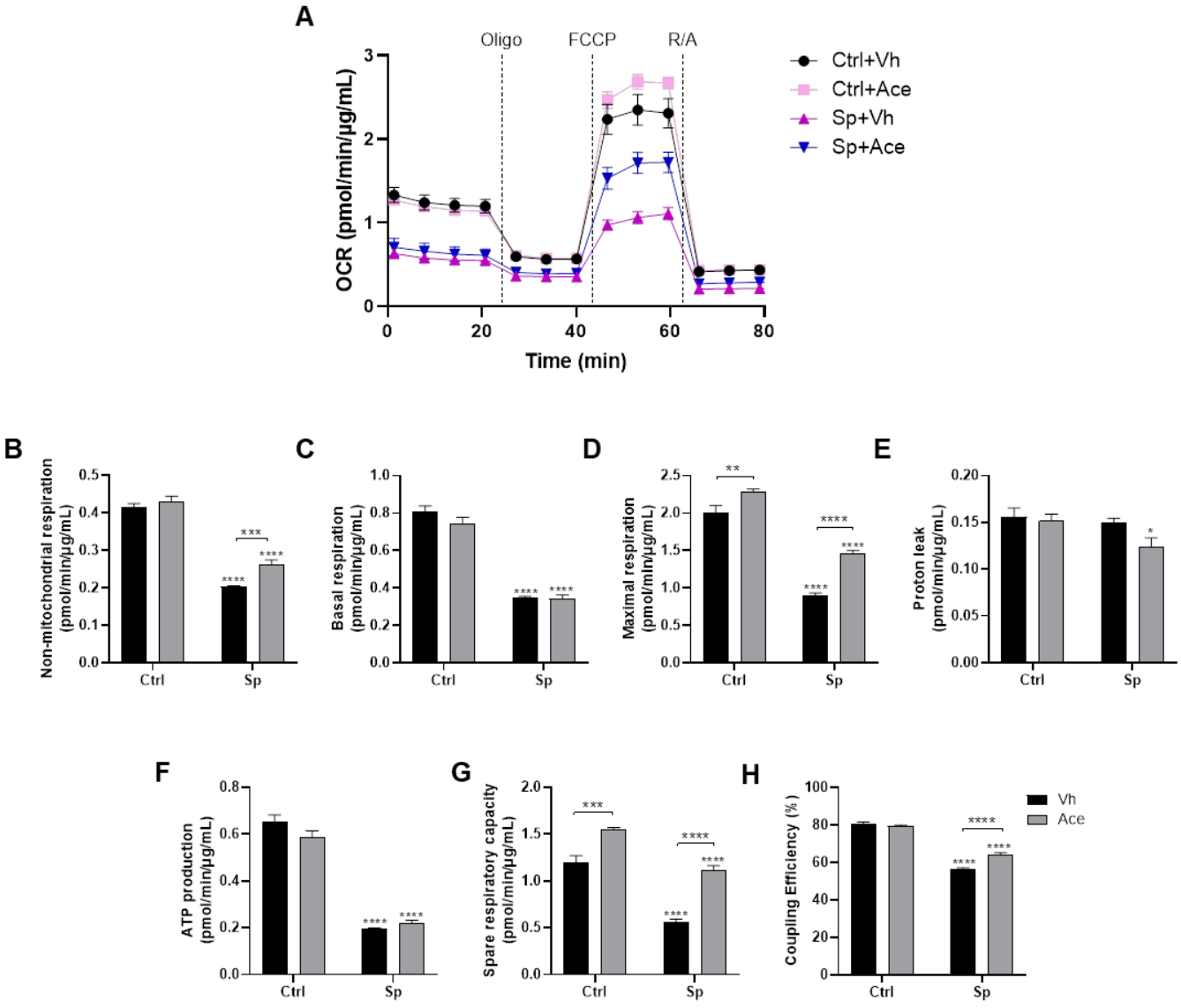


Figure 22: Acetate modulates oxygen consumption of macrophages stimulated or not with *S. pneumoniae*.
Mitochondria stress test of MPI cells pre-treated or not with acetate, and then stimulated or not with *S. pneumoniae* for 24 h. (**A**) OCR was measured in Seahorse after injection of oligomycin (Oligo), FCCP and rotenone and antimycin (R/A). (**B-H**) Calculation for each graphic was done using the formula described in the materials and methods. Bars and lines showing the mean, and error showing the SEM of sextuplicates. Results are representative of three independent experiments. Statistical analysis was done using Two-way ANOVA corrected with Sidak's multiple comparisons test (*p < 0.05, **p <

0.01, ***p < 0.001, ****p < 0.0001). Comparison was made among respective control group or as indicated.

We then assessed the effect of acetate on glycolysis. We first checked the expression of glycolytic genes from RNA-Seq data obtained from macrophages pre-treated or not with acetate and stimulated with *S. pneumoniae*. Surprisingly, acetate led to the up-regulation of almost all glycolytic genes (Figure 23A). Next, we assessed the glycolytic activity of macrophages pre-treated or not with acetate and stimulated or not with *S. pneumoniae* for 24h. The glycolytic profile of macrophages was altered by all conditions in different degrees (Figure 23B). Acetate alone increased glycolysis but had no impact in the other parameters (Figure 23C-F). On the other hand, *S. pneumoniae* stimulation increased glycolysis, glycolytic capacity, and glycolytic reserve, as expected during a pro-inflammatory stimulus (Figure 23C-E). In line with glycolytic gene expression, acetate, in the presence of *S. pneumoniae*, increased glycolysis and also the glycolytic capacity of macrophages (Figure 23C and D). Moreover, acetate together with *S. pneumoniae* did not increase glycolytic reserve when compared to *S. pneumoniae* stimulation alone (Figure 23E), and this can be explained by the increase in glycolytic capacity and glycolysis in a similar scale in both groups. Additionally, none of the conditions altered the non-glycolytic acidification (Figure 23F). Hence, acetate increased glycolysis and in the presence of *S. pneumoniae* this effect was amplified.

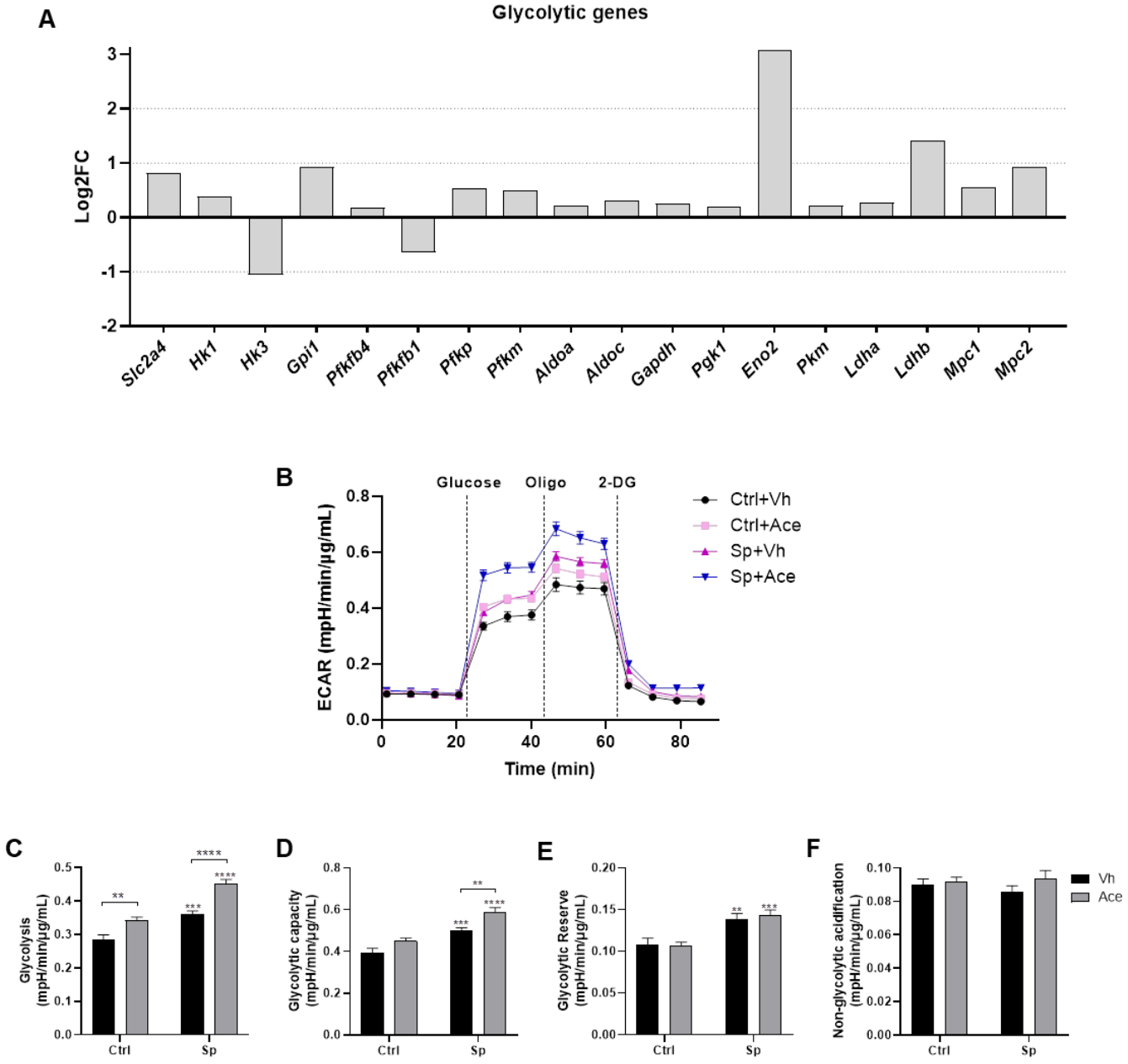


Figure 23: Acetate enhances glycolytic genes expression and glycolysis.
(**A**) Log_2 fold change of glycolytic genes expression from lysate of acetate pre-treated cells stimulated with *S. pneumoniae* over vehicle pre-treated cells stimulated with *S. pneumoniae*. Data obtained from RNA-Seq. All represented genes had p-value < 0.01 in the comparison between the two groups. (**B**) Glycolysis stress assay of MPI cells pre-treated or not with acetate, and then stimulated or not with *S. pneumoniae* for 24 h. ECAR was measured in Seahorse after injection of glucose, oligomycin (Oligo) and 2-Deoxy-D-glucose (2-DG). (**C-F**) Calculation for each graphic was done using the formula described in the materials and methods. Bars and lines showing the mean, and error showing the SEM of sextuplicates. Results are representative of three independent experiments. (**C-F**) Statistical analysis was done using Two-way ANOVA corrected with Sidak's multiple comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Comparison was made among respective control group or as indicated. Abbreviations: Solute carrier family 2 facilitated glucose transporter member 4 (*Slc2a4*), hexokinase (*Hk1, Hk2*), glucose-6-phosphate isomerase (*Gpi1*), 6-phosphofructo-2-kinase (*Pfkfb4, Pfkfb1*), ATP-dependent 6-phosphofructokinase platelet and muscle types (*Pfkp*, *Pfkm*), fructose-bisphosphate aldolase (*Aldoa, Aldoc*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*),

phosphoglycerate kinase 1 (*Pgk1*), gamma-enolase (*Eno2*), (*Pkm*), L-lactate dehydrogenase (*Ldha, Ldhb*), mitochondrial pyruvate carrier (*Mpc1, Mpc2*).

Nowadays several studies are showing the participation of cellular metabolism in immune function. The vast majority of studied link the modulations in metabolism to the production of IL-1β or IFN-γ (Balmer et al. 2016; Trompette et al. 2018; Tannahill et al. 2013; Gomes et al. 2021; Buck, O'Sullivan, and Pearce 2015; Galván-Peña and O'Neill 2014). As mentioned before, in the context of macrophages, it is shown that proinflammatory stimulus decreases cellular respiration due to an impairment in the TCA cycle. The breakage in TCA cycle results in accumulation of succinate, which increases IL-1β production (Tannahill et al. 2013). Also, it was described by Lu and colleagues, and supported by others that pro-inflammatory stimulus increases glycolysis and leads to an accumulation of pyruvate, which enhanced IL-1β production (Lu, Forbes, and Verma 2002; Tannahill et al. 2013). In our context, no difference in basal respiration was observed among *S. pneumoniae*-conditioned macrophages versus acetate-treated macrophages stimulated with *S. pneumoniae*. Furthermore, acetate even increased some parameters of mitochondrial respiration, such as maximal respiration and spare respiratory capacity. Therefore, it is unlikely that mitochondrial respiration is linked to IL-1β production. On the other hand, the increased glycolysis led by acetate could play a role in the production of this cytokine. Thus, to assess if increased glycolysis mediated by acetate was inducing IL-1β production, we stimulated cells in the presence or absence of glucose, or in the presence of different concentrations of 2-DG, an analogous of glucose-6-phosphate that blocks glycolysis. Due to the number of groups in the analyses and the magnitude of IL-1β production triggered by *S. pneumoniae* plus acetate, we observed only a trend in acetate pre-treatment without stimulus to increase IL-1β, and a trend in reducing IL-1β levels without glucose and with 10mM of 2-DG (Figure 24). In contrast, deprivation of glucose reduced IL-1β production in *S. pneumoniae* stimulated macrophages, and the effect was amplified in acetate treated macrophages stimulates with *S. pneumoniae* (Figure 24A *left panel*). In

accordance, the blockage of glycolysis by 2-DG had a dose-dependent effect in reducing IL-1β production in *S. pneumoniae* stimulated cells in the presence or absence of acetate (Figure 24A *right panel*). Hence, acetate-induced increase in IL-1β production was mediated by glycolysis. To address the effect of acetate induced IL-1β via glycolysis in the effector function of macrophages, we assessed the killing ability of acetate treated macrophages in the presence of 2-DG. The blockage of glycolysis resulted in a similar percentage of viable bacteria observed in the vehicle group, and it abolished the effect of acetate (Figure 24B). Therefore, the effector function of macrophages relies on the production of IL-1β mediated by glycolysis.

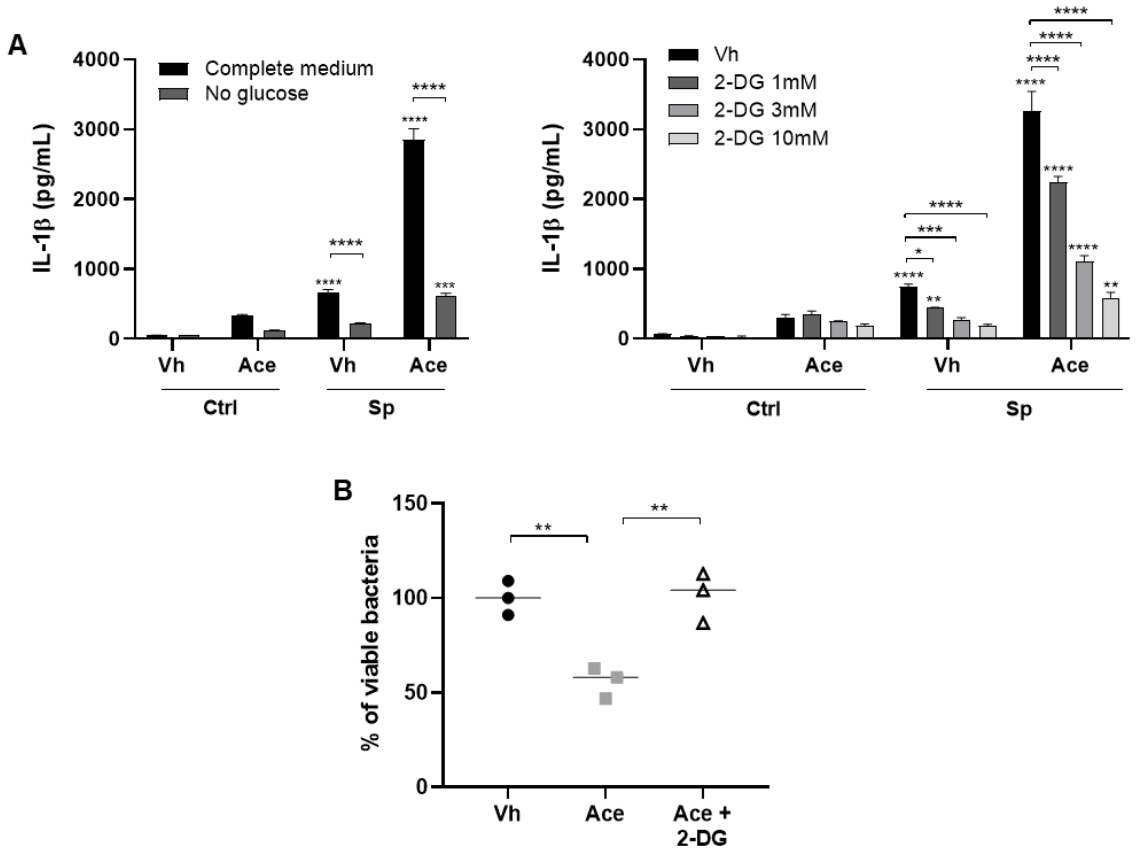


Figure 24: Glycolysis is responsible for acetate-induced IL-1β production.
ELISA for IL-1β from supernatant of MPI cells pre-treated or not with acetate in the presence or not of (***left panel*** **A**) glucose or (***right panel*** **A**) 2-DG and then stimulated or not with *S. pneumoniae* for 24 h. (**B**) Killing assay of macrophages previously activated with *S. pneumoniae* in the presence or absence of acetate and 2-DG 10 mM for 24 h. Then incubated with live *S. pneumoniae*. Later cells were lysed, and viable intracellular bacteria were assessed. (**A**) Bars showing the mean and error showing the SEM, and (**B**) data showing the median of triplicates/quadruplicates. Results are representative of three independent experiments. Statistical analysis was done using (**A**) Two-way ANOVA corrected with Sidak's multiple comparisons test and (**B**) One-Way ANOVA (*p < 0.05, **p

< 0.01, ***p < 0.001, ****p < 0.0001). Comparison was made among respective control group or as indicated.

Acetate induces *Il1b* transcription via glycolysis-HIF-1α axis

As acetate-induced IL-1β production was mediated by glycolysis, we decided to investigate the mechanism by which glycolysis was acting. The best described mechanism by the literature relies on hypoxia inducible factor 1α (HIF-1α) activation (Lu, Forbes, and Verma 2002; Tannahill et al. 2013). HIF-1α is a transcription factor responsible for the response to hypoxia. HIF-1α gene is expressed in all immune cells, however under normoxia or stead-state condition, HIF-1α protein is constantly degraded by prolyl hydroxylases (PHDs). During hypoxia or upon pro-inflammatory stimulus HIF-1α degradation is inhibited, and the protein goes to the nucleus to exert its functions (Corcoran and O'Neill 2016). The stabilization of HIF-1α can be driven by many factors. In the context of hypoxia, PHD is inhibited due to the absence of its co-factor, oxygen. In the context of inflammation, PDHs can be inhibited by ROS, TNF-α, TGF-β, IL-6 and others (Sena et al. 2013; Albina et al. 2001; Deng et al. 2013). Additionally, metabolic changes can also increase HIF-1α protein concentrations, like accumulation of pyruvate due to enhanced glycolysis, and high concentrations of succinate originated from TCA arrest (Tannahill et al. 2013; Lu, Forbes, and Verma 2002). Upon stabilization HIF-1α can promote the transcription of different sets of genes, such as metabolic (*Slc2a4*, *Ucp3*, *Pfk*, *Pgk1*, *Aldoa*, and *Pdk1*) and inflammatory genes (*Il1b*, *Cxcr4* and *Inos*) (Takeda et al. 2010; W. Liu et al. 2012; Gomes et al. 2021). Therefore HIF-1α is an important link between cellular metabolism and immune response. In the enrichment analysis performed with RNA-Seq data, we observed an upregulation in the response to hypoxia. As HIF-1α is the main agent involved in hypoxia and it can be activated by increased glycolysis, we assessed HIF-1α expression and HIF-1α target genes expression. Interestingly, HIF-1α was upregulated in all conditions, with a substantial upregulation in the

acetate pre-treated macrophages stimulated with *S. pneumoniae* (Figure 25A). In accordance, except for *Pdk1*, all HIF-1α target genes were upregulated upon treatment with acetate in the presence or absence of *S. pneumoniae* (Figure 25B). On the other hand, stimulation with *S. pneumoniae* alone upregulates 5 out of 8 genes (Figure 25B). The analysis of HIF-1α target genes allow us to infer that HIF-1α was activated upon stimulation and treatment. To investigate whether HIF-1α activation was mediated by glycolysis, we treated the cells with 2-DG and assessed the expression of its targets. The blockage of glycolysis abrogated the effect of acetate and *S. pneumoniae* on HIF-1α target genes expression (Figure 25C). Meaning that glycolysis was mediating HIF-1α activation by acetate.

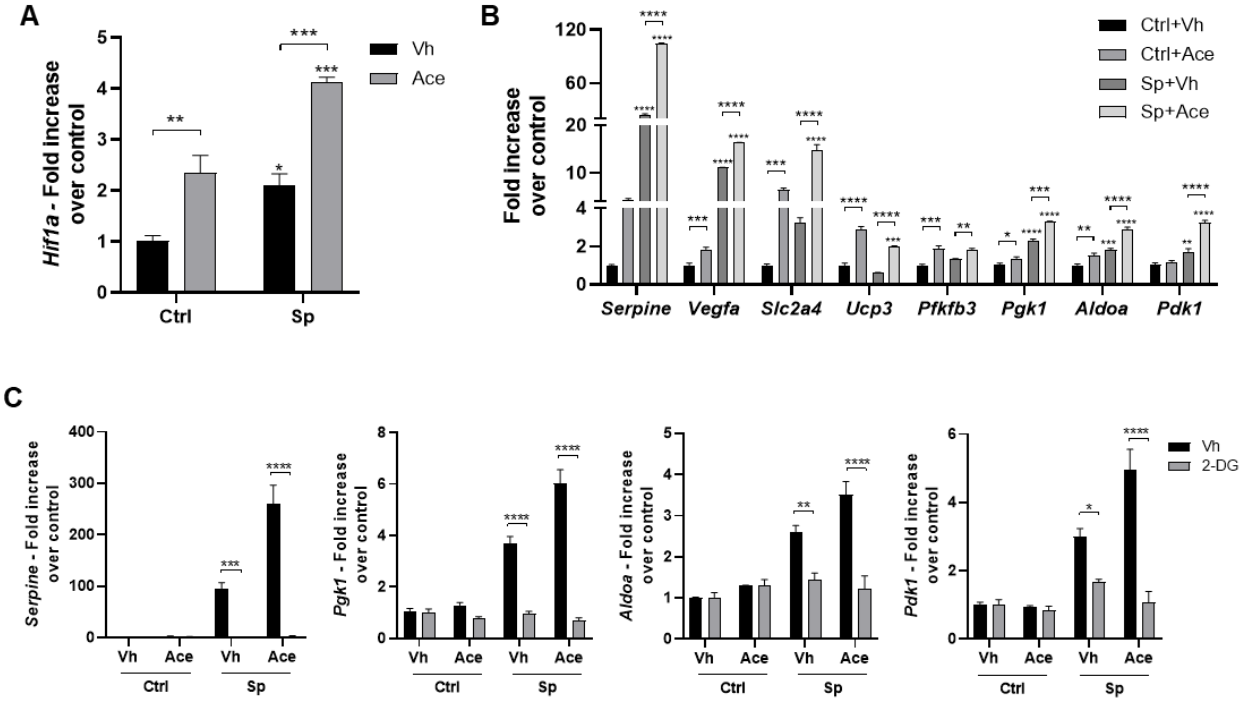


Figure 25: Acetate treatment increased HIF-1α gene expression and protein activation via glycolysis.
(**A** and **B**) *Hif1a* and HIF-1α target genes expression assessed by RT-PCR from the lysate of MPI cells pre-treated or not with acetate and stimulated or not with *S. pneumoniae* for 18h. (**C**) HIF-1α target genes expression assessed by RT-PCR from the lysate of MPI cells pre-treated or not with acetate in the presence or absence of 10mM of 2-Deoxy-D-Glucose (2-DG) and stimulated or not with *S. pneumoniae* for 18h. Bars showing the mean, and error showing the SEM of triplicates. Results are representative of three independent experiments. Statistical analysis was done using Two-way ANOVA corrected with Sidak's multiple comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Comparison was made among respective control group or as indicated. Abbreviations: Vascular endothelial growth factor A (*Vegfa*), solute carrier family 2, facilitated glucose transporter member 4 (*Slc2a4*), uncoupling protein 3 (*Ucp3*), 6-phosphofructo-2-kinase

(*Pfkfb3*), phosphoglycerate kinase 1 (*Pgk1*), fructose-bisphosphate aldolase (*Aldoa*) and pyruvate dehydrogenase kinase 1 (*Pdk1*).

Next, we addressed if HIF-1α activated by acetate was leading to *Il1b* transcription and consequent secretion. For this, we used *Hif1a* siRNA to knock down HIF-1α expression, and siRNA scramble as a control. These cells were pre-treated or not with acetate and stimulated or not with *S. pneumoniae*. As expected, HIF-1α knockdown cells presented decreased HIF-1α gene expression and the effect of acetate on HIF-1α target genes was also abrogated indicating that the silencing process was successful (Figure 26A and B). Moreover, we observed that *Il1b* gene expression in acetate pre-treated macrophages trend to decrease upon HIF-1α silencing. Additionally, *Il1b* expression was reduced in HIF-1α silenced macrophages stimulated with *S. pneumoniae* in the presence or absence of acetate (Figure 26C *left panel*). Interestingly, HIF-1α knockdown only decreased IL-1β concentrations in acetate pre-treated macrophages stimulated with *S. pneumoniae* (Figure 26C *right panel*). Hence, IL-1β production enhanced by acetate relies on glycolysis-HIF-1α activation.

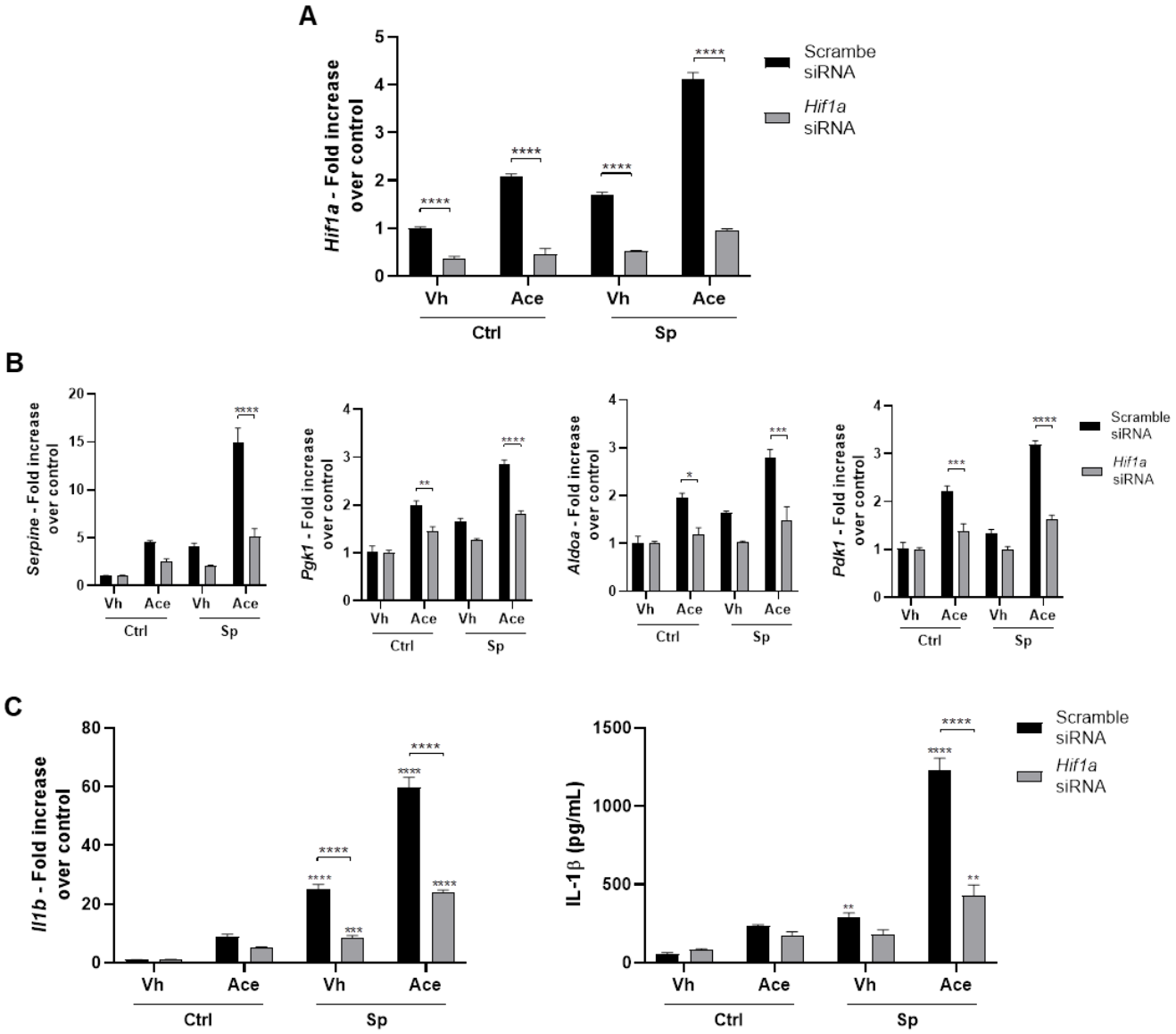


Figure 26: HIF-1α is responsible for IL-1β expression and production triggered by acetate.
(**A** and **B**) *Hif1a* and HIF-1α target genes expression assessed by RT-PCR from the lysate of MPI cells transfected with scramble siRNA or *Hif1a* siRNA, pre-treated or not with acetate and stimulated or not with *S. pneumoniae* for 18h. (**C**) Il1b gene expression assessed by RT-PCR from the lysate and IL-1β concentrations assessed by ELISA from the supernatant of MPI cells transfected with scramble siRNA or *Hif1a* siRNA, pre-treated or not with acetate and stimulated or not with *S. pneumoniae* for 18h. Bars showing the mean, and error showing the SEM of triplicates. Results are representative of three independent experiments. Statistical analysis was done using Two-way ANOVA corrected with Sidak's multiple comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Comparison was made among respective control group or as indicated.

Discussion

Emerging literature suggests that SCFAs play an important role in pulmonary immune defense against respiratory pathogens (Machado, Sencio, and Trottein 2021). Considering the impact of pulmonary infections, especially pneumococcal pneumonia, on the worldwide mortality it is important to consider new therapeutics that consists in the control of bacterial loads and inflammation. Here, we present an important contribution for acetate's mode of action, and we decipher the mechanism by which acetate boosts macrophages' killing ability. To summarize, we showed that acetate supplementation (in drinking water) to naïve mice increased the secretion of proteins involved in host defense in the lungs. This proteomic analysis confirmed that acetate can distally impact the functions of pulmonary cells. In line, acetate supplementation preventively armed the lungs to defend against *S. pneumoniae*, resulting in a better clearance and reduced bacterial dissemination to distal organs. In accordance with this protective effect, macrophages also presented an activated profile with enriched defense pathways upon acetate treatment. We showed that acetate boosted the killing ability of macrophages by enhancing NO production. The increase in NO concentration was mediated by IL-1β secretion, the sole pro-inflammatory cytokine increased by acetate. Surprisingly, the production of IL-1β upon acetate treatment was independent of FFAR2, ACSS1 and ACSS2. Instead, IL-1β production was mediated by the glycolysis-HIF-1α axis and its secretion was dependend on NLRP3 inflammasome.

SCFAs, especially butyrate, are known to induce the production of antimicrobial peptides by intestinal epithelial cells. It was shown that mice receiving supplementation with SCFAs had increased production of RegIIIγ and β-defensin 1, 3 and 4 by intestinal epithelial cells (Zhao et al. 2018). Upon binding to FFAR2, SCFAs activated mTOR and STAT3 which lead to higher expression of these AMPs. Apart from this study with all three SCFAs, all other studies were focused only on butyrate. Colonocytes from humans and rabbits presented increased production of LL-37 upon butyrate treatment (Raqib et al. 2006a; Schauber et al. 2003). In line, porcine intestinal epithelial cells also presented higher production of the AMPs

β-defensins and cathelicidins via inhibition of HDAC by butyrate supplementation (Xiong et al. 2016; Zeng et al. 2013). Although most studies are with intestinal epithelial cells, butyrate was also shown to increase the expression of the AMPs calprotectin, S100A8, S100A9 and cathelicidins via HDAC inhibition in murine macrophages (Schulthess et al. 2019; J. Zhang et al. 2020). In accordance, an enhanced production of LL-37 was also observed in monocytes from humans that received oral supplementation of phenylbutyrate (Mily et al. 2013). Interestingly, two other studies showed that butyrate also induced the expression of cathelicidins in human lung epithelial cell line (Steinmann et al. 2009; Kida, Shimizu, and Kuwano 2006). The observed effect of butyrate in macrophages and lung epithelial cells are in line with our finding that acetate supplementation increased the production of S100A7 in the pulmonary space. The AMP S100A7 is described to be produced by bronchial epithelial cells and alveolar macrophages, thus acetate could be acting by direct targeting these cells (Andresen et al. 2011). In accordance, haptoglobin and complement, proteins involved in host defense, were also present in higher concentrations in the pulmonary space of mice supplemented with acetate.

It is also described that macrophages treated with all three SCFAs presented higher expression and secretion of lysozyme. In this context, lysozyme production induced by butyrate was mediated by HDAC inhibition as observed by Schulthess for AMPs and cathelicidins (J. Zhang et al. 2020). Therefore, the increase in lysozyme concentrations found in the pulmonary cavity of mice supplemented with acetate might come from alveolar macrophages.

Although there is no description about the impact of SCFAs in the production of surfactant proteins, SCFAs are described to enhance the mucosal barrier in the intestine of rats (Shimotoyodome et al. 2000). In addition, they were also shown to induce the production of mucin *in vitro* (Willemsen 2003). Therefore, SCFAs, specifically acetate, could have a similar

effect in the lung mucosal site and enhance barrier properties by increasing the secretion of surfactant proteins.

Despite the considerable number of studies showing the ability of butyrate to induce AMPs production, this is the first time that acetate alone is shown to increase the production of AMPs and proteins associated to host defense. In addition, this is also the first study covering the impact of acetate supplementation in the concentration of proteins in the pulmonary cavity of mice. Interestingly, acetate was found in the lungs of mice and humans, and it was shown to be sensed by alveolar macrophages and alveolar epithelial cells which express FFAR2 (Q. Liu et al. 2021). Therefore, acetate could direct target pulmonary cells to modulate the secretion of proteins involved in host defense. Although we did not evaluate the role of proteins and peptides enhanced by acetate in pathogen elimination, we hypothesized that they could contribute to arm the lungs to better respond face an infection. Additionally, these alterations in protein secretion might help to explain why acetate has been shown to be beneficial in a different set of pulmonary infection models (Ji et al. 2021; Sencio et al. 2020; Antunes et al. 2019; Galvão et al. 2018).

The protective effect of acetate against respiratory infections was already described by some research groups. It was demonstrated that acetate was beneficial during RSV infection, and our group showed that acetate also protected IAV-infected mice from secondary bacterial infection (Antunes et al. 2019; Ji et al. 2021; Sencio et al. 2020). In addition, acetate was also shown to ameliorate pneumonia caused by *Klebsiella pneumoniae* (Galvão et al. 2018). Our results confirm these findings, as we observed that acetate supplementation also protected mice from *Streptococcus pneumoniae* by reducing bacterial loads in the lungs, and by lowering bacterial dissemination from the lungs.

Alveolar macrophages have a crucial role in host defense against respiratory infections, and they were shown to mediate the effect of acetate in face of RSV, *K. pneumoniae* and *S. pneumoniae* secondary infection (Ji et al. 2021; Vieira et al. 2017; Sencio et al. 2020).

However, the mechanisms by which macrophages protect mice from bacterial infection is barely described. Here, we showed for the first time that acetate markedly modulated macrophages' profile and we describe the mechanism by which acetate boosts their bactericidal activity. In line with the literature, we observed that acetate-treated macrophages presented improved killing ability. Although this phenotype was already described, we unravel a different mechanism by which acetate boosts macrophages' defense. We showed that acetate increased the production of nitric oxide, increasing the intracellular killing of *S. pneumoniae*. Alternatively, Zhang and colleagues showed that acetate improved the killing activity of macrophages and this effect was linked to increased production of lysozyme (J. Zhang et al. 2020). Another recent study demonstrated that acetate enhanced the killing of *Salmonella enterica* serovar Typhimurium by BMDM via inflammasome activation (Tsugawa et al. 2020). Therefore, there is a growing body of evidence that acetate can boost macrophages to improve bacterial clearance by different mechanisms.

To the best of our knowledge, this is the first time that acetate is shown to increase NO production by macrophages. Interestingly, propionate and butyrate were shown to reduce NO production in neutrophils stimulated with LPS and RAW 264.7 cells stimulated with lipoproteins from *Staphylococcus aureus*, while acetate had no effect (Vinolo et al. 2011; J. W. Park et al. 2019). In line, another study also showed that propionate and butyrate reduced NO production in RAW 264.7 cells stimulated with LPS. However, in this context, acetate also decreased NO production (T. Liu et al. 2012). Therefore, the stimulus used to induce nitric oxide seems to be important for the effect of acetate.

The production of NO is crucial for the killing of *S. pneumoniae* as demonstrated by Marriot et al. (2004). NO is described to kill bacteria by different mechanisms, it can have a direct or an indirect role. The direct effect of NO in the bacteria can be through the interaction of NO with reactive oxygen intermediates, such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), or through the generation of dinitrogen tetroxide (N_2O_4), dinitrogen trioxide (N_2O_3),

nitrogen dioxide (NO_2), S-nitrosothiols (RSNO) and peroxynitrite ($OONO^-$). These reactive species kill bacteria by causing damage to the DNA, impairing the activity of metabolic enzymes, depleting iron and binding to proteins essential for the vital functions of the bacteria (Jones et al. 2010). Apart from the direct effect, NO can induce macrophages apoptosis which is directly linked to *S. pneumoniae* clearance *in vitro* and *in vivo* (Marriott et al. 2004; Dockrell et al. 2003; 2001).

Due to the importance of NO production in our context, we investigated the mechanism of acetate effect, by assessing the secretion of pro-inflammatory cytokines known to trigger NO production. Acetate has been described to decrease and/or increase the production of pro-inflammatory cytokines regarding the context (Xu et al. 2019; T. Liu et al. 2012; Tsugawa et al. 2020; Antunes et al. 2019). Upon stimulation with *S. pneumoniae,* acetate had a dual effect in the production of cytokines, increasing IL-1β, and decreasing TNF-α and IL-12 by MPI cells. In line, alveolar macrophages collected from mice (primary cells) and treated with acetate displayed the same profile of cytokine production, reinforcing MPI cell line as a good model to study alveolar macrophages. These results are interesting not only because they orientated our research on IL-1β, but also because they show the potential of acetate in modulating inflammatory response. The inflammatory response in pneumococcal pneumonia is a determinant feature for successful bacterial clearance or exacerbated tissue damage. The secretion of high concentrations of pro-inflammatory cytokines, especially TNF-α, is correlated to tissue damage and barrier breakage (Petecchia et al. 2012; Hardyman et al. 2013; Mazzon and Cuzzocrea 2007). Additionally, pneumonia can evolve to sepsis and the higher production of cytokines can culminate in cytokine storm (von Dossow et al. 2005; Gotts et al. 2019). Therefore, the observed reduction in pro-inflammatory cytokines by acetate *in vitro* might be transposed to a beneficial effect in the context of pneumococcal pneumonia. On the other hand, production of IL-1β by alveolar macrophages enhanced by acetate can be beneficial, since it can improve macrophages' function in an autocrine manner, and it can also

activate neutrophils (Marriott et al. 2012). Moreover, IL-1β can induce Th1 and Th17 antimicrobial responses via production of NO and AMPs (Netea et al. 2010; Weiss et al. 2019; Rafa et al. 2013). The observed *in vitro* effect of acetate makes it an interesting target to treat infectious diseases that trigger high inflammatory responses, as it could control exacerbated inflammation and limit bacterial proliferation. Of interest, a recent study showed that intact gut microbiota is associated with lung immune tone, once SCFAs originated from murine and human gut microbiota could reach the lungs, prime alveolar macrophages and induce the production of IL-1β (Q. Liu et al. 2021). Hence, supplementation with acetate, prebiotics or probiotics that produces acetate can be a potential target to boost pulmonary defense. In this context, our host laboratory has developed multiple approaches including the use of fiber-enriched diets and the use of several commensal strains selected for their ability to produce acetate.

IL-1β was the sole pro-inflammatory cytokine produced by alveolar macrophages and in MPI cells that was enhanced by acetate, and it was indeed the responsible for higher production of NO induced by acetate. As already mentioned in the results section, the ability of IL-1β to induce NO production was already demonstrated in different contexts, such as *in vitro* stimulation of PBMC and hepatocytes (Rafa et al. 2013; Kitade et al. 1996).

Regarding the role of IL-1β in acetate-mediated macrophages' killing ability, we investigated the mechanism by which acetate was enhancing IL-1β secretion. Conflicting results have been published regarding the ability of acetate to modulate IL-1β production. Acetate was shown to decrease the production of IL-1β by BMDM stimulated with LPS and nigericin or ATP, by RAW 264.7 cells stimulated with LPS and by pulmonary cells submitted to hypoxia (Xu et al. 2019; T. Liu et al. 2012; Q. Zhang et al. 2021). In BMDM, the effect was attributed to activation of FFAR2 receptor, leading to decreased calcium mobilization and NLRP3 ubiquitination, resulting in the blockage of NLRP3 inflammasome (Xu et al. 2019). In contra part, acetate's effect observed in RAW 264.7 cells was related to the inhibition of NF-

κB activation by acetate (T. Liu et al. 2012). It is also described in the literature that acetate can increase IL-1β production by recruited peritoneal macrophages stimulated with LPS and MSU, by neutrophils stimulated with *Clostridium difficile* or *Aggregatibacter actinomycetemcomitans* and by BMDM stimulated with *Salmonella* Typhimurium (Vieira et al. 2015; Fachi et al. 2020; R. O. Corrêa et al. 2017; Tsugawa et al. 2020). The effect of acetate on peritoneal macrophages stimulated with LPS and MSU and on neutrophils stimulated with *C. difficile* was mediated by FFAR2 and inflammasome activation (Vieira et al. 2015; Fachi et al. 2020). On the other hand, the effect observed in neutrophils stimulated with *A. actinomycetemcomitans* and in BMDM stimulated with *S.* Typhimurium were independent of FFAR2. In neutrophils, the authors suggested that acetate increased IL-1β production by inhibition of HDAC, while in BMDM it is suggested that acetate can directly bind to ASC to promote inflammasome assembly and activation (R. O. Corrêa et al. 2017; Tsugawa et al. 2020). In our hands, acetate alone seemed to induce less IL-1β in the *Ffar2*$^{-/-}$ alveolar macrophages compared to the WT cells, however, when stimulated with *S. pneumoniae*, alveolar macrophages from KO and WT mice produced similar concentrations of IL-1β. Thus, our results are in line with those presented by Corrêa et al. (2017) and Tsugawa et al. (2020) that acetate does not depend on FFAR2 to increase IL-1β production.

Based on these studies, acetate can improve the production of IL-1β by inhibiting HDAC or by directly binding to ASC. To the best of our knowledge, mechanisms by which acetate could inhibit HDAC activity are still ignored. Some authors suggest that the observed increase in histone acetylation led by acetate is rather caused by the accumulation of acetyl CoA originated from acetate's metabolism (Qiu et al. 2019; Soliman and Rosenberger 2011). Therefore, acetate could be converted into acetyl CoA by ACSS2 in the cytoplasm or directly in the nucleus and be used as a substrate for histone acetylation, resulting in increased transcription of *Il1b*. In line, acetate's metabolism could also trigger IL-1β production by another pathway. As acetate can be converted into acetyl CoA by ACSS1 inside the

mitochondria, it can fuel and even lead to an unbalanced TCA cycle, culminating in the accumulation of metabolites such as succinate. Succinate accumulation could block the degradation of HIF-1α, which increases *Il1b* transcription (Tannahill et al. 2013). Surprisingly, in our experimental setting, this was not the case, as depletion of ACSS1, ACSS2, and ACSS1/2 did not alter the production of IL-1β.

As mentioned before, NLRP3 inflammasome has been shown to mediate the effect of acetate on IL-1β production (Xu et al. 2019; Tsugawa et al. 2020; Q. Zhang et al. 2021). We observed that NLRP3 had at least a partial contribution in IL-1β production in our setting. Although we did not decipher how acetate activates NLRP3 inflammasome, we came out with some hypothesis. Based on the literature, the only described mechanism that could explain the direct effect of acetate on NLRP3 inflammasome activation was through the direct binding of acetate to ASC, as shown for butyrate and propionate (Tsugawa et al. 2020). On the other hand, indirect mechanisms such as increased glycolysis, HIF-1α activation, and fatty acid oxidation, observed by us could also explain the activation of NLRP3. Indeed, it is described that increased glycolysis activates NLRP3 inflammasome via hexokinase 1 in BMDM (J. S. Moon et al. 2015). HIF-1α was shown by several groups to activate NLRP3 inflammasome by increasing its gene expression (Huang et al. 2019; Gupta et al. 2017; Jiang et al. 2020). Although no mechanism is described, FAO is also shown to activate NLRP3 (J.-S. Moon et al. 2016; Hohensinner et al. 2021).

To fully understand the impact of acetate on macrophages, we also address the metabolic state of these cells upon acute injection of acetate and 24 h after acetate treatment, in the presence or absence of *S. pneumoniae*. Researches in the field of immunometabolism have shown that the metabolism of macrophages is dramatically affected by several pro-inflammatory stimuli. Besides, acetate can be used as a source of energy by fueling different metabolic pathways, such as TCA cycle and lipid synthesis. Interestingly, the increased oxygen consumption and decreased extracellular acidification observed by us upon acute

injection of acetate was also described in cell lines of human colon cancer and in memory OT-I T cells (Sahuri-Arisoylu et al. 2021; Balmer et al. 2016; 2020). We can infer that acetate could be entering in the TCA cycle and increase the respiration. This could lead to higher production of ATP and consequently reduced need of glycolysis to meet energy demand. Although interesting, this result does not tell us a lot about the steps involved in respiration and glycolysis. Curiously, injection of *S. pneumoniae* did not impact the respiration, nor the glycolysis of macrophages. This was unexpected, as pro-inflammatory stimuli, especially LPS, are described to immediately modify respiration and glycolysis of macrophages (M. K. S. Lee et al. 2019; Lauterbach et al. 2019). Therefore, to have the full picture of *S. pneumoniae* conditioned macrophages metabolism, we evaluated some parameters of respiration and glycolysis after 24 h of stimulation. Our results were similar to the ones described in the literature. *S. pneumoniae*, as other pro-inflammatory stimuli, increased the glycolytic activity of macrophages, and decreased the respiration (Viola et al. 2019). This phenomenon is called Warburg effect, and it happens under stress conditions or scarce oxygen levels, to allow the cell to meet energy demand via glycolysis, while respiration is limited.

In the metabolic analysis, we also observed that acetate regulates the respiration of macrophages. There are many possibilities, raised by us and others, to explain how acetate might modulate cellular respiration. The first mechanism that comes to our mind is the conversion of acetate into acetyl CoA to enter the TCA cycle, working as a fuel (Daïen et al. 2021; Rowlands, Klugmann, and Rae 2017; Mashimo et al. 2014). Another possibility is that acetate could be the substrate for the acetylation of mitochondrial proteins, resulting in higher activity and consequently enhanced respiration (Sahuri-Arisoylu et al. 2021). One more possibility was recently showed by Balmer and colleagues (2020), in which acetate directly binds to proteins and increases their activity. In this study, the authors observed that the TCA cycle and mitochondrial respiration were boosted by the higher activity of glutaminase (GSL), which furnished higher levels of α-ketoglutarate to the TCA cycle. The enhanced activity

observed in GSL was a result of the direct binding of acetate. In our case, we did not observe an increase in basal respiration, neither in acetate treated cells alone, nor in the presence of *S. pneumoniae*. Nevertheless, we did observe an increase in the maximal respiratory capacity and spare respiratory capacity in both conditions. This result indicates that in case of energy demand, the cells can boost the respiration. Generally, the source of fuel to enhance respiration comes from fatty acid oxidation, and this goes in the encounter to our observation in the enrichment analysis, which showed an upregulation of FAO pathway. The same was observed with $CD4^+$ T cells and B cells, which presented an increased maximal respiration upon acetate treatment, due to the increased pool of TCA metabolites led by acetate (Daïen et al. 2021; Kim et al. 2016). The increase in maximal respiration and in FAO pathway is intriguing, because acetate also enriched the pathway of lipid synthesis which goes in a opposite direction of FAO. Interestingly the literature shows the synthesis of lipids and fatty acids can occur at the same time as the FAO, and this was shown to happen in different contexts. Acetate was shown to be converted into acetyl CoA which fueled the TCA cycle, increasing maximal respiration and it was also used as substrate for fatty acid synthesis, increasing the lipid content in B cells (Kim et al. 2016). In addition, adipocytes submitted to cold temperatures and hepatocytes treated with vanadate also presented increased fatty acid synthesis at the same time as increased FAO (Yu et al. 2002; Guzmán and Castro 1990).

Apart from the modulation in cellular respiration, acetate also upregulated the glycolytic profile of macrophages in the presence and absence of *S. pneumoniae*. Our results are in accordance with other studies focusing on $CD8^+$, $CD4^+$ T cells and B cells, which presented higher glycolysis upon acetate exposure (Balmer et al. 2016; Kim et al. 2016). Balmer showed that acetate expanded the pool of acetyl CoA in $CD8^+$ T cells and it promoted the acetylation of GAPDH. The increased activity of this enzyme resulted in higher glycolysis and an optimal memory of $CD8^+$ T cells. Whilst Kim and co-authors showed that acetate increased the production of ATP by mitochondrial respiration in B cells. As a result, mTOR was activated,

leading to increased glycolysis and increased secretion of IgA. In accordance, we also showed that acetate-induced glycolysis affected cellular immune response. Nevertheless, we did not figure out the whole mechanism by which acetate was increasing IL-1β via glycolysis. In our setting, we observed an upregulation of most glycolytic genes by acetate, however it is still not clear how acetate increased glycolytic genes expression. Acetylation of GAPDH, as observed by Balmer and colleagues (2016), and acetylation of histones, to increase glycolytic gene expression, are dependent on the conversion of acetate to acetyl CoA. As we knocked out the enzymes responsible for this conversion (ACSS1 and ACSS2) and we did not observe any difference on IL-1β production (a result of increased glycolysis), it is unlikely that glycolysis was enhanced by acetylation of proteins. The other possibility, according to the literature, would be the activation of mTOR. However, in B cells, mTOR was activated by enhanced ATP production by the mitochondria, which reduced AMPK, an inhibitor of mTOR (Kim et al. 2016). In our setting, acetate did not modulate ATP production by mitochondria. Hence, it is likely that this mechanism does not explain the increased glycolytic activity of macrophages upon acetate treatment. Therefore, the only mechanism that we can suggest is the activation of proteins by direct binding of acetate as suggested by Balmer and colleagues (2020). These proteins could be transcription factors, enhancing the expression of glycolytic genes, or they could also be enzymes involved in glycolysis. Nevertheless, it is important to note that HIF-1α is a transcription factor of several glycolytic genes. We observed that HIF-1α activation was responsible for the enhanced expression of *Pgk1* and *Aldoa*, and it could also be responsible for the increased transcription of many others. Thus, the increased expression of glycolytic genes could be rather a positive feedback loop (acetate increases glycolysis that activates HIF-1α, which increases glycolytic gene expression leading to increased glycolysis), than a direct effect of acetate by itself.

Most publications about macrophages' immunometabolism describe the cause-consequence relation of increased glycolysis and/or impaired TCA cycle with IL-1β

production (Lu, Forbes, and Verma 2002; Tannahill et al. 2013; Gomes et al. 2021). In both metabolic changes, the transcription factor HIF-1α was the responsible to promote the transcription of *Il1b* mRNA, resulting in higher production and secretion of this cytokine. Akin to these findings, we observed that glycolysis increased by acetate leads to HIF-1α activation and increased *Il1b* transcription.

Despite the clear impact of SCFAs in cellular metabolism, studies that evaluate immune response upon SCFA treatment often neglect this pathway. Evidence showing that acetate, propionate, and butyrate can impact the metabolism of immune cells are recent. In addition, the evaluation of cellular metabolism requires rather complex or expensive techniques, such as mass spectrometry-based techniques or kits to quantify metabolites, and seahorse analysis to measure extracellular acidification or oxygen consumption. Most of the studies covering the cellular immunometabolism upon SFCAs treatment are with lymphocytes. SCFAs showed a wide range of effects in the metabolism of $CD8^+$ T cells. Acute exposure to acetate was shown to increase glycolysis resulting in improved $CD8^+$ T cell effector function with a pro-inflammatory phenotype (Balmer et al. 2016). In line, butyrate was also shown to boost the cytotoxic effector function of T cells. This effect was associated with an increased glycolysis and maximal mitochondrial respiration, due to increased FAO of butyrate and FFAR3 activation (Trompette et al. 2018). On the other hand, long exposure with acetate restricted the effector function of $CD8^+$ T cells and increased their viability, due to increased glutaminolysis and boosted mitochondrial respiration (Balmer et al. 2020). Interestingly butyrate showed a similar effect than acetate in memory CD8+ T cells. Butyrate increased glutaminolysis and fatty acid oxidation to boost mitochondrial respiration, shifting naïve CD8+ T cells to memory cells with long-term survival (Bachem et al. 2019). In the group 2 innate lymphoid cells the effect of butyrate was completely different from the one observed in CD8+ T cells. Butyrate, but not acetate, reduced GATA3, respiration and glycolytic capacity, and this was associated to a modulation of ILC2 functions resulting in reduced allergy (Lewis et al. 2019).

Remarkably, acetate, butyrate, and propionate had similar effects in B lymphocytes. These SCFAs fueled the TCA cycle, increased the glycolysis and resulted in plasma cell differentiation with higher secretion of IgA and IgG (Kim et al. 2016). On the other hand, acetate was shown to induce B1a cell differentiation into IL-10–producing B cell, while butyrate and propionate inhibited this differentiation. Acetate acted by fueling the TCA cycle and increasing protein acetylation (Daïen et al. 2021).

To the best of our knowledge, there is only one study with macrophages, which shows that butyrate reduces glycolysis without affecting mitochondrial respiration. This is likely to be induced by mTOR inhibition and increased autophagy and bacterial killing (Schulthess et al. 2019). Interestingly, the observed effect of butyrate in macrophages profile has no similarity with that of acetate observed by us, except by the increased ability to kill bacteria. Schulthess and colleagues (2019) associated the whole profile of butyrate-differentiated macrophages to the inhibition of the HDAC3, without establishing a connection among cellular metabolism and immune/effector function as we did. It is important to highlight the different impacts in cellular metabolism, even when the same cell type and SCFA is used. Many parameters should be considered such as concentration, time of exposition and stimulation.

Different from what has been published so far, our study demonstrates for the first time that acetate can impact macrophage's metabolism and thus, modulate its functional activity. Although we were not able to dissect how acetate triggers those changes, we did a broad investigation on the current known mechanisms to properly discard them. As a result, we uncovered that acetate via increased glycolysis and NLRP3 inflammasome was enhancing the production of IL-1β and nitrite to boost bacterial killing by macrophages, independently of FFAR2, ACSS1 and ACSS2.

To conclude, our data unravel a new mechanism through which acetate, a major product of the gut microbiota, could distally arm the lungs to fight against bacterial intruders. We

showed that alveolar macrophages can be the targets of acetate although other cellular candidates might also play a part in anti-bacterial defense. Whatever the mechanisms, our data confirm that acetate is an interesting metabolite endowed with immune regulatory functions. There is, at the moment, a huge interest in optimizing strategies aimed to enhance the power of the gut microbiota. In the context of respiratory infections, it is very likely that strategies based on the use of prebiotics (fiber-enriched diets) and/or probiotics (acetate producing bacteria) will be instrumental to fight against respiratory infections. Increased levels of acetate produced by the gut microbiota could arm alveolar macrophages to produce more IL-1β and more IFN-β, as shown by us and by Ji and co-authors (2021), potentiating the bacterial and viral clearance during respiratory infections. The mechanisms showed by our study highlight the vast range of acetate's mode of action and we show here that there is a long way to go to clear and fully understand how acetate modulates cellular responses. Aside, we also showed some key points to focus on when searching mechanisms for intracellular *S. pneumoniae* killing by macrophages. It is known that NO production is essential in the killing of *S. pneumoniae*, however it is extremely important to boost NO production without enhancing the pro-inflammatory response of the cells. Strategies to boost host defense while pro-inflammatory parameters are controlled can be useful for different kind of infections that drive exacerbated inflammatory response, which results in tissue damage.

Conclusion

In the present study we found that:

- Acetate supplementation to naïve mice armed the lungs to increase the secretion of host-defense proteins in the broncho-alveolar space and to reduce pulmonary bacterial loads and bacterial dissemination in mice infected with *S. pneumoniae*
- Acetate-conditioned macrophages presented increased *S. pneumoniae* killing, an effect that depended on NO production
- Acetate reduced the production of the pro-inflammatory cytokines (TNF-α, IL-12 and IL-6) and increased the production of IL-1β by alveolar macrophages
- The increased NO production triggered by acetate was mediated by IL-1β that acted in an autocrine manner to boost NO
- Acetate increased IL-1β production independently of FFAR2, ACSS1 and ACSS2
- Increased IL-1β secretion induced by acetate was mediated by NLRP3 inflammasome
- Acetate did not modulate basal respiration of macrophages, but it increased their capacity to respond to energy demand
- Acetate increased the glycolytic profile of macrophages, which was the responsible for HIF-1α activation and further transcription of *Il1b* gene.

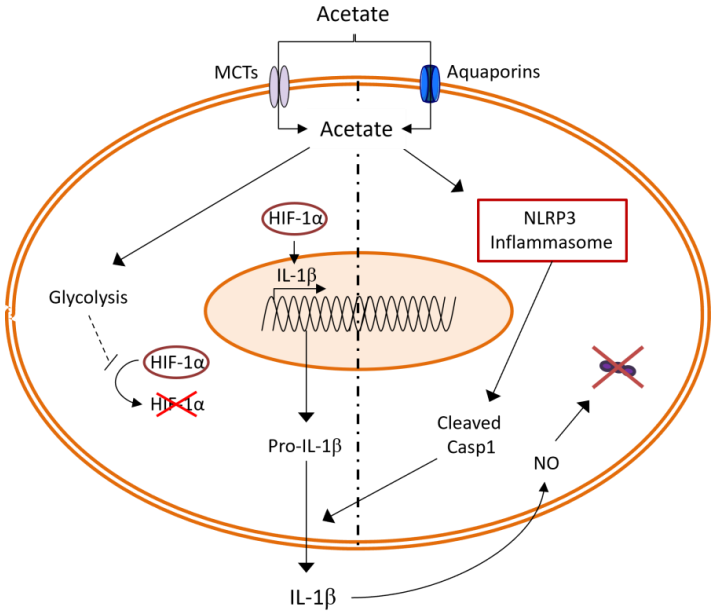


Figure 27: Summary of the effect of acetate on macrophages stimulated with *S. pneumoniae*.

Perspectives

Putting together, our main findings are in line with the literature, describing the beneficial effect of acetate in the clearance of bacteria *in vivo* and *in vitro*. The mechanism described here differs from what is published so far and brings some intriguing questions about acetate's mode of action. It is still not clear whether acetate can directly inhibit HDACs, or its product, acetyl CoA, indirectly inhibit HDAC. Based on the literature, it is more likely that acetate's effect depends on its conversion to acetyl CoA, however this is not shown. We have seen an up regulation of many genes, such as genes responsible for glycolysis, FAO, lipolysis, NLRP3 inflammasome and HIF-1α. Our objective is to investigate the ability of acetate to inhibit HDAC. For this, we plan to assess HDAC activity in wild type and *Acss1/2*$^{-/-}$ MPI cells stimulated with *S. pneumoniae* in the presence of acetate. In this experiment, we would be able to address two questions: 1-Whether acetate can inhibit HDAC activity; 2-Whether the inhibition is lost on *Acss1/2*$^{-/-}$ cells. This experiment could also give us a hint on the glycolytic profile induced by acetate. If acetate has an ability to inhibit HDAC by itself, this could explain the increased glycolytic gene expression and the increase in glycolysis induced by acetate. Although acetate was shown to directly bind to GSL, we do not have any clue on which enzyme from glycolytic pathway acetate could bind. Therefore it would be hard to assess whether acetate binds to any enzyme from the glycolytic pathway.

We also mentioned that acetate could activate NLRP3 via increased glycolysis, FAO, HIF-1α activation or direct binging. Therefore, we plan to assess NLRP3 inflammasome activation by acetate in the presence of 2-DG (glycolysis inhibitor), etomoxir (FAO inhibitor), and in HIF-1α deficient cells. We also envisage in silico docking to see if acetate could directly bind to ASC, and in order to confirm it, we plan to modify (mutate) the potential bind sites in the ASC protein (if we find any) and assess inflammasome assembly by confocal microscopy or immunopreciptation.

It is also our interest to confirm these results *in vivo*. First, we aim to administer anti-IL-1β antibody to mice infected with *S. pneumoniae* and supplemented or not with acetate, to see

whether this blockage would impair the reduction in bacterial loads upon acetate treatment. A similar approach could be applied to assess the participation of NO, glycolysis and NLRP3 activation on acetate-mediated bacterial clearance.

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Annexes

Annex I: Supplementary data

Proteins with different expression among naïve mice receiving water (Vh) versus 200 mM of acetate on drinking water for 5 days (Ace).

Gene name	**Vh 1**	**Vh 2**	**Vh 3**	**Vh 4**	**Vh 5**	**Ace 1**	**Ace 2**	**Ace 3**	**Ace 4**	**Ace 5**
C8g	6,0	2,6	3,0	4,6	0,0	10,6	11,6	8,7	14,7	11,4
Tubb3	3,7	2,7	1,8	3,6	3,8	1,5	1,3	0,5	1,1	1,2
Gclc	16,0	13,2	9,0	6,9	12,9	5,3	5,0	3,5	2,5	1,6
Lta4h	11,0	10,5	9,0	9,2	7,2	5,3	3,3	3,5	0,0	6,5
Tubb6	17,1	12,6	11,0	15,7	15,0	8,6	9,8	4,6	5,6	10,0
Pkm	97,0	86,9	93,0	71,3	83,0	70,9	52,8	64,4	54,0	68,1
Rab15	1,6	2,1	0,0	1,3	2,2	2,8	4,2	2,7	3,9	3,5
Azgp1	11,0	10,5	6,0	13,8	4,3	16,0	18,2	19,2	19,6	17,8
Aldh1a7	47,8	32,0	36,2	29,6	23,7	48,9	55,7	44,9	60,8	53,9
Ppic	1,0	1,3	0,0	4,6	0,0	3,5	8,3	8,7	9,8	4,9
Pir	4,0	1,3	3,0	0,0	0,0	8,9	11,6	7,0	12,3	3,2
Me1	9,0	6,6	3,0	4,6	4,3	1,8	1,7	0,0	2,5	0,0
Npc2	3,0	7,9	0,0	6,9	0,0	10,6	14,9	17,4	14,7	6,5
Hmgb1	3,0	2,6	0,0	2,3	0,0	5,2	4,8	7,0	4,7	11,1
Sod1	53,0	40,8	48,0	59,8	45,8	62,0	71,0	81,9	112,9	84,4
Gstm7	1,2	1,4	0,4	2,3	1,6	3,6	1,8	3,7	2,6	3,4
Akr1b7	2,3	1,3	1,8	0,5	0,0	3,7	3,8	2,5	2,0	3,3
Cct5	2,0	2,6	3,0	2,3	7,2	0,0	1,7	0,0	0,0	0,0
Rpl7a	2,0	2,6	6,0	2,3	10,0	1,8	0,0	0,0	0,0	0,0
Prdx4	1,5	1,7	3,0	3,0	0,2	3,8	5,5	2,6	5,3	5,6
Psmd9	3,0	2,6	0,0	0,0	0,0	8,9	8,3	5,2	4,9	3,2
Stard10	1,0	1,3	0,0	0,0	0,0	3,5	5,0	1,7	9,8	4,9
Rab33b	1,0	1,3	0,0	0,0	1,4	1,8	1,7	1,7	2,5	1,6
Hprt1	3,0	4,0	3,0	6,9	2,9	1,8	1,7	1,7	2,5	1,6
Tst	9,0	7,9	12,0	6,9	1,4	14,2	11,6	17,4	17,2	11,4
Hp	63,0	47,4	81,0	87,4	47,2	99,3	89,1	101,0	120,3	82,7
Cap1	31,0	22,4	30,0	13,8	15,7	10,6	9,9	7,0	4,9	11,4
Inmt	110,0	83,0	93,0	55,2	85,9	65,6	38,0	38,3	51,5	68,1
Ppp2r1a	3,3	4,4	2,0	5,4	1,4	0,6	0,5	0,6	0,0	1,1
Por	2,0	1,3	3,0	2,3	4,3	1,8	0,0	0,0	0,0	0,0
Rps10	1,0	2,6	3,0	2,3	4,3	0,0	0,0	1,7	0,0	0,0
Ube2k	0,0	1,3	0,0	0,0	0,0	3,5	1,7	1,7	2,5	1,6
Ppp2r1a	6,7	8,8	4,0	10,7	2,9	1,2	1,1	1,2	0,0	2,2
Tuba4a	28,7	21,0	18,8	18,1	29,9	16,9	13,6	15,8	6,9	17,1
Eef1a1	56,0	60,6	63,0	57,5	74,4	46,1	46,2	50,5	39,3	60,0
Rab35	1,6	2,0	0,0	2,3	2,2	2,7	2,6	2,7	3,8	4,1
Kininogen	0,4	0,3	0,4	2,2	3,9	5,2	8,4	3,6	2,4	6,8
Gm20441	1,5	0,3	1,7	1,1	0,3	1,3	3,1	1,5	2,8	3,0
Tagln2	33,0	18,4	21,0	16,1	21,5	28,4	33,0	29,6	34,4	45,4
Prep	85,0	59,3	57,0	20,7	45,8	39,0	18,2	17,4	4,9	17,8

Lasp1	9,5	6,6	12,0	2,3	0,0	16,0	12,8	12,2	17,2	9,7
Arl3	2,0	1,3	0,0	2,3	0,0	3,5	5,0	1,7	2,5	3,2
Hemopexin	5,5	5,3	4,6	5,4	6,4	7,9	12,3	10,4	7,4	7,0
Gsto1	51,0	55,3	84,0	89,7	31,5	81,5	92,4	106,2	120,3	90,8
Hpx	141,5	160,6	184,4	139,4	128,2	176,4	222,0	217,8	203,7	156,8
Cbr1	21,6	24,0	34,8	22,1	10,9	33,0	32,3	36,9	45,7	30,5
Lcp1	24,2	39,7	18,1	23,0	22,4	6,2	17,5	8,0	17,3	16,4
Qsox1	33,0	39,5	30,0	27,6	20,0	17,7	29,7	8,7	9,8	16,2
Cmpk1	15,0	10,5	6,0	11,5	8,6	16,0	16,5	15,7	19,6	11,4
Pls1	6,4	4,4	3,2	0,1	4,6	0,2	0,0	0,2	0,0	0,3
Ube2v1	3,0	1,3	0,0	0,0	2,8	4,1	3,2	4,0	12,1	7,5
Psmd6	0,0	1,3	3,0	2,3	2,9	1,8	0,0	0,0	0,0	0,0
Capg	1,0	0,0	3,0	2,3	2,9	5,3	8,3	5,2	14,7	3,2
Cct4	7,0	4,0	3,0	4,6	12,9	0,0	3,3	1,7	0,0	1,6
Eif5a	8,9	9,1	11,8	9,0	5,6	5,2	8,2	3,4	4,8	6,3
Igh	0,5	0,0	0,0	0,0	0,0	2,2	0,4	0,4	3,5	0,8
Ube2n	10,0	5,3	3,0	0,0	5,7	7,1	6,6	13,9	14,7	13,0
Ttr	44,0	39,5	36,0	41,4	21,5	33,7	57,8	59,2	66,3	53,5
Ppia	55,0	38,2	39,0	59,8	37,2	46,1	57,7	71,4	83,4	77,9
Tf	543,0	485,1	658,9	653,8	625,8	822,6	775,6	696,7	815,8	581,3
Klk1b22	14,6	37,1	3,0	4,6	17,7	5,3	1,7	0,0	0,0	0,0
Klk1b5	2,0	10,9	2,9	0,7	2,2	1,7	1,1	0,0	0,0	0,0
Sftpa1	17,0	7,9	12,0	18,4	10,0	28,4	29,7	27,9	29,5	14,6
Sftpd	21,0	27,7	39,0	39,1	34,3	44,3	56,1	41,8	68,7	35,7
AP02072	1,0	6,6	0,0	11,5	27,2	37,2	24,8	50,5	7,4	34,1
Alpha-globin	0,6	0,3	0,2	0,4	0,3	0,3	0,5	0,9	0,7	0,7
Serpinb3a	0,0	1,3	0,0	2,3	0,0	1,8	1,7	3,5	2,5	3,2
Csrp1	9,0	11,9	6,0	2,3	1,4	16,0	11,6	8,7	9,8	13,0
Vcl	29,0	36,9	0,0	32,2	25,8	12,4	11,6	5,2	0,0	11,4
Ranbp1	5,0	6,6	3,0	0,0	1,4	5,3	5,0	10,4	7,4	6,5
Fga	6,0	5,3	0,0	2,3	0,0	7,1	18,2	10,4	19,6	3,2
Apoh	13,0	26,3	15,0	20,7	2,9	17,7	36,3	26,1	29,5	27,6
Lcn2	3,0	0,0	3,0	4,6	1,4	3,5	3,3	8,7	7,4	4,9
Tns1	10,0	7,9	6,0	0,0	4,3	1,8	0,0	0,0	0,0	0,0
Klk1b27	8,8	20,5	3,0	0,0	18,9	2,4	0,0	0,0	0,0	0,0
Coro1b	6,0	6,6	3,0	0,0	2,9	0,0	0,0	0,0	0,0	1,6
Bpifb9b	4,1	8,9	2,0	0,0	3,9	0,0	0,0	0,0	0,0	1,1
Gpd1	3,2	1,3	6,0	0,6	0,0	0,0	0,4	0,0	0,0	0,0
Fasn	2,0	32,9	0,0	4,6	11,4	0,0	0,0	0,0	0,0	1,6
Vomeromodulin	2,0	4,3	1,0	0,0	1,9	0,0	0,0	0,0	0,0	0,5
Chmp4b	1,0	0,0	0,0	0,0	0,0	1,8	0,0	1,7	2,5	3,2
Rhof	1,0	0,0	0,0	0,0	0,0	1,8	1,7	1,7	0,0	1,6
Fabp5	6,0	2,6	0,0	0,0	8,6	7,1	5,0	13,9	9,8	8,1
Ces1b	7,9	5,0	7,0	2,8	2,2	2,8	1,1	2,7	1,5	1,1

Annex II: Article published at Infection and Immunity

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MINIREVIEW

Short-Chain Fatty Acids as a Potential Treatment for Infections: a Closer Look at the Lungs

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ABSTRACT Short-chain fatty acids (SCFAs) are the main metabolites produced by the gut microbiota via the fermentation of complex carbohydrates and fibers. Evidence suggests that SCFAs play a role in the control of infections through direct action both on microorganisms and on host signaling. This review summarizes the main microbicidal effects of SCFAs and discusses studies highlighting the effect of SCFAs in the virulence and viability of microorganisms. We also describe the diverse and complex modes of action of the SCFAs on the immune system in the face of infections with a specific focus on bacterial and viral respiratory infections. A growing body of evidence suggests that SCFAs protect against lung infections. Finally, we present potential strategies that may be leveraged to exploit the biological properties of SCFAs for increasing effectiveness and optimizing patient benefits.

KEYWORDS bacteria, gut, respiratory infection, short-chain fatty acid, virus

Short-chain fatty acids (SCFAs), namely, acetate (C_2), propionate (C_3), and butyrate (C_4), are the main metabolites produced by the gut microbiota (in a 60:20:20 proportion, respectively). These compounds are derived from the anaerobic fermentation of nondigestible polysaccharides, such as resistant starches and dietary fibers. The SCFA concentration in the intestine can be as high as 10 to 100 mM, and the SCFAs exert many physiological functions (1–4). For example, butyrate and (to a lesser extent) the other two SCFAs are major energy sources for colonocytes and act as key factors in intestinal epithelial cell growth and function. SCFAs regulate inflammatory responses, and SCFA supplementation can reduce the severity of intestinal disorders such as colitis (5, 6). It is important to note that the effects of microbiota-derived SCFAs are not limited to the intestinal compartment. In fact, SCFAs can cross into the blood and act at distal sites, such as the lungs (7). Many studies have reported that SCFAs protect against infections. Various modes of action have been reported in this context, ranging from a direct effect on the growth and/or virulence of microorganisms to an indirect effect on the host immune system.

Infections. Despite the advent and widespread use of vaccines, antibiotics, antiviral drugs, and antifungal drugs, microbial infections still constitute a major public health issue, exemplified by the recent coronavirus disease 19 (COVID-19). In 2019, a report from World Health Organization stated that 26% of deaths worldwide are caused by communicable diseases. With 3 million deaths per year, lower respiratory tract infections constitute the fourth leading cause of death overall and the deadliest communicable disease. Nowadays, there is an increasing number of infections caused by multiresistant microorganisms that can mutate and spread easily. Given the impact of infections on mortality and morbidity rates worldwide, it is extremely important to study these diseases. As mentioned above, we lack effective means of preventing and treating many infections. The rise in antibiotic resistance, the large number of bacterial serotypes, and viral shift and drift are major concerns for physicians and researchers.

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Annex III: Article published at Frontiers in Immunology

frontiers in Immunology

ORIGINAL RESEARCH
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Acetate Improves the Killing of *Streptococcus pneumoniae* by Alveolar Macrophages *via* NLRP3 Inflammasome and Glycolysis-HIF-1α Axis

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Short-chain fatty acids (SCFAs) are metabolites produced mainly by the gut microbiota with a known role in immune regulation. Acetate, the major SCFA, is described to disseminate to distal organs such as lungs where it can arm sentinel cells, including alveolar macrophages, to fight against bacterial intruders. In the current study, we explored mechanisms through which acetate boosts macrophages to enhance their bactericidal activity. RNA sequencing analyses show that acetate triggers a transcriptomic program in macrophages evoking changes in metabolic process and immune effector outputs, including nitric oxide (NO) production. In addition, acetate enhances the killing activity of macrophages towards *Streptococcus pneumoniae* in an NO-dependent manner. Mechanistically, acetate improves IL-1β production by bacteria-conditioned macrophages and the latter acts in an autocrine manner to promote NO production. Strikingly, acetate-triggered IL-1β production was neither dependent of its cell surface receptor free-fatty acid receptor 2, nor of the enzymes responsible for its metabolism, namely acetyl-CoA synthetases 1 and 2. We found that IL-1β production by acetate relies on NLRP3 inflammasome and activation of HIF-1α, the latter being triggered by enhanced glycolysis. In conclusion, we unravel a new mechanism through which acetate reinforces the bactericidal activity of alveolar macrophages.

Keywords: alveolar macrophages, short chain fatty acid, immunometabolism, *Streptococcus pneumoniae*, innate immunity, nitric oxide, IL-1β

Annex IV: Articles published as collaborator

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