Innate Immune Modulation by Fluoroquinolones:

The Influence of Gastrointestinal Hormones

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This thesis is submitted for the degree of Doctor of Philosophy

Biomedical and Life Sciences

Lancaster University

June 2022

DSTL/PUB142007

[dstl]

Biomedical & Life Sciences | Lancaster University

Abstract

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As well as targeting bacteria directly, antibiotics have various harmful effects on host cells, often attributed to loss of commensal bacteria. Fluoroquinolone antibiotics are particularly well known for this, and have been attributed to symptoms such as tendon rupture and nerve damage. Whilst many clinical effects of fluoroquinolones are well documented, less so are the direct effects they have on immune cells and their subsequent responses. Antibiotics are known to affect weight and feeding, processes known to be modulated by intestinal hormones such as Cholecystokinin.

Fluoroquinolones, typically Ciprofloxacin and Levofloxacin are used as treatments for severe acute respiratory infections, such as inhalation Anthrax and Pneumonia. We therefore focussed on the effects they can have on macrophages, key cells in lung disease. We treated bone marrow-derived macrophages with Levofloxacin and Ciprofloxacin, and studied key parameters such as activation and polarisation via flow cytometry. Moreover, we treated both wild-type and CCK knockout mice with human equivalent dose regimes of these antibiotics to assess alteration of various innate immune cell subsets in the uninfected state. We also extracted bacterial DNA from the faeces of treated mice to investigate any dysbiosis that occurs.

Fluoroquinolones were found to affect Macrophage polarisation both *in vitro* and *in vivo*, causing a ‘boost’ in M1 polarisation following BMDM IFNγ stimulation, while following 14 day treatment in vivo we also saw an increase in M1 polarisation in the lung. Interestingly we found profound CCK mediated weight loss in both Ciprofloxacin and Levofloxacin treated mice versus controls, as well as dysbiosis after only 24hrs of treatment. The role of CCK in this mechanism gives rise to the possibility of introducing CCK receptor antagonist co-treatment with fluoroquinolone treatment in order to mitigate and reduce harmful side effects.

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Acknowledgements

It is no overstatement to say that this thesis would not have been possible without support of many kinds from many different people.

I would like to first thank my supervisor, Dr John Worthington. I couldn't have asked for a better supervisor. Your guidance and mentorship has inspired and challenged me, whilst your sense of humour and personality has made it an absolute pleasure to be your student. I will always appreciate the faith you showed in me.

I would like to thank my secondary supervisors for their invaluable input to my project. Thank you Dr Rachael Rigby and Dr Alexandre Benedetto for offering advice, time, equipment and a different viewpoint when I needed it. I'd like to express my gratitude to Dr Riccardo D'Elia for introducing me to the fascinating and vital world of DSTL. It was you and your colleagues at DSTL that brought my work into the real world, and out of the bubble of academia. I hope this thesis will be a contribution, however small, to the critical work you do.

Lab work would have been far less enjoyable without the wonderful members of the Worthington lab past and present. Adura, thank you for advice and calming presence when I needed it. Megan, thank you for your technical and emotional support, though I'm sure you'd agree it was a two-way deal!

Thank you Jayde, without you I would have never grasped many of the more technical and complex aspects of my project. The breadth of knowledge you have, and the time you dedicate to helping others will never cease to amaze me.

Thank you to the lab's brilliant animal technicians past and present, Eva, Jan, David and Christine. Your support of all kinds throughout my project is truly appreciated.

I would also like to thank my current supervisor, Dr Lucy Jackson-Jones. Being in your lab has improved the way I think about immunology, and has helped make this thesis what it is.

I struggle to put into words how grateful I am to my family. Mum, Dad, Euan, there is no way I'd be where I am today without you. Mum, you made me a scientist. I have been, and will always be inspired by you.

I'm so grateful to have had such a wonderful and supportive group of friends around me throughout my PhD. Thank you to James and Nadin, you two have been so valuable to me and have kept me sane against all odds. Thank you to Sheila and Caroline, you made me realise I could do this! Thank you to you guys in C18 for giving me the comic relief when I needed it, and for your support in many forms.

Author's Declaration

I declare that the work contained within this thesis is my own unless stated otherwise, and that it has not been submitted for any degree previously.

Alex Hardgrave

Experimental Contributions

Some data collection was performed by undergraduates/interns carrying out projects under my supervision, as follows:

Brandon Johnson collected the data used for figures 4.28 and 4.31

Ivy Maminimini collected the data for and contributed to the generation of figures 5.12, 5.13 and 5.14

Catherine Jackson collected the data for and contributed to the generation of figure 5.25, and generated figures 5.24 and 5.26

Chapter 1: Introduction

Infectious diseases have, and continue to greatly contribute to global mortality, particularly in the developing world. According to World Health Organisation data, infectious/parasitic diseases and respiratory infections combined caused over 8 million deaths in 2016. Whilst this is a considerable decrease from the over 11 million deaths they caused in 2000, they still represent almost 15% of all global deaths (WHO, 2018). Infections of the respiratory tract form such a large portion of infectious disease mortality that they are given a separate classification by the WHO. In addition, whilst other infectious diseases are predicted to decrease from 11.2% of global deaths (2015) to 8.3% by 2030, respiratory infections are only predicted to decrease by 0.5%, and projections indicate they will be the 4th greatest cause of death by 2030 (Mathers and Loncar, 2006).

1.1 Acute Infections of the Respiratory Tract

Unsurprisingly, one of the most common types of infection is that of the respiratory tract. This is due to the fact that people inhale approximately 500 million litres of air in their lifetime, a considerable portion of which contains aerosolised bacteria, or microbe contaminated dust (Madigan et al., 2015). Also, the ease of person-person transmission is a key factor aiding the prevalence of respiratory infections. An intense sneeze ejects between 10^4 and 10^6 bacteria from the mouth at approximately 100 metres/second in small droplets. These droplets quickly evaporate in the air, leaving mucus in which aerosolised bacteria and viruses can easily become trapped (Madigan et al., 2015).

The respiratory tract is divided into upper and lower sections, and infections are categorised by the section in which they inhabit. The upper respiratory tract consists largely of the nasal and oral cavities, as far down as the pharynx and larynx. The lower respiratory tract starts at the trachea, and continues down through the bronchi to the alveoli.

Infections of the respiratory tract tend to very in their typical severity based on whether they are located in the upper or lower section. On the whole, infections of the upper section tend to be more mild and acute, for example the common

cold. In contrast, infections in the lower respiratory tract are generally more chronic and more severe, even fatal. The most well-known example of this type of infection is *Streptococcus pneumoniae* pneumonia, with others including influenza, and *Staphylococcus aureus* infection (Madigan et al., 2015).

This dichotomy of severity between infections of the upper and lower respiratory tract is further indicated by global death statistics. Lower respiratory tract infections alone are the number one cause of death in lower income countries, ahead of diarrhoeal diseases and ischaemic heart disease. In 2016 almost 3 million deaths were caused by lower respiratory tract infections, whereas upper respiratory tract infections caused only 6000 deaths (WHO, 2018).

The main symptom of a lower respiratory tract infection is pneumonia; so much so that the terms are used almost synonymously. More precisely, Pneumonia is defined as 'an acute lower respiratory tract infection, together with new radiographic shadowing' (British Thoracic Society Standards of Care, 2001). Symptoms of pneumonia include fever (>38°C), decreased chest expansion when breathing, and an increased respiratory rate (Hoare and Lim, 2006). Whilst the two most common causes of pneumonia are viral (Rhinovirus and Influenza), the most common bacterial cause is *Streptococcus pneumoniae*. There are many bacteria known to cause pneumonia, both gram positive and negative, ranging from common species such as *S. aureus* to more rare and severe pathogens such as *Bacillus anthracis* (Wunderink and Waterer, 2017, Penn and Klotz, 1997) and *Burkholderia pseudomallei*.

Burkholderia pseudomallei is an environmental aerobic gram-negative bacillus that is highly prevalent within Northern Australia and Southeast Asia. It is the causative agent of Melioidosis, a serious disease resulting in 40% mortality even when treated. This is comparable to the most severe form of anthrax, inhalation, despite being less well-known (CDC, 2020). It is characterised by abscesses and pneumonia, and is leading cause of sepsis in endemic countries (Wiersinga et al., 2012). *B. pseudomallei* has recently gained increased attention in the western world due to its potential for use as a bioterrorism agent. Determined by factors such as mortality/morbidity rates and ease of dissemination, *B.*

pseudomallei is a category B bioterrorism agent, the second highest priority defined by the Center for Disease Control (CDC, 2017).

1.2 The Immune Response to Lung Infection

It stands to reason that given the vast number of pathogenic microbes that enter the lungs through the respiratory tract, the immune system must provide a robust response. If no such system were in place, potentially lethal infections could take hold almost daily. As with the rest of the body, lung infections are met by distinct innate and acquired adaptive responses.

The first barriers potential invaders encounter are literally that: physical barriers. Before bacteria even reach the epithelial wall, they encounter a layer of mucus, and a process called mucociliary clearance. Mucus is a jelly-like semisolid substance, consisting of 97% water. The other 3% comprises of mucins, other proteins, salts, lipids and debris sloughed from the epithelium (Fahy and Dickey, 2010). Mucins are long complex glycoprotein molecules characterised by the presence of a large 'mucin domain' rich in serine and threonine, and are secreted by multiple cell types, such as goblet cells and mucous cells (Thornton et al., 2008). They can be either cell-tethered or secreted, depending on which of the 17 MUC genes encodes them, 10 of which code for cell-tethered and 7 for secreted mucins (Hiemstra et al., 2015). In the human respiratory tract, the mucus layer is a mixture of MUC5AC and MUC5B in similar quantities, and a small amount of MUC2 mucins (Thornton et al., 2008). Whilst MUC2 is uncommon in lung mucus, it is a major protective component of gut mucus, with MUC2-deficient mice spontaneously developing colon cancer (Velcich et al., 2002).

Rather than just acting as a stationary barrier preventing bacterial entry into the epithelium, mucus actively moves bacteria and debris out of the lungs by a process known as mucociliary clearance. This process occurs by epithelial cilia (flagellum-like outwardly extending protuberances) actively beating upwards in a 'power stroke', pushing the bottom layer of mucus upwards out of the lungs at the rate of approximately 1mm/min. It has been shown that the rate of ciliary

beating increases in response to several factors including increased hydration (Knowles and Boucher, 2002). Other stimuli include agonists of adenosine, cholinergic and adrenergic receptors (Salathe, 2007), and various chemical irritants (Shah et al., 2009). The importance of this ciliary movement is illustrated by the harmful phenotypes of disorders that affect it. This disorder is known as primary ciliary dyskinesia (PCD), and is caused by several, mainly autosomal recessively inherited genetic abnormalities. PCD is most often diagnosed in newborns, but can be presented in adulthood, with patients typically suffering chronic respiratory symptoms. In addition, sputum samples of PCD patients are often found to contain bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Haemophilus influenza* (Knowles et al., 2013, Popatia et al., 2014).

As well as by the delicate movements of cilia, mucus is also transported upwards by the process of coughing. It is not always positive however, as excessive coughing can be an indication of an underlying disorder (Rubin, 2010).

With the exception of physical barriers such as the mucus layer, innate immune responses require the host to recognise the fact that they are in the presence of a pathogen. As a result, the immune system can detect various features of an invading pathogen, generally relying on their unfamiliarity/ presence of foreign matter. These various features include LPS (Lipopolysaccharides/endotoxins), bacterial-specific carbohydrates, or chunks of bacterial DNA (Boyton and Openshaw, 2002). These various recognisable features are termed pathogen-associated molecular patterns (PAMPs), and are characterised by being evolutionarily conserved, present on whole pathogen classes (and therefore assumed essential for their survival), and identifiable as non-self by the host (Mogensen, 2009).

Cells of the innate immune system recognise PAMPs using receptors known as pattern recognition receptors (PRRs), of which four types have been discovered: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and NOD-like receptors (NLRs) (Takeuchi and Akira, 2010). Of the four, the TLR family is the best

characterised. Ten human and thirteen mouse TLRs have been described, all of which binding to a different ligand (i.e. PAMP). Some of these ligands include flagellin from bacteria (TLR5) (Hayashi et al., 2001) and viral dsRNA (TLR3) (Alexopoulou et al., 2001).When a TLR recognises a PAMP it upregulates specific genes, activating a signalling cascade. This signalling cascade can produce different effector molecules depending on the specific TLR (fig 1.1). For example, MyD88-dependant signalling pathways free NF-κB to move into the cell's nucleus and activate proinflammatory cytokine expression. Alternatively, this can occur through the activation of the MAP kinase cascade. An example of this kind of pathway occurs upon activation of TLR1 (Takeuchi and Akira, 2010). TLR3 activates a different signalling pathway, dependant on TRIF. This type of pathway leads to the expression of type 1 IFNs via IRF3 translocation into the nucleus (Kawasaki and Kawai, 2014).

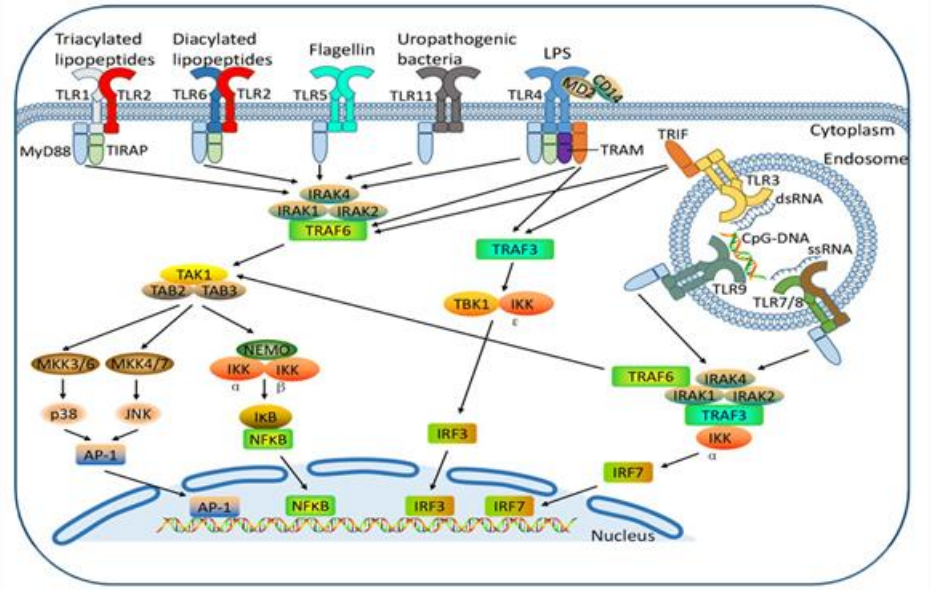


Figure 1.1: Signalling cascades of membrane and endosome-bound TLRs.
Taken from Du et al. (2016)

In response to PAMPs and subsequent intracellular TLR signalling cascades, a variety of effector molecules are released. Many of these molecules are chemokines such as GM-CSF, which allow the epithelial cells to initiate and modulate a response by inducing the formation of innate immune cells (Eddens and Kolls, 2012). Other downstream effects of TLR signalling include dendritic cell stimulation by TLR2, 4, 7 and 8 agonists, and their subsequent release of IL-23 (Roses et al., 2008). IL-23 goes on to activate γ/δ T cells as well as invariant natural killer T (iNKT) cells, both of which produce IL-17, a proinflammatory cytokine (Chen et al., 2011, Lockhart et al., 2006).

1.3 Immune Cells in the Lung

The release of these proinflammatory cytokines by epithelial barrier cells leads to a primary innate immune response, of which a critical component are macrophages. Macrophages are present throughout the human body, and are the most abundant type of immune cell in the lung at homeostasis, i.e. not during an infection (Murray and Wynn, 2011). They are an incredibly diverse cell type, and perform many functions, ranging from phagocytosing ('eating') pathogens, to clearing cell debris. They were discovered by Russian zoologist Ilya Metchnikoff at the end of the 19th century, and their name literally means 'big eaters' (Tauber, 2003, Byrne et al., 2015). Macrophages are dispersed throughout the body, composing phenotypically distinct subtypes highly specialised for the environment in which they are located. These tissue macrophages are embryonically derived, developing distinctly depending on their niche (Yona et al., 2013). They are supported, during inflammation especially, by circulating monocytes (essentially macrophage precursors) that can be recruited to specific sites and mature to form monocyte-derived macrophages (Epelman et al., 2014).

The majority of macrophages in the lung form one of two distinct phenotypes, alveolar macrophages (AM) and interstitial macrophages (IM). Though both the same type of cell, in broadly the same location, they have distinct functions.

As the name suggests, AMs are located within the alveoli, in the airway itself. AMs exist in a complex, dynamic environment that is different from that of most cells of the innate immune system, and as a result are not as easily classified as other macrophages (Hussell and Bell, 2014). They are unlike most macrophages in the body, in that they are not easily defined by their characteristics. This is because of the uniquely dynamic environment in which they reside; they must be equally dynamic in function and phenotype in order to maintain homeostasis, and fight infection (Hussell and Bell, 2014). During homeostasis, AMs have a vital immunoregulatory role. The lung environment is exposed to many potentially inflammatory stimuli, such as debris and non-harmful antigens from commensal bacteria. Suppression and regulation of this inflammation is achieved via tissue-wide mechanisms, which in turn affect how AMs function distinctly from other macrophage populations. One such mechanism is mediated by type 2 alveolar cells, which express CD200 on their surface. This binds with CD200R on AMs, suppressing inflammatory activity (Snelgrove et al., 2008). AM activity is also suppressed by alveolar epithelial cells expressing the integrin αvβ6, which tethers transforming growth factor β (TGF β) (Munger et al., 1999). AMs themselves also play a role in regulating inflammation in the lung. They have been shown to induce CD4+ T cells to forkhead box P3 (FOXP3)+ Regulatory T cells (Tregs), via secretion of TGFβ and retinoic acid (Coleman et al., 2013) (fig 1.2).

IMs are less well studied than AMs, located within the lung tissue itself , and are often thought of as the ‘second line of defence’ in the lung compared to the AMs’ ‘first line of defence’ (Schyns et al., 2018). Whilst not as inherently suppressive as AMs due to their more ‘sheltered’ environment, they still carry out vital immunoregulatory functions, typically via more specialised and efficient activities, such as the release of anti-inflammatory cytokines (Franke-Ullmann et al., 1996). They also have a role in allergy prevention, inhibiting Th2 responses to environmental antigen via the release of IL-10. This prevents DC maturation and migration, normally induced via airborne LPS, and subsequent asthmatic pathology (Bedoret et al., 2009). Interestingly, IMs have been shown to contribute to immune tolerance by generating FOXP3+ Tregs like their alveolar counterparts. As with AMs this was shown to be induced by the

release of TGF-β and retinoic acid, though this process was inhibited by the activation of IMs by inhaled allergens (Soroosh et al., 2013).

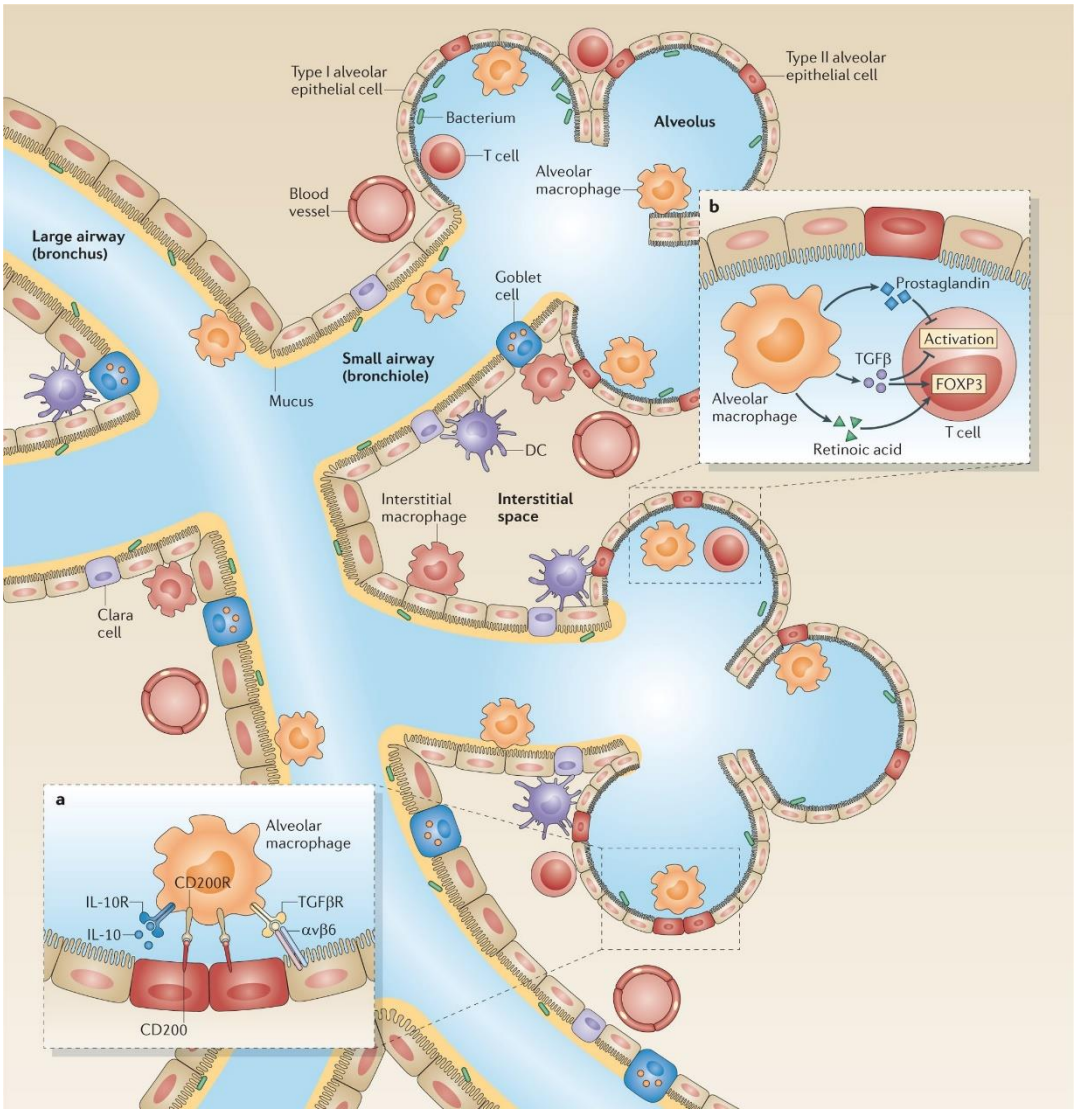


Figure 1.2: Overview of immune responses in the alveoli of the healthy lung*. A) Regulation of AMs by IL-10 and TGFβ, via CD200r. B) Modulation of Treg functionality by AMs via retinoic acid mediated stimulation, and suppression by TGFβ and prostaglandin. Taken from Hussell and Bell (2014)*

Until recently, it was thought that lung macrophages were recruited from the blood, in the form of circulatory monocyte precursors. This has, however, been shown to be not the case. Alveolar macrophages are in fact tissue-resident macrophages, and their populations are not supplemented by blood-derived monocytes. Rather, they are derived from their own embryonic progenitor cells in the yolk sac that are 'implanted during embryonic development (Schulz et al., 2012, Gomez Perdiguero et al., 2015), and proliferate locally in a self-sufficient

manner at steady state (Jenkins et al., 2011, Yona et al., 2013). This is particularly pertinent in inflammation-sensitive, Th2-biased environments such as the lung, as it means the tissue-specific characteristics of the resident macrophages are retained, rather than risking the potentially damaging inflammatory effects of non-tissue specific monocyte-derived macrophages. Hashimoto et al. (2013) showed that even in the event of challenge, these macrophage populations rarely rely on 'outside support' from circulatory cells. Even after depletion of tissue-resident macrophages, recovery can occur independent of circulatory monocytes or haematopoietic progenitors. Only genotoxic insult caused tissue-resident macrophages to rely on circulating donor cells for repopulation, after which, the ability to self-maintain was retained. Interestingly, alveolar macrophage function is not established in the womb. In fact, their phenotype is established in response to cytokines-in particular GM-CSF, a few days postnatally (Guilliams et al., 2013).

It has been thought that interstitial macrophages were also tissue derived, and long lived. In fact, they are shorter lived than AMs, with less capacity to self-renew after depletion (Evren et al., 2020). This is thought to be due to the fact that circulating monocytes can be recruited to the lung and rapidly differentiated into interstitial macrophages via M-CSF (Chakarov et al., 2019), something which AMs, and other tissue resident macrophages cannot do.

Another dynamic environment in the body in which resident macrophages play an important homeostatic immune role is the intestine (Bain and Mowat, 2014). However, in contrast with the long-lived tissue-resident paradigm, it was shown that intestinal macrophages rely on replenishment by monocytes from the blood despite being derived from embryonic precursor cells like tissue resident macrophages in other tissues (Bain et al., 2014). More recently, it has shown to be more complex than initially thought. In fact there are multiple transcriptionally distinct subsets of resident macrophages in the gut, which can be identified based on Tim-4 and CD4 expression. Shaw et al. (2018) found that only Tim-4^{-}CD4^{-} macrophages displayed the high monocyte replenishment as shown by Bain et al, whereas Tim-4^{+}CD4^{+} macrophages were self-renewing and maintained locally. This shows that in multiple dynamic tissues of the body, resident embryonically derived macrophages are not homogenous populations.

Rather they are often divided into multiple populations, each falling somewhere different on the spectrum of self-reliance.

As well as their function and location within the lung, AMs and IMs differ with regards to their phenotype. It is widely known that mononuclear phagocytes are phenotypically heterogeneous dependant on location (Geissmann et al., 2010), and lung macrophages are no exception. This allows for AMs and IMs to be distinguished from both non-lung macrophages and one another via the expression levels of various cell surface markers. Universal macrophage markers can be used to identify AMs and IMs (though not specifically) to varying degrees of success. CD64 is expressed in AMs and IMs, as is F4/80, albeit at a lower level (Austyn and Gordon, 1981, Misharin et al., 2013b). IMs can be distinguished from AMs via the expression of CD11b, which AMs lack. AMs express high levels of CD11c, though research is unclear as to whether IMs express CD11c, and to the same level (Misharin et al., 2013b, Guth et al., 2009, Zaynagetdinov et al., 2013). Other more specific markers highly expressed in AMs include the M2 marker CD206, as well as CD200r (Misharin et al., 2013b, Snelgrove et al., 2008). AMs also express Sialic acid-binding immunoglobulin-like lectin F (Siglec-F), a classical eosinophil marker (Feng and Mao, 2012).

In response to stimulus, such as those occurring in infection, macrophages have the ability to polarise towards either an M1 (classical) or M2 (alternative) phenotype (fig. 1.3). It is largely this ability that has contributed to the diversity of macrophages' functions throughout the body. This concept of M1/M2 was coined by Mills et al. (2000), mirroring the Th1/ Th2 paradigm, whereby different stimuli cause helper T cells to become activated differently in order to more effectively fight different pathogens. M1 and M2 polarisation is called as such because of the very different functions of the two types. M1 classically activated macrophages, as the name suggests, carry out functions 'classically' associated with macrophages- killing and engulfing pathogens, as well as infected and cancerous cells. In contrast, M2 alternatively activated macrophages carry out a variety of other functions less typically associated with macrophages, such as tissue repair and immunoregulation (Ley, 2017, Byrne et al., 2015). Although it was found to be an oversimplification, at the time of

discovery, M1 and M2 macrophages were essentially defined as pro-inflammatory and anti-inflammatory, respectively. It was found that M1 and M2 macrophages could be distinguished by the way in which the metabolise arginine (Mills et al., 1992). M2 macrophages were found to preferentially use the arginase pathway to metabolise arginine into orthinine, another amino acid with several uses related to cell repair (Mills, 2001). In contrast, M1 macrophages preferentially produced Nitric Oxide (NO), via the induction of inducible nitric oxide synthase (iNOS/NOS2)(Nathan, 2006). NO, along with its subsequent metabolites, is cytotoxic and vital to carry out the killing mechanisms associated with macrophage function (Hibbs Jr et al., 1988).

Classically activated (M1) macrophages carry out their phagocytic proinflammatory effector role as part of cell-mediated immune responses. Their activation is mediated via cytokines, secreted by other immune cells, as well as pathogen-associated stimuli. The main cytokine responsible for M1 macrophage stimulation is IFNγ, one of the main sources of which are NK cells. NK cells act early, responding to infection or other stresses by releasing proinflammatory IFNγ to prime macrophages as well as other cells. This is a short-lasting effect however, and so must be supported by adaptive the immune system to ensure enough longevity to maintain active macrophages throughout an infection. This is largely carried out by Th1 cells (Mosser and Edwards, 2008). Whilst M1 macrophages can be activated by IFNγ solely, it is typically

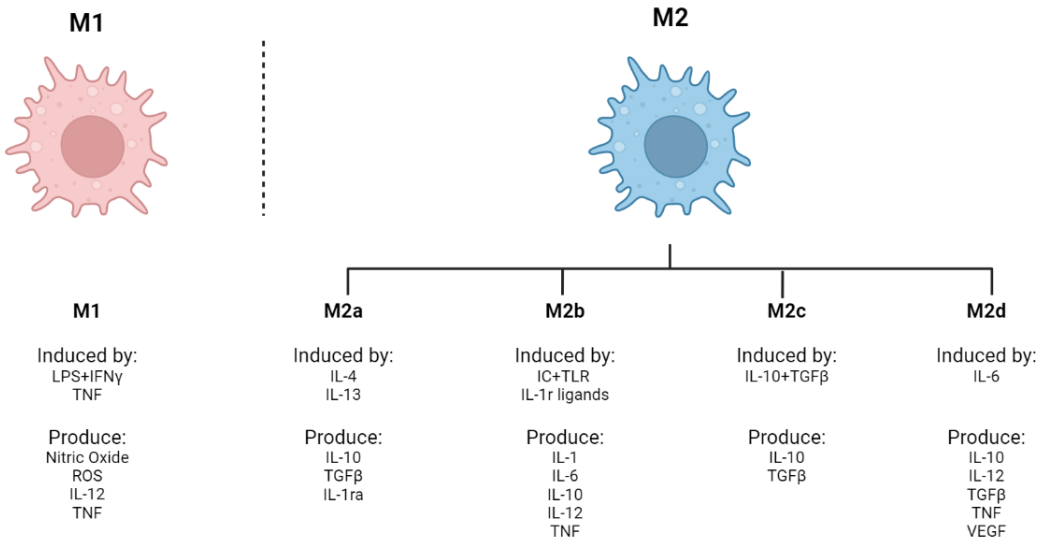


Figure 1.3: Summary of M1 and M2 Macrophage phenotypes*; their induction, their function, and the cytokines they produce.*

accompanied by a co-stimulus. This is often termed ‘priming’, with IFNγ priming macrophages for activation by other molecules. One such molecule is Lipopolysaccharide (LPS), a component of bacterial cell membranes that acts a pathogen-associated molecular pattern (PAMP). This is recognised by pattern recognition receptors (PRRs) on macrophages, specifically Toll-like receptor 4 (TLR4) (Kawasaki and Kawai, 2014). Another such molecule is tumour necrosis factor alpha (TNFα). TNFα activates macrophages already primed by IFNγ, and has been shown to further increase activation compared to IFNγ alone (Parameswaran and Patial, 2010).

Whilst M1 macrophages are fairly unified, carrying out a common function in response to relatively common stimuli, M2 macrophages are somewhat more complex. Simply defined as ‘anti-inflammatory’ initially, M2 macrophages carry out several diverse functions, often counteracting the proinflammatory effects of their classically activated counterparts. Until relatively recently, M2 activation was thought to occur via the cytokines IL-4 and IL-13. These can be released by multiple cell types, such as Th2 cells and B cells, epithelial and tumour cells as well as other macrophages (Gordon and Martinez, 2010). Recently, the paradigm and accompanying nomenclature changed however. It was demonstrated that the phenotype and function of M2 Macrophages varies to such an extent that they have been further subdivided into M2a, M2b, M2c and M2d (Martinez and Gordon, 2014, Murray et al., 2014). The Il-4 and IL-13 – activated phenotype previously encompassing all M2 macrophages now being defined as M2a.

M2a macrophages, alternately named M(IL-4) are active in response to helminth and fungal infections via IL-4 and IL-13 as previously discussed. They can be distinguished from M1 and other M2 subtype macrophages by the expression of several cell surface markers. These include arginase-1 (Arg1), CD206 (macrophage mannose receptor), Ym1 and MHC-II amongst others. CD206 in particular performs multiple roles. As the name suggests, it has an affinity towards glycoproteins high in mannose, acting as a scavenger receptor for these proteins in homeostasis (Azad et al., 2014). In infection, CD206 can act as a PRR, due to the fact that mannose-containing structures are commonly found on the surface of pathogenic microbes (Medzhitov, 2007). The main

function of M2 macrophages is to promote tissue repair and growth, and as such have often been known as wound healing macrophages (Rőszer, 2015, Yao et al., 2019).

M2b macrophages are activated by the presence of LPS, as well as immune complexes (ICs) (antigen-bound antibodies), which gives rise to their alternative name, M(Ic)s. They have also been referred to as regulatory macrophages, as they perform a wide range of immunoregulatory functions primarily via secretion of IL-10, as well as IL-12 (Wang et al., 2019). This definition has been discouraged however due to the regulatory properties of other macrophage subsets (Murray et al., 2014). Key identifying markers of M2b macrophages include, as expected, IL-10high, as well as IL-12low, CD86, and IL-6 (Wang et al., 2019, Rőszer, 2015).

M2c macrophages are perhaps the most complicated macrophage subtype to define, because they can be activated via multiple stimuli. This means that following the previously mentioned recent activation-based nomenclature, they in fact comprise 3 subtypes. They can be activated by IL-10 (M(IL-10)), anti-inflammatory Glucocorticoid hormones (M(GC)) or Glucocorticoids in combination with TGFβ (M(GC-TGFβ) (Rőszer, 2015, Murray et al., 2014). M2c macrophages' function is to clear apoptotic cells via phagocytosis in response to anti-inflammatory stimuli. They express Arg1, as well as CD206 and uniquely amongst macrophages, CD163. They also secrete IL-10 and TGFβ, amongst other cytokines (Zizzo et al., 2012). As they are activated by their secretory products, they have the ability to create a positive feedback loop, amplifying their own activity.

M2d macrophages are the interestingly not included in all lists of M2 macrophage subtypes, likely due to them being the most recently defined. They are activated by IL-6 and TLR antagonists, (specifically adenosine), and promote angiogenesis. In cancer, this has the effect of promoting tumour growth (Ferrante et al., 2013, Wang et al., 2010). They share little markers with other M2 macrophages, except IL-10 and IL-10r. Markers unique to the M2d subset include NOS2 and VEGF (Ferrante et al., 2013, Yao et al., 2019)

In inflammatory lung disease, the distinction between M1 and M2 phenotype macrophages becomes blurred. The alveoli are a very complex and dynamic microenvironment; the requirements of which are changing constantly (Hussell and Bell, 2014). As a result both AMs and IMs develop phenotypes to match, showing plasticity of structure and function.

1.4 Immune Cells in Respiratory tract Infections

Alveolar macrophages play a major role in the initial immune response to respiratory tract infections. In *Streptococcus pneumoniae* infections AMs act to clear pathogens after opsonisation in response to Th1-type cytokines induced during the infection (Gordon et al., 2000). However, transcriptomic analysis showed that key M1-related genes, such as inducible nitric oxide synthase (iNOS) were not induced, and rather were replaced with M2-related genes such as arginase during *S. pyogenes* infection (Goldmann et al., 2007). This shows that as expected in the complex alveolar microenvironment, macrophage activation phenotype is atypical.

In early *M. tuberculosis* infections, an M1 phenotype is induced in AMs, leading to high iNOS levels (Chacon-Salinas et al., 2005). This is actually beneficial to *M. tuberculosis*, as it is then able to enter these M1 macrophages via phagocytosis and survive long term within the endosome, replicating many times. The bacteria are able to prevent the production of a phagolysosome, thus enabling this long-term survival. They further manipulate the lung immune system by promoting a Th1 (and subsequently macrophage-rich) environment. In a normal infection scenario, this would aid in clearing an infection, but in the case of *M. tuberculosis* works to further the establishment of the disease (Byrne et al., 2015).

Macrophages are vital immune cells in lung disease, both directly fighting infection and aiding other types of immune cell to fight infection. Other cells are also involved, such as antigen presenting cells, which work to initiate and direct the immune system to respond to an infection or damage. In the lung, as with all barrier sites, key antigen presenting cells are dendritic cells (DCs). A main

role of DCs is to migrate into the lymphatic system from the site of infection and activate the adaptive immune system, more specifically CD4+ and CD8+ T cells (Banchereau et al., 2000). Furthermore, they are defined as “any mononuclear phagocyte that has the ability to take up antigen, process it for presentation on MHC-I or II, migrate to the nearest draining lymph node and efficiently and effectively activate and polarise naïve T cells” (Cook and MacDonald, 2016). Their role in instigating adaptive immune responses is shown in models of DC depletion, where a lack of DCs has led to loss of T cell-mediated immunity to bacteria, viruses and parasites (Bar-On and Jung, 2010).

Like DCs, neutrophils also bridge the innate and adaptive response in a lung infection (Liu et al., 2017). They have a complex role, carrying out a wide range of functions involving killing pathogens and clearing debris. They can phagocytose pathogens directly, or create neutrophil extracellular traps (NETs) which, as the name suggests, trap invading cells and destroy them (Rosales et al., 2016). In response to an infection neutrophils migrate to the lungs, often to such an extent that neutrophils can consist of up to 60-80% of BAL (Bronchoalveolar lavage) cells (Boyton and Openshaw, 2002). This neutrophil recruitment and subsequent migration occurs in response to several events. TLR signalling, specifically that in response to bacterial products, such as TLR4 and TLR5 (LPS and flagellin respectively) results in the production of chemokines, which induce chemotactic migration of neutrophils to the site of infection (Craig et al., 2009). The specific molecular mechanisms of neutrophil recruitment are tissue specific and respond to different stimuli, to better target the response to the type of infection (Rossaint and Zarbock, 2013).

Immune responses in the lung are obviously not limited to the innate response however. Though often neglected, lymphocytes also play a major role in protecting the lung environment from infection, especially in co-ordination with innate immune cells. As with all adaptive immunity, this depends on recognition of the pathogen by B and T cell receptors. Once detected, B cells in the lung respond quickly with IgG antibodies. T cells are also involved, with CD4+ T helper cells stimulating both innate immune cells and B cells. CD8+ cytotoxic T cells work to lyse pathogens, typically with perforin granules (Boyton and Openshaw, 2002).

Though the immune system is largely preserved between individuals, differences do occur. A notable and well-documented example of this is in mice. Different strains of laboratory mice are known to differ from one another in the nature of their immune responses. More specifically, C57BL/6 mice are known to be more aligned with a Th1-like response, whereas BALB/c align more towards a Th2 response. This is characterised largely by distinct cytokine production. C57BL/6 mice produce higher levels of IFNγ and lower levels of IL-4, with the reverse true in the case of BALB/c mice (Mills et al., 2000). It has been shown that immune differences between mouse strains are not limited to T cell responses. Watanabe et al. (2004) showed that strain differences extend to the innate immune response. Macrophages from C57BL/6 mice secreted greater levels of TNF and IL-12 in response to LPS and MALP-2 stimulation than those from BALB/c mice. In addition, BALB/c macrophages did not release NO and lysosomal enzymes in response to the stimulus. They found that there were no differences in neutrophil activity or enzyme secretion with LPS and MALP-2 stimulation. C57BL/6 and BALB/c mice also differ in their abilities to resolve inflammatory pathologies, such as in the lung. One example of this is allergic airway inflammation. After allergen inhalation, BALB/c and C57BL/6 mice both develop a Th2-driven inflammatory response. C57BL/6 mice exhibit greater eosinophilia, whereas BALB/c mice display a significant increase in airway smooth muscle mass (Van Hove et al., 2009). This increase in airway smooth muscle mass is thought to translate to the pathology of chronic asthma, indicating that as well as BALB/c mice exhibiting a Th2 bias, they are also prone to overreactivity. More recent research into this phenomenon, however, has indicated that it does not occur due to the two strains' differing inflammatory responses. Parkinson et al. (2021) showed that, the differences in airway remodelling are likely due to compositional differences in the airway extracellular matrix between species. These differences were found to be present both during steady state and during an allergic response.

The differing immune responses of C57BL/6 and BALB/c mice has been shown to in turn have an effect on their microbiome. Fransen et al. (2015) showed that BALB/c mice produce a greater variety and quantity of IgA antibodies than C57BL/6 mice in both the serum and faeces. This increase in IgA correlated

with increased Shannon index microbial diversity in BALB/c mice, indicating a greater abundance of bacterial species present. It was hypothesised that this increased level of polyreactive IgA led to an increase in the internalisation of IgA-coated bacteria into the peyer's patches. This subsequently causes a positive feedback loop, further increasing IgA levels in BALB/c mice. Interestingly, these polyreactive IgA antibodies were already present in germ free mice, indicating their increased levels are genetic, rather than being initiated by increased microbial diversity. It is because of this difference in between C57BL/6 and BALB/c mice, that the strain used in *in-vivo* experiments must be taken into account.

In most acute lung infections, the immune system is able to effectively fight the infection and no treatment is required. There are cases, however, where the immune system is not sufficient alone to fight the infection. In these cases, drugs must be prescribed to supplement the patient's immune response. Several factors may contribute to this, such as the existence of a comorbidity that has affected the immune system. Other comorbidities include heart, lung liver and other conditions. Other main reason antibiotics are required is the presence of severe symptoms, indicating the infection is that of a pathogen that is more difficult to fight (NICE, 2008). The drugs prescribed in these situations are antibiotics.

1.5 Antibiotics

Antibiotics, medicines discovered and designed to inhibit or destroy microorganisms, are some of the most widely known and widely used therapies in the modern world.

One of the ubiquitous events in scientific history was the accidental discovery of Penicillin and its antimicrobial properties by Alexander Fleming in 1928. Fleming found that a fungal contamination on a plate of bacteria had destroyed the bacterial colonies it touched (Fleming, 1929). The *Penicillium* fungus that contaminated the plate was shown to have antimicrobial properties, and was developed into the drug penicillin, which is still in use today.

Interestingly, the use of antibiotics has existed for much longer than most people would think. Traces of the antibiotic Tetracycline have been found on skeletal remains on several archaeological sites. This includes skeletons dating from 350-550AD, from ancient Sudanese Nubia (Nelson et al., 2010), and Roman-era skeletons in Egypt (Cook et al., 1989). The only way tetracycline could have entered the bone in such a way is if it was ingested *in vivo*, rather than post mortem contamination. One explanation as to how tetracycline got into the bones is via *Streptomycetes*-contaminated grain. The tetracycline likely had a protective effect, as no bone infection was detected on the Egyptian samples, and there were historically low documented rates of infectious disease in Sudanese Nubia (Cook et al., 1989, Armelagos, 1969).

Rather than tetracycline being the only antibiotic used historically, it is more likely simply the only antibiotic that would still be detectable. Unlike other antibiotics, tetracyclines are strong chelators, enabling them to irreversibly bind to minerals in bone and enamel. No other antibiotics do this, and therefore would not be preserved to the same extent (Aminov, 2010). Because of this, knowledge about the historic use of other antimicrobials is generally limited to documented rather than physical evidence. An example of this includes historical reports of the antimicrobial properties of ‘red soil’ in Jordan, which is still being used as an inexpensive alternative treatment today. It was discovered that actinomycete bacteria in the soil produce actinomycins, polypeptide antibiotics (Falkinham et al., 2009).

1.6 Fluoroquinolone Antibiotics

The fluoroquinolones (or more generally, quinolones) are a large family of antibiotics over 5 decades old, which is still being expanded with the ongoing development of new drugs. The first quinolone antibiotic was nalidixic acid, discovered in 1962, and first used clinically in 1967 (Emmerson and Jones, 2003). Due to the lack of a fluoro group, nalidixic acid was not a true fluoroquinolone; the first of which was flumequine, developed in 1976. This was the first clue that modifying the quinolones' chemical structure had potential to improve their efficacy, as before this, no new quinolones had shown any real improvement over nalidixic acid, especially against gram-positive organisms (Appelbaum and Hunter, 2000). Today, two of the most potent and commonly used fluoroquinolones are Ciprofloxacin and Levofloxacin (fig. 1.4).

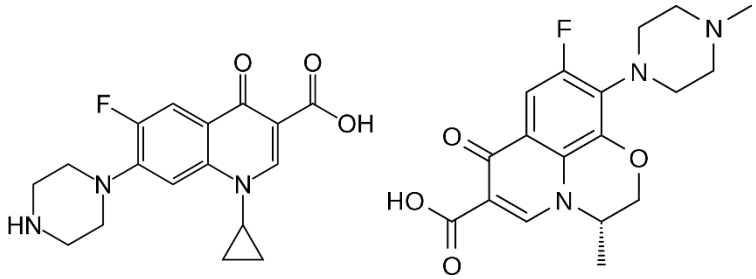


Figure 1.4: Chemical structures of Ciprofloxacin (left) and Levofloxacin (right).

Ciprofloxacin is a second-generation fluoroquinolone, first used clinically in 1987. It works by targeting DNA gyrase and a type II topoisomerase, topoisomerase IV, necessary to separate bacterial DNA, inhibiting cell division (Pommier et al., 2010, Drlica and Zhao, 1997). It is rapidly bactericidal, and is mainly used to effectively treat gram-negative bacteria, such as *Vibrio cholera*, *Legionella pneumophila*, and *Yersinia enterocolitica*. It has shown some efficacy at treating certain gram-positive bacteria, such as penicillin-resistant *Streptococcus pneumonia*. Specific conditions it is used to treat include urinary tract infections, lower respiratory tract infections and typhoid fever (FDA, 2009).

Table 1.1: Selected organisms shown to be susceptible to Ciprofloxacin treatment, either via clinical or in vitro studies (FDA, 2009)

Gram-Positive Microorganisms	Gram-Negative Microorganisms
Bacillus anthracis (post-exposure prophylaxis)	*Vibrio cholerae*
Staphylococcus haemolyticus	*Legionella pneumophila*
Staphylococcus aureus	*Salmonella enteritidis*
Streptococcus pneumoniae	*Klebsiella oxytoca*
Enterococcus faecalis	*Aeromonas hydrophila*
	Edwardsiella tarda
	Vibrio vulnificus
	Neisseria gonorrhoeae

Ciprofloxacin is used by militaries and at-risk professions as post-exposure prophylaxis (PEP) for anthrax (*Bacillus anthracis*). This reduces the chance of infection occurring after an individual has been exposed to the bacterium, typically through excavation, or potentially bioterrorism. The efficacy of Ciprofloxacin for this purpose has been shown through animal studies, as due to the rarity and severity of anthrax infection, human trials would be unethical and infeasible (Friedlander et al., 1993). Ciprofloxacin was used as PEP after the 2001 US anthrax bioterror attacks, with over 9300 people recommended to complete 60 days of antibiotic prophylaxis. No one who received Ciprofloxacin prophylaxis went on to develop inhalation anthrax (FDA, 2009, Jernigan et al., 2002, Belongia et al., 2005).

Levofloxacin is a third generation fluoroquinolone, approved for clinical use in 1996. It works in the same was as all fluoroquinolones: by inhibiting the DNA gyrase and topoisomerase IV (Drlica and Zhao, 1997). It is a broad-spectrum antibiotic, used to treat mild, moderate and severe infections caused by a range of both gram-positive and gram-negative bacteria (FDA, 2017). It is an improvement over ciprofloxacin in that it is able to treat gram-positive infections much more effectively.

Conditions Levofloxacin is used to treat include nosocomial and community-acquired pneumonia, skin infections, complicated urinary tract infections, and bacterial exacerbations of bronchitis. Bacterial species Levofloxacin is used

against include methicillin susceptible *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Escherichia coli*, *Klebsiella pneumonia*, and *Streptococcus pneumonia* (including multidrug-resistant). Levofloxacin is also used to treat pneumonic and septicaemic plague caused by *Yersinia pestis*, as well as prophylaxis for the disease. Like Ciprofloxacin, Levofloxacin is used as PEP for people exposed to inhalation Anthrax, albeit with no human trials for ethical and feasibility reasons (FDA, 2017).

Table 1.2: Selected organisms shown to be susceptible to Levofloxacin treatment, either via clinical or in vitro studies (FDA, 2017).

Gram-Positive Microorganisms	**Gram-Negative Microorganisms**
Bacillus anthracis (post-exposure prophylaxis)	*Yersinia pestis*
Enterococcus faecalis	*Legionella pneumophila*
Staphylococcus aureus	*Serratia marcescens*
Streptococcus pneumoniae (inc. multi-drug resistant)	*Escherichia coli*
Clostridium perfringens	*Chlamydophila pneumoniae*
Streptococcus pyogenes	*Bordetella pertussis*
	Enterobacter aerogenes
	Klebsiella oxytoca

Whilst fluoroquinolones are tremendously effective at treating very serious infections in medical emergencies, they are not without their side effects. Their widespread side effects are so well known that several fluoroquinolones have been taken off the market because of the frequency of adverse effects, such as norfloxacin, sparfloxacin and gatifloxacin (Roberts, 2016). These adverse effects are not just known amongst clinicians, with campaign groups emerging across the world by former patients who have experienced symptoms caused by fluoroquinolone treatment. Known as being ‘floxed’, these symptoms can widely vary, and often continue long after treatment courses have stopped. They are so widely reported, with hundreds of thousands of adverse effect reports and 6,575 deaths, that the FDA now recognises FQAD (fluoroquinolone-associated disability) as a syndrome (Marchant, 2018).

1.7 Adverse Effects of Fluoroquinolones

Some of the most widely reported adverse effects of fluoroquinolones are tendon-related, such as tendonitis or even tendon rupture. A longitudinal study looked at the association between fluoroquinolone prescription and tendon ruptures & other severe collagen-associated adverse effects such as retinal detachment and aortic aneurysms (Daneman et al., 2015). The study followed up over 1.7 million patients between 1997 & 2012, with over 650,000 prescribed fluoroquinolones. Fluoroquinolone prescription was found to increase the risk of tendon rupture and aortic aneurysm. Another cohort study focussed on aortic aneurysm/dissection and fluoroquinolone use, following over 360,000 fluoroquinolone prescriptions between 2006 and 2013. Compared to amoxicillin (a comparably used antibiotic), fluoroquinolone use was linked to a 66% increase in incidence of aortic aneurysm or dissection within 60 days of commencing treatment (Pasternak et al., 2018).

One emerging hypothesis explaining how antibiotics can directly cause adverse effects in the host is via mitochondrial damage. This would go some way to explain the widespread nature of reported adverse effects, as mitochondrial function directly affects all cells. The physiology and evolution of mitochondria can explain to an extent why they can be affected by antimicrobials. The well-known endosymbiont hypothesis states that mitochondria are bacterial in origin; previously existing as independent prokaryotic cells to which endocytosis occurred, which continued to function as respiratory organelles (Martin et al., 2001). As mitochondria inevitably still retain some resemblance to their cellular ancestors, this has the potential to lead to becoming a target for antimicrobials, especially those designed to arrest respiration.

Kalghatgi et al. (2013) showed how several classes of antibiotic, including β-lactams and fluoroquinolones cause oxidative damage not only in bacterial cells (as intended), but also in mammalian cells at clinical-equivalent doses. More specifically, they induced overproduction of reactive oxygen species and mitochondrial dysfunction *in vitro* and *in vivo*.

Further studies have shown that in human tendon cell models, oxidative damage caused by fluoroquinolone treatment can be mitigated by mitochondria-targeted antioxidants far greater than non-specific ones (Lowes et al., 2009). This shows that mitochondria are responsible for the oxidative stress causing cellular damage. In addition to oxidative damage, ciprofloxacin has been demonstrated to affect mitochondrial function by cleaving mitochondrial DNA, subsequently causing loss of cellular functions reliant on it. This process has been shown to occur via the inhibition of topoisomerase II, which is the intended mechanism of fluoroquinolones, albeit in bacterial cells, not those of the host (Hangas et al., 2018).

Interestingly, in addition to tendon-related disorders, antibiotic-induced neurological conditions have been reported for some years, particularly in patients prescribed fluoroquinolones (Reeves, 1992). More recent research has attributed this to mitochondrial dysfunction, with clinical and *in vivo* evidence attributing it to psychosis and mental disorders such as autism and depression (Ben-Chetrit et al., 2013, Rossignol and Frye, 2012, Stefano et al., 2017).

1.8 The Harmful Cellular Effects of Antibiotic Use

Whilst the side effects of fluoroquinolones have been well documented clinically over the years, we are beginning to understand the severe adverse effects of fluoroquinolone use at a cellular level. Antibiotics in general have been shown to cause adverse effects via the dysbiosis they cause- where commensal bacteria in the gut are depleted as well as the target organisms.

Lankelma et al. (2017) studied the effect of antibiotic-mediated microbial disruption on the immune response to Melioidosis. Lankelma et al infected C57BL/6 mice with *B. pseudomallei* with some animals pre-treated with broad-spectrum antibiotics. Animals were sacrificed at different timepoints, and their infection and immune response measured (fig 1.5).

The effects of antibiotic treatment on Melioidosis and the immune response were multiple and varied. A two way relationship was seen between the microbiota and Melioidosis; the gut microbiome composition was altered significantly by Melioidosis infection, and a microbiome disrupted by antibiotics

lead to increased infection rates. More specifically, animals with a disrupted microbiome showed increased proinflammatory cytokine release in response to Melioidosis. Results were variable, but significant increases of plasma TNFα and IFN-γ levels were seen in microbiota disrupted mice 72hrs post infection. Another specific effect of microbial disruption was that alveolar macrophages displayed impaired phagocytosis. This is significant, due to the 'first line of defence' role that the alveolar macrophages play in *B. pseudomallei* infection (Wiersinga et al., 2006).

An interesting finding of Lankelma et al. (2017) was that whilst antibiotic gut microbiome disruption did affect the immune response to Melioidosis, it did not significantly impact survival or organ injury. Whilst there was a trend towards decreased survival and elevated clinical observation score in microbiome-disrupted animals, this trend did not reach significance. This was also the case with organ damage. At the 72hr timepoint post-infection all but one markers of cellular damage were elevated in microbiome-disrupted mice, but this increase was not significant.

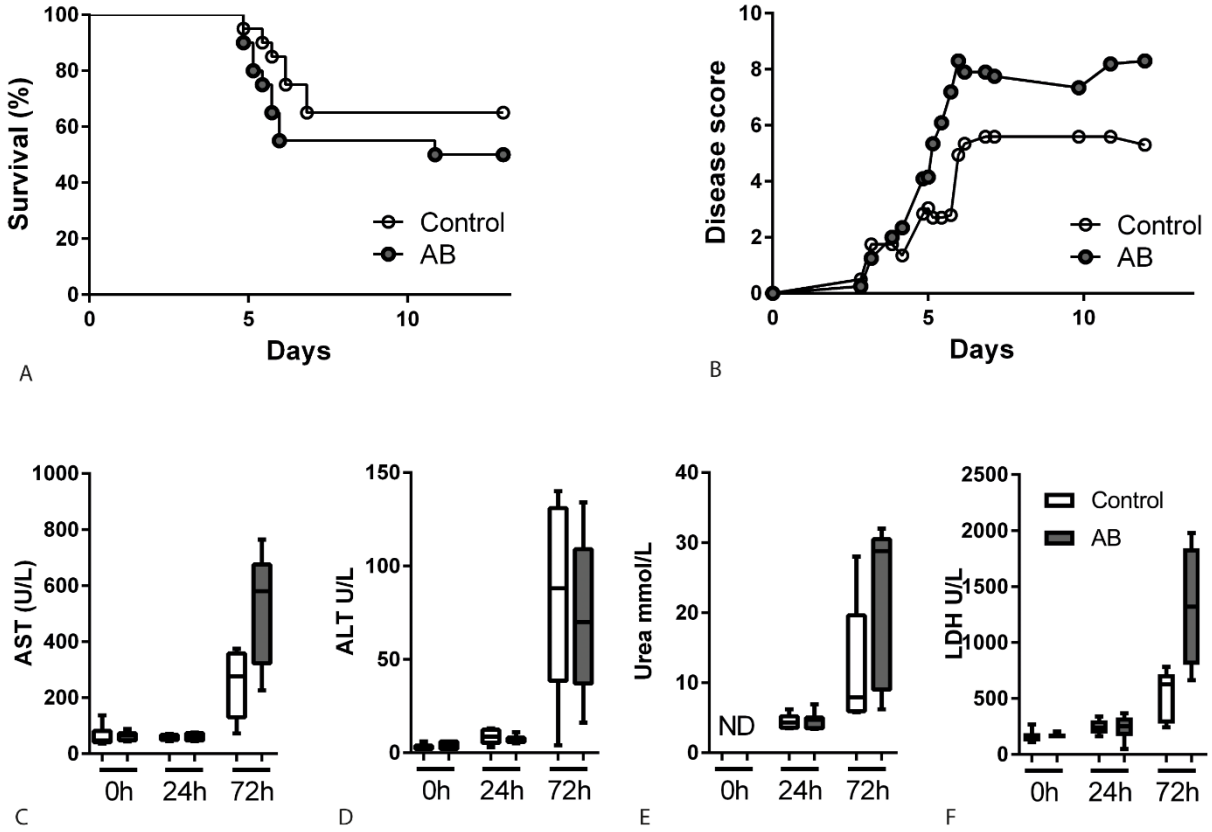


Figure 1.5 The effects of antibiotic-induced dysbiosis on animal survival and organ/cell damage*. A&B: Survival and disease score of control and antibiotic-treated mice inoculated with 150 CFU of B.pseudomallei. C-F: Markers of liver damage (Aspartate aminotransferase, AST, C; alanine aminotransferase, ALT, D), renal damage (urea, E), and general damage (lactate dehydrogenase, LDH, F) 0, 24, and 72h after inoculation. Taken from Lankelma et al. (2017).*

Whilst Lankelma *et al* comprehensively describe the effects of antibiotic-induced dysbiosis on the immune response to infection; the mechanisms of these effects and the possibility of direct cellular effects are relatively unexplored.

Other studies have looked at how antibiotics may affect immune cell function in ways other than via dysbiosis. Research has shown how antibiotics themselves can affect cellular processes, either directly or indirectly. Yang et al. (2017) looked at how, rather than directly affecting immune cells, antibiotics such as ciprofloxacin alter the production of several metabolites in the host, which have subsequent effects on immune function. Antibiotics commonly function by affecting bacterial metabolism, and Yang et al. showed that host metabolism is also affected. Yang et al. divided metabolites into signatures upregulated during

antibiotic treatment, *E. coli* infection, and both infection and treatment. They treated *E. coli* cells with metabolites from each of these signatures, and subsequently assessed the Ciprofloxacin minimum inhibitory concentration (MIC) on these cells. In all groups, several metabolites caused a significant increase in MIC (infection and treatment 8/20, treatment only 8/20, infection only 3/10). This shows that independent to the microbiome and exclusively occurring in the presence of infection, these metabolic changes inhibit the efficacy of ciprofloxacin, making any infection less susceptible to treatment.

Whilst some research attributes the fluoroquinolones' effects to the dysbiosis they induce, some focuses on the direct effects of the drug on host cells. Some evidence points to the involvement of the mitochondria in this phenomenon. This project aims to explore these effects, focussing on different antibiotics that, although prescribed for the same conditions, may cause different effects on host cells, either directly or via dysbiosis. A combination of *in vivo* mouse studies and *in vitro* macrophage experiments will allow effects to be seen on both a reductionist cellular level, and a broad, complex organismal level.

1.9 Gastrointestinal Hormones and the Microbiome

One of the areas most prone to the off-target effects of antibiotic treatment is the gastrointestinal tract. This is because the microbiome is known to have a profound effect on gut health. Another key contributor to gut physiology and metabolism are gastrointestinal hormones. They have a complex two-way relationship with the microbiome, with both affecting and being affected by the other. Many effects that the microbiome has on the gut are hormonally mediated.

Gastrointestinal hormones are secreted by enteroendocrine cells dispersed throughout the GI tract, and have a wide variety of effects on organs both within the digestive system and beyond. One of the most well characterised effects of GI hormones is feeding and weight change, also a well-characterised side effect of antibiotic treatment.

Two main groups of GI hormones are the Gastrin family and the Secretin family, grouped as such because of their chemical structure (Poyner and Hay, 2012, Jens et al., 2007). Gastrin family hormones, namely Gastrin and Cholecystokinin were discovered in 1906 and 1928 respectively. Gastrin was initially found to cause the release of gastric acid, whilst CCK was found to stimulate the contraction of the gall bladder and release digestive enzymes (Edkins, 1906, Ivy and Oldberg, 1928). The Secretin family of hormones was named after Secretin, the first discovered member of the group, and is alternatively named the Secretin-Glucagon family. They all interact with secretin G protein coupled receptors and have diverse effects. For example, glucagon is released by the pancreas, and acts in the liver to increase blood glucose levels, reversing the effects of insulin (Bataille et al., 1988). Secretin has very different effects, though it works in the same locations in the body, namely the pancreas and liver. It is secreted in the duodenum, and has several functions. One is to neutralise the pH of acidic gastric chyme entering the small intestine, thus creating a more optimum environment for the function of digestive enzymes. Secretin also has a role in the regulation of water homeostasis, acting as a diuretic to increase urinary volume as an antagonist to vasopressin (Afroze et al., 2013).

GI hormones unsurprisingly have a very close relationship with metabolism. Because many GI hormones have effects on nutrient absorption and other metabolic processes, they must be tightly controlled and only be released when required. This leads to a complex two-way relationship, with hormones both controlling and responding to the state of metabolism. Because of this, enteroendocrine cells (EECs) must be highly adapted as chemosensors.

There are several types of EEC, including G-cells, I-cells, M-cells and S-cells. (Gribble and Reimann, 2016b). Until relatively recently, the dogma was that each type of EEC secreted one specific hormone only, and were classified as such. In fact, it has been found that each type of EEC can store multiple different hormones, and generally store them in individual storage vesicles. The cocktail of hormones each cell releases is variable, dependant on spatio-temporal, and metabolic factors (Fothergill and Furness, 2018) They are located throughout the GI tract, embedded within the intestinal epithelium. Different types of EEC are shaped differently; most are ‘open type’, spanning the whole epithelium and making direct contact with the lumen. Some EECs are classified as ‘closed-type’ in that they do not span the entire epithelium and therefore do not make direct contact with the lumen. One such example are D-cells, which release Somatostatin (Ku et al., 2003).

EECs respond to the ingestion of food by sensing changes of concentrations of nutrients such as carbohydrates, lipids and proteins within the gut lumen. This directly increases the secretion of various hormones. This phenomenon has been shown in several studies. Schirra et al. (1996) found that K-cells release GIP (gastric inhibitory polypeptide) in response to glucose ingestion, which acts to induce the secretion of insulin. GIP continued to be released after the glucose had been emptied from the stomach, showing that it continued to be detected in the duodenum.

In contrast, EECs also use nutrient sensing to detect the lack of food, and release appetite-promoting hormones such as Ghrelin and Motilin (Gribble and Reimann, 2016b). Ghrelin has been shown to increase in a fasting state, and decrease after feeding (Stengel and Taché, 2009). Interestingly, this increase

before a meal occurs to a greater extent when food is eaten at the same time every day (Brede et al., 2017).

As well as nutrients ingested in food, EECs also detect and respond to nutrients and metabolites released by the microbiota. This has been termed the 'microbiota-derived luminal metabolome', and is relatively dynamic. It is not only affected by the microbiome itself (i.e. species and quantity present), but by other factors such as bacterial metabolites and other secretions (Gribble and Reimann, 2019b). Studies in germ-free mice have demonstrated the extent to which GI hormones are affected by the microbiome. Wichmann et al. (2013) found that GLP-1 levels are increased in GF mice, because of increased expression of proglucagon. Colonisation of GF mice suppressed proglucagon expression and increased energy availability.

The most abundant microbial metabolites in the GI tract are short chain fatty acids (SCFAs), derived from the digestion of carbohydrates by gut bacteria (Martin et al., 2019). Because of this, SCFAs are one of the most common means by which microbes affect gut hormone release. SCFAs either bind to free fatty acid receptors 2/3 (FFAR2/3) on the surface of enteroendocrine cells (Offermanns, 2014), or activate nuclear histone deacetylases (HDAC) within the cells themselves (Waldecker et al., 2008). Another mechanism by which microbes are able to signal to and influence EECs are via bile salts. Bile acids are a product of cholesterol metabolism, and are released by the liver in order to aid in the digestion of lipids. Bacteria in the gut are able to metabolise these 'primary' bile acids using bile salt hydrolases, synthesising 'secondary' bile acids (Jones et al., 2008). Secondary bile acids also function as signalling molecules that can bind to the G protein-coupled Takeda G-protein receptor 5 (TGR5), or the nuclear farnesoid X receptor (FXR) on EECs. Bile salt hydrolase activity is variable, with different species and phyla of bacteria being capable of different levels of secondary bile acid synthesis. It is because of this that the composition of the gut microbiome can have such a great effect on bile acid-mediated gut hormone release (Jones et al., 2008).

Glucagon-like peptide 1 (GLP-1) is one such hormone that can be drastically altered by the microbiome via SCFA and bile acid secretion. Microbial SCFAs

stimulate the secretion of GLP-1 via both the FFAR2 and FFAR3 surface receptor (Tolhurst et al., 2012). The effect of microbial bile acids on GLP-1 are more complex. It has been shown that TGR5 mediated signalling upregulates the secretion of GLP-1 as well as the hormone PYY (Bala et al., 2014). Conversely, FXR activation has been seen to decrease GLP-1 secretion (Martin et al., 2019). Pathak et al. (2017) found there is in fact crosstalk between the two receptors. The activation of FXR by the agonist INT-767 was shown to upregulate the expression of TGR5 genes, thus increasing GLP-1 albeit indirectly. This mechanism was shown to be reliant on TGR-5, with mice only lacking FXR showing no difference in GLP-1 release, compared to reductions in secretion in *Tgr5*$^{-/-}$ and *Fxr*$^{-/-}$ *Tgr5*$^{-/-}$ animals.

The effect of microbial metabolites on CCK is less well studied. In pigs, ileal infusions of SCFAs have been shown to increase serum CCK levels, but conversely decrease CCK secretion in the pancreas (Sileikiene et al., 2008).

As well as metabolism and physiology, GI hormones and EECs also play a role in inflammation and the immune response. EECs are adapted to be highly sensitive sensors of many molecules such as bacterial metabolites as mentioned above. It is therefore no surprise that this sensory ability also plays a role in immunity and inflammation, especially given that inflammation often occurs as a result of dysbiosis (Zeng et al., 2017).

In non-infection gut inflammation, such as Crohn's disease and Ulcerative Colitis inflammatory bowel disease, some of the non-inflammatory symptoms include a loss of appetite and other signs of EEC dysfunction (Harrison et al., 2013). Furthermore, multiple studies of IBD patients have shown altered levels of several GI hormones (Worthington et al., 2018b). Specifically, increased levels of GLP-1 have been found in the plasma and serum of Ulcerative Colitis and Crohn's disease patients (Bendet et al., 2004). In addition, increased CCK levels have been measured in the plasma of Crohn's disease patients (M. K. Vu, 2000).

EECs have also been linked to intestinal infections, with several studies showing differences in hormone levels in infected patients. A particularly well-documented example of this is *Helicobacter pylori* infections. Patients suffering

from *H. pylori* infection have been shown to have reduced secretion of Ghrelin, thought to occur due to the impact *H. pylori* infection has on the intestinal mucosa, near to the EECs (Jeffery et al., 2011). Studies conflict, however, as to the nature of this disruption. Choi et al. (2016) found that after *H. pylori* eradication therapy, Ghrelin secretion significantly increased. In contrast, Martín-Núñez et al. (2021) have found that *H. pylori* eradication therapy in fact also reduced Ghrelin secretion. This study did not measure Ghrelin levels prior to infection however.

EECs have been shown to secrete a number of cytokines, showing their more direct influence on intestinal inflammation. Bogunovic et al. (2007) showed that EECs have TLRs on their surface that, upon activation, release multiple cytokines including TNFα, macrophage inflammatory protein-2 and TGFβ, as well as NF-κB and other transcription factors. Activation has been shown to occur in response to pathogen-specific stimuli, with flagellin and LPS causing the release of the proinflammatory cytokines IL-32 and CXCL1&3 (Selleri et al., 2008). This indicates a direct link between EECs and intestinal inflammation.

1.10 Cholecystokinin

One gut hormone of particular interest with respect to its interactions with the immune system is Cholecystokinin (CCK). As with other GI hormones mentioned previously, CCK is secreted by enteroendocrine cells in the small intestine. Its most well-known function is to control food intake by stimulating satiety, i.e. giving a signal of being ‘full’ (Fink et al., 1998). Several studies have demonstrated this function, one such example being Irwin et al. (2013), who treated mice with an enzyme- resistant CCK analogue, (pGlu-Gln)-CCK-8. They found that food intake and body weight was significantly reduced after treatment. Other related effects included reduced plasma glucose, and increased both plasma insulin levels and insulin sensitivity. CCK has also been observed to reduce food intake in rats and pigs, as well as other models (Gibbs et al., 1973, Baldwin et al., 1982). It is of no surprise that these effects have led to widespread interest in the use of CCK and CCK analogues to treat obesity. Christoffersen et al. (2020) studied the long-term effects of a CCK analogue (NN9056) on both lean domestic pigs and obese minipigs. Body weight and

food intake was reduced in both species, and did not cause adverse effects, showing its potential as an obesity therapy.

The effects of CCK are not limited to satiety signalling. Another well-documented function of CCK is to aid in digestion. This occurs via the modulation of the release of pancreatic enzymes to digest a variety of nutrients (Rehfeld, 2004). CCK also stimulates the release of bile from the gallbladder to further aid digestion, specifically that of lipids (Shaw and Jones, 1978). This works in tandem with satiety induction after a meal. The effects of CCK are more widespread than the digestive system however. As mentioned above, CCK causes the suppression of feeding, which is a process that involves conscious thoughts. Thus, CCK must have neurological effects. These neurological effects have been found to be farther reaching than simply hunger signalling. CCK has been shown to have a role in anxiety, with infusions of CCK-4 (tetrapeptide) found to acutely induce panic attacks in patients both with and without pre-existing anxiety disorders (Rotzinger and Vaccarino, 2003). Furthermore, when patients are treated with CCK-B receptor antagonists, CCK-4-induced panic attacks were prevented (Bradwejn et al., 1994). Similarly, the CCK has been linked to the induction of pain, with CCK-8 (octapeptide) infusion causing aversive visceral pain memory in rats (Cao et al., 2012). As with anxiety, CCK-modulated pain is also modulated by the CCK-B receptor, giving rise to the potential for CCK-B antagonists as pain relief (Bernard et al., 2021).

CCK, like other gut hormones, has been shown to have an effect on inflammation and immunity. For example, CCK octapeptide (CCK-8) has been shown to have multiple immunomodulatory effects on T cells. Zhang et al. (2014a) found that when murine CD4+ T cells were treated with CCK-8, Th1 and Th17 differentiation was inhibited and inducible Treg differentiation was upregulated.

Further anti-inflammatory effects of CCK have also been discovered. CCK pre-treatment has been recently shown to prevent LPS-induced colon barrier dysfunction in a rat model of sepsis (Saia et al., 2020). In addition, CCK affects proinflammatory macrophage function, inhibiting LPS-induced NOS2 expression in peritoneal macrophages (Saia et al., 2014b). Dendritic cell

function can also be regulated by CCK. Jia et al. (2014b) found CCK-A and B receptors on the surface of human plasmacytoid dendritic cells (pDCs). CCK-8 was shown to modulate and inhibit TLR-9 mediated activation of pDCs and subsequent downstream adaptive immune processes. Furthermore, in a mouse model, CCK-8 was also found to modulate DC cytokine production, and subsequently inhibit Th17 and Th1 polarisation, which inhibited arthritis formation (Li et al., 2011). This is in contrast with Zhang et al. (2014a) mentioned above, who found CCK-8 increased Th1 polarisation.

The aims of this thesis are to examine the immunomodulatory effects of fluoroquinolones, specifically Ciprofloxacin and Levofloxacin, and the potential role that Cholecystokinin plays in this and other negative effects of fluoroquinolone treatment. Using both *in vivo* and *in vitro* approaches will allow the role of the microbiome to be explored.

This widespread post-exposure prophylaxis use-case of fluoroquinolone antibiotics means that reducing negative off-target effects is of particular importance. Learning more about the mechanism by which fluoroquinolone treatment harms patients will enable future treatment regimens to be modified in order to counteract it.

Chapter 2: Materials and Methods

2.1 Animals

All animal experiments used mice that were co-housed, under standard laboratory husbandry conditions (21°C, 45–65% humidity, 12h dark/light cycle, food and water *ad libitum*). Experiments used BALBc (Envigo) or CCKLacZ (Lay et al., 1999b) mice with in house C57BL/6 wild-type controls. All experiments conformed to the Lancaster University Animal Welfare and Ethical Review Body (AWERB) and ARRIVE guidelines. All animal procedures were performed in accordance with the Animals (Scientific Procedures) Act, UK 1986; under Home Office project licenses PPL 70/8521 and PP4157153, utilising Schedule 1 methods

2.2 In Vivo Antibiotic treatments

Mice received ciprofloxacin or levofloxacin, both at doses of 100mg/kg. Animals were dosed at approximately 10am and 6pm every day via 100 µL oral gavage, and faecal samples and body mass of all animals were taken daily. Non-antibiotic control mice received a vehicle mock dose of MilliQ water.

2.3 Cell Counting

Cell counts were carried out using a Countess Automated Cell Counter (Invitrogen) and disposable counting slides (NanoEnTek) to determine the cellularity (cell number) of each sample. Samples were diluted 1/10 in media if required, and diluted 1/2 in 0.4% Trypan blue (NanoEnTek).

2.4 Bone Marrow Isolation and Macrophage Derivation Culture

Femurs and tibias of mice were removed and cleaned in a sterile changing station. In a class 2 biosafety cabinet, all remaining tissue was cleaned from the bones. Using sterile dissecting scissors, the end of each bone was removed. The bone marrow was flushed out of the bone using a 25g needle and syringe containing 2.5ml of Dulbecco's Modified Eagle Medium (DMEM) w/10% Foetal Calf Serum (FCS) (Gibco and Sigma), and homogenised by passing through a 100µm cell strainer (Fisher). Cell suspension was pooled and resuspended in red blood cell lysis buffer (Sigma). After 2mins, DMEM

w/10% FCS was added and cells were centrifuged at 400g for 5 minutes and resuspended in 1ml DMEM w/10% FCS.

For culture, cells were plated out in 6 well plates (Corning) at $1x10^6$ cells per well in 4ml full Bone marrow derived macrophage (BMDM) Medium (DMEM w/GLUTAMAX, 10% FCS, 20ng/ml Macrophage Colony Stimulating Factor (M-CSF) and cultured at 37°C in 5% CO_2. On day 4 and 6 of incubation, 2ml of medium was removed from each well, and replaced with fresh medium. On Day 7 of incubation, cells were ready for analysis or further treatment.

2.5 Bone Marrow-Derived Macrophage Polarisation

Media and cells were removed from each well, and 2ml pre-warmed PBS w/ 3mM Ethylenediaminetetraacetic acid (EDTA) and 10mM Glucose added to aid detaching adherent cells. Plates were returned to the incubator for 15 mins. Cells were agitated and the cell suspension removed with a micropipette. Cells were replated out in BMDM media, allowed to settle, and then stimulated with 20ng/ml Interferon Gamma (IFNγ) (Peprotech), 20ng/ml Interleukin 4 (IL-4) (Peprotech), or 100ng/ml Lipopolysaccharide (LPS) (Sigma) as indicated. These are standard doses, extensively shown in literature to induce a model of type 1 (IFNγ, LPS) or type 2 (IL-4) immune response *in vitro.* This is a more reproducible, ethical, and convenient method than live pathogen challenge.

2.6 IgG Phagocytosis assay

IgG FITC-labelled beads (Cayman Chemical) were added to BMDM suspensions to a final dilution of 1/500. Cells were then incubated for 30-60 minutes at 37°C in 5% CO_2. To remove background FITC fluorescence from surface-bound beads, cells were quenched in trypan blue quenching solution (Cayman Chemical) for 2 minutes. Cells were then centrifuged (400g for 5 minutes) and washed in PBS (1% BSA), before analysis via flow cytometry.

2.7 Lactate Dehydrogenase Cytotoxicity Assay

Before performing the assay, it was optimised according to the manufacturer's instructions to determine the appropriate concentration of cells per well. 100µl BMDMs were plated in BMDM culture media into a 96 well plate at $1x10^4$ cells/well, and incubated overnight at 37°C in 5% CO_2. Cells were incubated for 45 minutes with 10µl of fluoroquinolone to a desired range of final concentrations. Additional cells were incubated with 10µl qH_2O (spontaneous lysis control) or 10µl 10X Lysis buffer (maximum lysis control) (Thermo).

After incubation, 50µl of sample medium was transferred from each well into a separate corresponding plate. 50µl of Reaction Mixture (Thermo) was then added to each well and mixed using a multichannel pipette. The plate was then incubated in the dark for 30 minutes at room temperature as per the supplied protocol, after which 50µl of stop solution (Thermo) was added to each well.

Absorbance was then measured at 490nm (680nm reference) using an Infinite M200 pro plate reader (Tecan). In order to calculate LDH activity, 680nm absorbance was subtracted from 490nm absorbance. To determine % cytotoxicity, LDH activity of spontaneous lysis was subtracted from that of treated cells, divided by total LDH activity (maximum LDH activity- spontaneous LDH activity), and multiplied by 100.

2.8 Intestinal Cell Isolation

Intestines were cleaned of any faeces, and all mesenteric fat removed. They were opened longitudinal and digested in gut digest medium (50ml Roswell Park Memorial Institute Medium (RPMI) (Sigma), 10% FCS (Gibco), 0.5mg/ml Dispase (Gibco), 0.5mg/ml Collegenase (Gibco)) in a shaking incubator for 30mins at 37°C. After incubation, the digestion medium was passed through a 100µm cell strainer and agitated to separate remaining tissue from cells. Resulting cell suspensions were placed on ice.

2.9 Broncho-Alveolar Lavage

Immediately post sacrifice via CO_2, the trachea of each mouse was exposed, and broncho-alveolar lavage (BAL) performed. This involved making a small

incision in the trachea, inserting a blunt needle and repeatedly inflating the lungs with 1ml of PBS before recovery via needle and stored on ice.

2.10 Lung Cell Isolation

The right lobe of the lung was finely diced using scissors and placed in 2ml lung digest medium (PBS (Sigma), 0.1mg/ml Liberase TM (Roche), 50µg/ml DNAse I (Roche)) for 30 minutes in a vigorously shaking incubator at 37°C. Digestion was stopped after 30mins by adding 5mM EDTA (Fisher). Digestion medium was passed through a 100µm cell strainer to produce a single cell suspension. If required, red blood cell lysis was then performed by resuspending in RBC lysis buffer (Sigma) for 1 minute. An equal volume of RPMI was added to stop lysis. Cells were then centrifuged at 400g for 5 minutes and resuspended in 1ml RPMI.

2.11 Mesenteric Lymph Node and Spleen Processing

Mesenteric lymph node (MLN)s and spleens were processed in a class 2 biosafety cabinet by disaggregating with a syringe plunger through a 40µm cell strainer (Fisher) and flushing with RPMI. Spleen cells were resuspended in RBC lysis buffer (Sigma) for 1 minute to lyse red blood cells and 1ml RPMI was added to stop lysis. All samples were centrifuged at 400g for 5 minutes and resuspended in RPMI.

2.12 Ex- Vivo Stimulation

For intracellular staining, samples were incubated overnight in full stimulation medium with 1/500 Cell Stimulation Cocktail w/protein transport inhibitors (Invitrogen). The following day they were stained using the intracellular staining protocol listed below with an intracellular stain panel (see appendix).

2.13 Flow Cytometric Cell Analysis

All centrifugation as part of the flow cytometry protocol was carried out at 400g for 5 minutes unless stated otherwise. Samples were processed in 1.5ml Eppendorf tubes or round bottom 96 well plates (Corning). If required as part of the stain panel, cells were first suspended in 100µl of a live/dead stain (either Zombie Aqua (Biolegend) or 7AAD (eBioscience)) and incubated in the dark for 15 minutes. For all stain panels, samples were centrifuged and resuspended in 25µl PBS 1% BSA containing 1/200 anti-mouse CD16/32 (eBioscience), and incubated on ice for 15 minutes to block non-specific FC receptor binding.

For surface marker staining, an equal volume of surface antibody mastermix (2x) (see appendix) was added to each sample, mixed thoroughly, and incubated on ice for 30 minutes in the dark. All antibodies were used at a concentration of 1.5µg/ml with the exception of Brilliant Violet antibodies, which were used at 5µl per million cells.

For intracellular staining, samples were resuspended in 50µl Fix/Perm Buffer (eBioscience) and incubated on ice for at least 30 minutes (up to 18 hours/overnight). 200µl 1X Perm buffer (eBioscience) was then added to each

sample, before centrifuging and resuspending in 100µl 1X Perm buffer. Intracellular antibody mastermix (see appendix) was then added, and incubated for 30 minutes on ice in the dark.

Samples were centrifuged and resuspended in 200µl PBS (1% BSA) and pipetted into FACS tubes before being analysed on a Cytoflex flow cytometer (Beckman Coulter) following compensation using VersaComp Antibody Capture Beads (Beckman Coulter).

2.14 Mitochondrial Staining

Samples were centrifuged at 400g for 5 minutes and resuspended in MitoTracker mastermix (see appendix), and incubated at 37°C for 30 minutes. The samples were then centrifuged and resuspended in 200µl PBS (1% BSA), and transferred into FACS tubes. Samples were then analysed on a Cytoflex flow cytometer (Beckman Coulter).

2.15 Gating Strategy

In order to quantify populations of the various immune cell subsets required, the following gating strategy was utilised (Fig.2.1), taken from Yu et al. (2016). In order to better suit some of the specific samples used in this experiment gate positioning was modified. BMDM gating is also shown (Fig.2.2).

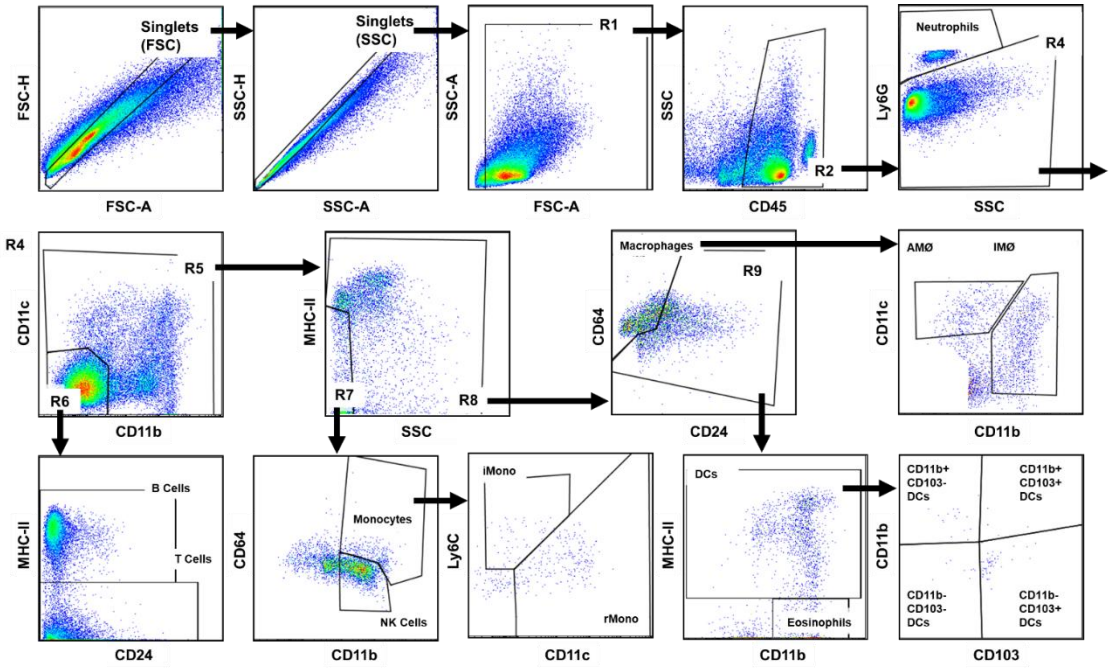


Figure 2.1: Flow cytometry gating strategy, adapted from Yu et al. (2016)*. Singlets were determined via height vs area correlation of both forward and side scatter. Gates are labelled with their respective cell populations, apart from gates R1-9, which contain multiple cell populations that are subsequently gated afterwards. Populations gated are: Neutrophils, Alveolar macrophages (AMΦ), Interstitial macrophages (iMΦ), Dendritic Cells (CD11b/CD103 + and -), B Cells, T Cells, Natural Killer (NK) Cells, inflammatory monocytes (iMono) and resident monocytes (rMono).*

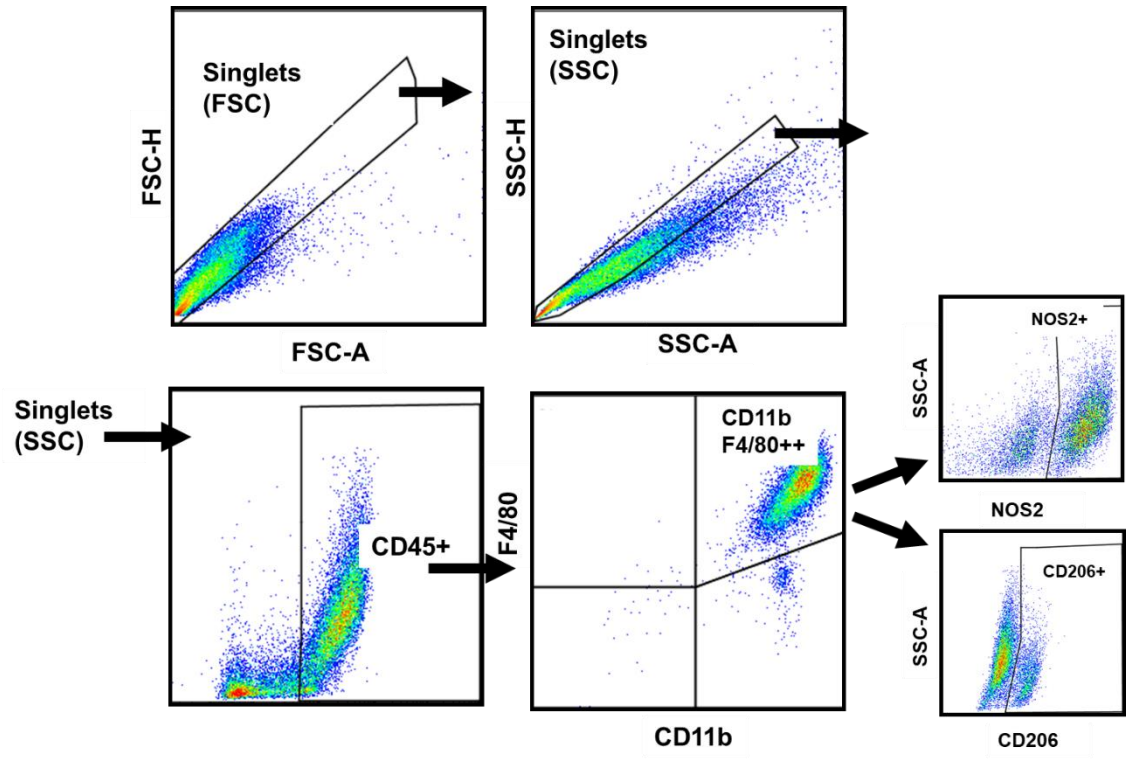


Figure 2.2: Bone Marrow-derived macrophage flow cytometry gating strategy*. Singlets were determined via height vs area correlation of both forward and side scatter. Macrophages were assessed as CD45+ CD11b F4/80++ cells. Macrophage polarisation was then determined via NOS2 (M1) or CD206 (M2) positivity.*

2.16 Cytometric Bead Array

Cytokine levels were assessed by Cytometric Bead Array (CBA), specifically a mouse inflammation flex kit (BD Biosciences) for IL-4, IL-9, IL-5, IL-10, IL-13, IL-17 IFNγ, and IL-4. Supernatants were incubated with antibody-coated capture beads for 1 hour at room temperature according to manufacturer's instructions. Cytokines were quantified using a detection reagent conjugated to PE and compared to standards of known concentration. Samples were acquired using a FACScanto flow cytometer (BD Biosciences) and analysed using FCAP Array (version 3) software (BD Biosciences).

2.17 Histology

Sections of small and large intestine, caecum, and the left lobe of the lung were collected in Carnoy's solution (60% Absolute Ethanol, 30% Chloroform, 10% Glacial Acetic Acid) for histological analysis.

After 24h, samples were removed from Carnoy's solution and placed in 70% ETOH. Prior to embedding, samples were prepared by placing into cassettes and dehydrating down an alcohol gradient (1hr x 90% ETOH, 2x1hr 100% ETOH), followed by 2 x 45 minutes in Xylene and 40min/overnight in molten paraffin wax (Fisher). Samples were embedded in Paraffin wax and cut to 5µm using a Leica RM 2235 manual microtome.

To assess goblet cells, samples were stained using PAS Alcian Blue (Thermo Fisher). After dewaxing (2 x 5 minutes Xylene), samples were rehydrated down an alcohol gradient (1min, 100%, 90%, 70%, 50%, H_2O), and stained in 1% Alcian Blue for 5 minutes. They were washed for 1 minute in distilled H_2O and 5 minutes in running H_2O. They were then stained in 0.5% Periodic Acid for 5 minutes, washed as above, then stained in Schiff's reagent for 15 minutes and washed as above. Samples were counterstained in Mayer's haematoxylin for 1 minute, washed as above, and dehydrated (30 seconds 70% ETOH, 30 seconds 100% ETOH) and cleared (1 minute in Xylene).

Pathology was also assessed using a Haematoxylin and Eosin (H&E) stain. Samples were dewaxed and rehydrated as above and stained in Harris Haematoxylin for 5 minutes. After washing in running tap water for 1 minute,

samples were differentiated using 1% hydrochloric acid in 70% ethanol for 10 seconds and washed again for 5 minutes. They were then stained in 1% Eosin for 4 minutes, washed for 1 minute, dehydrated (1min dH_2O, 30sec 50%, 70%, 90%, 100% x2) and cleared (5 minutes in Xylene). All samples were mounted using DPX mountant (Sigma) and coverslipped.

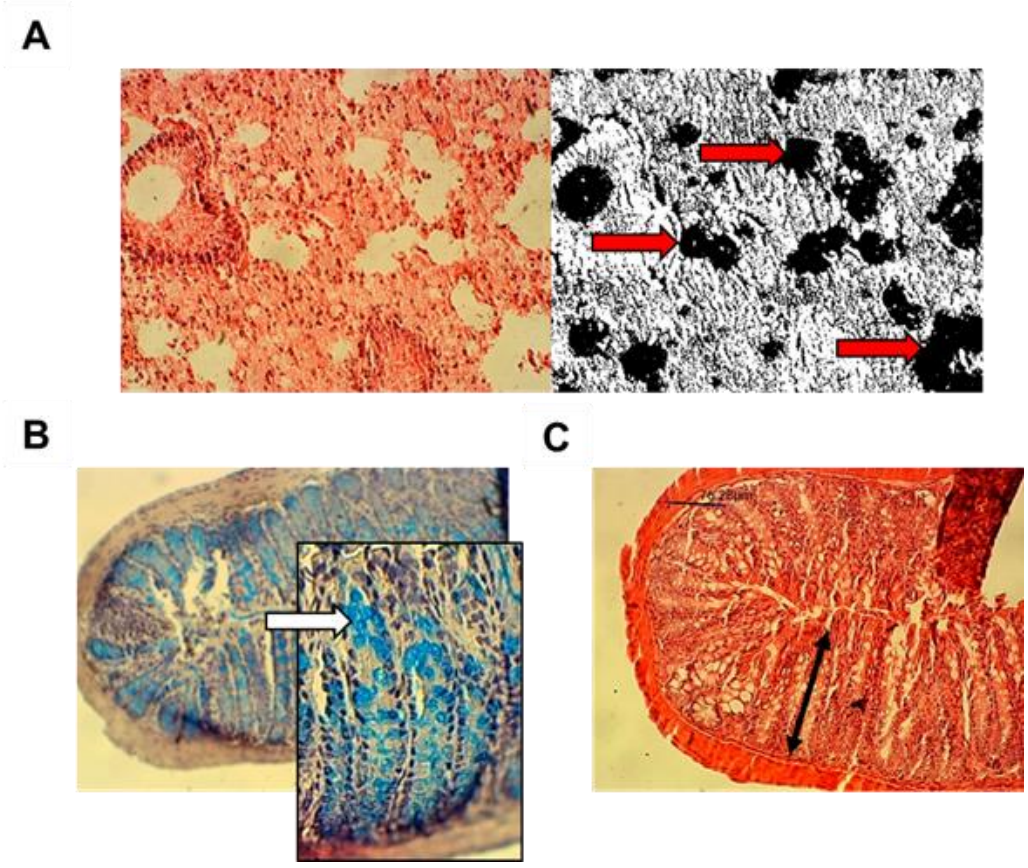


Figure 2.3: Histological analysis rationale*. A: Lung sections were converted into black and white using the threshold function, then inverted air gaps to black (see arrows). The analyse particles function was used to quantify air gaps. B: Goblet cells per colon crypt were counted, indicated by arrow in this figure. C: Colon crypt depth measured using scale bar created in the microscope software.*

2.18 Denaturing Gradient Gel Electrophoresis (DGGE)

2.18.1 Faecal DNA Extraction

DNA was extracted from mouse faeces using a modified version of the QIAamp® Fast DNA Stool Mini Kit protocol (Qiagen). The whole faecal sample was homogenised in 1ml InhibitEx buffer by agitating with an inoculation loop. Samples were then heated at 95°C for 30minutes, with the rest of the protocol unmodified (human DNA analysis protocol version), with the exception of the

final incubation before sample elution. In this case, 100µl of buffer ATE was pipetted onto the column, and incubated for 5 minutes at room temperature, before elution via centrifugation. DNA concentration was then quantified using a Qubit (Invitrogen) photometer.

2.18.2 PCR Amplification of 16s DNA

A PCR mastermix was prepared consisting of 12 µl REDTaq ReadyMix PCR Reaction Mix (Sigma), 1µl Forward Primer (341 6F+GC), 1µl reverse primer (518 R), 1µl BSA (20mg/ml), 2µl nuclease free water per sample. The mastermix was added to 100ng of extracted sample DNA in 10µl nuclease-free water, giving a total PCR volume of 27µl per sample. Samples were vortexed, and then ran on a TC-3000 PCR machine (Techne). PCR Parameters were as follows: 1 cycle of 95°C for 5 mins, 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and 1 cycle of 72°C for 10 mins.

2.18.3 Post-PCR DNA Purification

Post-PCR DNA purification was performed using an adapted version of the MinElute PCR Purification Kit (Qiagen). The protocol was modified via the addition of 10µl 3M Sodium Acetate (Sigma) to each sample instead of the provided pH indicator prior to loading them onto the MinElute columns. For the final step, 20µl of Buffer EB was added to the column membranes. DNA concentration was then quantified using a Qubit (Invitrogen) photometer.

2.18.4 Gel Pouring and Running

Denaturing Gradient Gel Electrophoresis was carried out using the DCode Universal Mutation Detection System (Bio-Rad). Glass gel plates were first cleaned in acetone; rubber spacers were greased and sandwiched between the plates, which were clamped into the plate holder. The seal was then tested with water to ensure no leakage.

To form the gradient in the gel, two stock gel solutions were prepared, with 100% and 0% denaturant respectively. The 100% denaturant gel was first

made (8.4g Urea, 5ml Acrylamide, 8ml Formamide, 0.4ml TAE 50x), heated to 50°C and stirred till the urea dissolved. The 0% denaturant gel was then made (5ml Acrylamide, 14.6ml qH_2O, 0.4ml TAE 50x). Low (30%) and high-grade (70%) denaturant gel media are required for the DGGE gel. These were made from the stock media (High grade (70%): 11.2ml of 100% denaturant solution, 4.8ml 0% denaturant, Low grade (30%): 4.8ml 100% denaturant, 11.2ml 0% denaturant). Both gel medias were cooled to -20°C for approx. 30 minutes to slow down polymerisation. After cooling, 150µl 10% Ammonium Persulfate and 8.5µl Tetramethylethylenediamine (TEMED) were added to each solution. The gel was then poured using a two chamber gradient gel mixer, attached to a peristaltic pump (Bio-Rad), tube via 21G needle. Once poured, the comb was inserted into the top of the plates, and TEMED added to the top of the gel to catalyse polymerisation. Once set, the comb was removed, and the wells flushed with 1X TAE to remove any excess TEMED. When the tank temperature reached 60°C, the gel was inserted into the tank. For each sample, 150ng of purified DNA in 12µl with 4µl loading dye was loaded into the well. The gel was then run overnight at 63v at 60°C. The following day, the plates were removed from the gel, which was stained with 20ml 1/500 SYBR Gold (Fisher) in the dark for 90 minutes at room temperature. The gel was then imaged using a Chemidoc (Bio-Rad) or Typhoon 9500 (GE Healthcare).

2.19 Statistical Analysis

Statistical analysis was carried out using Microsoft Excel and Prism 8 (Graphpad). Significance testing was carried out using one and two-way ANOVA followed by Sidak's multiple comparison test, or REML Mixed-effects analysis followed by Sidak's multiple comparisons test. DGGE analysis was carried out using CLIQS 1D Pro and R (VEGAN, ECODIST and MASS packages). CLIQS 1D pro was used for band matching, creating a band matrix for further analysis. In R, clustering analysis was performed using the NMDS script, with significance testing using ADONIS. Image analysis was carried out using FIJI (ImageJ).

Chapter 3: Investigating the direct modulation of macrophages by 2nd and 3rd generation fluoroquinolones

3.1 Introduction

Key cells in the innate immune response to respiratory infections are macrophages (Byrne et al., 2015). They are an incredibly diverse cell type, and perform many functions, ranging from phagocytosing pathogens and clearing cell debris to antigen presentation and cytokine release. Macrophages are dispersed throughout the body, composing phenotypically distinct subtypes highly specialised for the environment in which they are located. These tissue macrophages are embryonically derived, developing distinctly depending on their niche (Yona et al., 2013). They are supported, during inflammation especially, by circulating monocytes that can be recruited from the bone marrow to specific sites and mature to form monocyte-derived macrophages (Epelman et al., 2014).

As antibiotics are commonly prescribed to treat respiratory infections, it stands to reason to test their off-target effects on immune cells, as they play a major role in the host's response to infection. Furthermore, both Yang et al. (2017) and Lankelma et al. (2017) found impaired macrophage phagocytosis as a key marker of the immune response being affected, both by antibiotics directly and via the induced microbial dysbiosis. Lankelma et al. showed that broad spectrum antibiotic-induced dysbiosis impairs the ability of alveolar macrophages to phagocytose pathogens *in vivo*; while Yang et al. directly linked reduced phagocytosis in a macrophage cell line to the fluoroquinolone ciprofloxacin induced reductions in cellular respiration.

In order to further investigate direct roles of both 2nd and 3rd generation fluoroquinolones on macrophage phenotype, we utilised the Bone marrow-derived macrophage (BMDMs) system to gain a more reductionist, *ex vivo* view of the effects of ciprofloxacin and levofloxacin on the innate immune response. Once the culture of BMDMs was optimised, experiments involved the addition of fluoroquinolones to the growth medium at both the developing and mature macrophage stage prior to polarisation towards M1 and M2 phenotypes respectively (Fig.3.1).

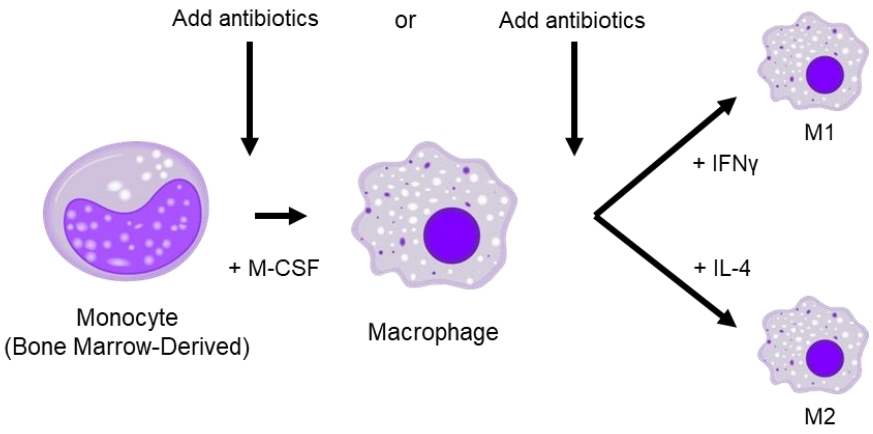


Figure 3.1. *Ex-Vivo* BMDM Experimental Design

Schematic to demonstrate the experimental design of BMDM experiments. Bone marrow cells were isolated from the femurs of wild-type mice and driven into macrophages using macrophage colony stimulating factor (M-CSF) in antibiotic free growth medium. Fluoroquinolones were added either immediately at day 0 to assess effects on macrophage development, or at day 7, to assess effects on mature macrophages prior to the polarisation to M1 or M2 phenotype via the addition of the cytokines IFNγ or IL-4 respectively.

The main objectives of the work in this chapter were:

I. **To investigate the effect of the fluoroquinolones ciprofloxacin and levofloxacin on mature macrophage M1/M2 polarisation**
II. **To investigate the effect of the fluoroquinolones ciprofloxacin and levofloxacin on developing macrophage M1/M2 polarisation**
III. **To investigate the effect of the fluoroquinolones ciprofloxacin and levofloxacin on macrophage activation, cytokine profile and phagocytosis phenotypes**
IV. **To investigate the toxicity of the fluoroquinolones, ciprofloxacin and levofloxacin on macrophages**

3.2 Fluoroquinolones do not alter the ability of mature macrophages to polarise to the M1 or M2 phenotype

Ex-vivo Bone Marrow-derived macrophages were used in order to create a reductionist, sterile experimental macrophage model to examine the effects of 2nd and 3rd generation fluoroquinolones ciprofloxacin and levofloxacin respectively. Polarisation is a key aspect of macrophage activation and function, enabling a specialisation to one of several phenotypes, all with different characteristics and pro/anti-inflammatory functions (Mills et al., 2000). To determine this, BMDMs were grown for 7 days as previously described. For the final 24 hours of the experiment, 30µM Ciprofloxacin or Levofloxacin were added to the cells, which were also polarised with 20ng/ml IFNγ (M1) or IL-4 (M2), with polarisation determined via flow cytometry of markers NOS2 (M1; (Bogdan et al., 2000)) or CD206 (M2 (Azad et al., 2014)). Antibiotics were initially dosed in w/v concentrations, then converted to equivalent molar concentrations to ensure comparability with other antibiotics with very different molecular weights. In preliminary experiments, doses were titrated within a range indicated by other *in vitro* studies (Dalhoff *et al.* 2005) to determine whether a dose-dependent effect was seen. It was not, and as such the current dose was decided upon.

On examining the purity of the BMDM population the absence of antibiotics, usually present in most cultures, did not seem to inhibit development of macrophages as a high (>96%) purity was seen in all experiments (Fig.3.2A). This high percent purity was not altered following overnight incubation with either ciprofloxacin or levofloxacin (Fig.3.2A). We next examined if fluoroquinolones were able to polarise mature macrophages to the M1/M2 phenotype alone, i.e. in the absence of IFNγ of IL-4. We found no significant change in NOS2 following either ciprofloxacin or levofloxacin treatment as compared to the no antibiotic controls (Fig. 3.2B). Equally, we found no significant change in CD206 following either ciprofloxacin or levofloxacin treatment as compared to the no antibiotic controls (Fig. 3.2B). Finally, we examined the influence of fluoroquinolones on mature M1/2 polarisation in the presence of the cytokine drivers IFNγ and IL-4 respectively. As expected, when

BMDMs were stimulated with IFNγ, M1 polarisation (%NOS2+) increased. Similarly, when they were stimulated with IL-4, M2 polarisation also increased (%CD206+) (Fig. 3.2B and C). However, addition of either ciprofloxacin or levofloxacin with IFNγ/IL-4 did not affect the extent to which BMDMs polarised to either M1 or M2 as compared to the no antibiotic controls (Fig. 3.2B and C). Collectively, these data indicate that mature BMDMs purity and potential to be polarised to the M1 or M2 phenotype are unaltered in the presence of fluoroquinolones.

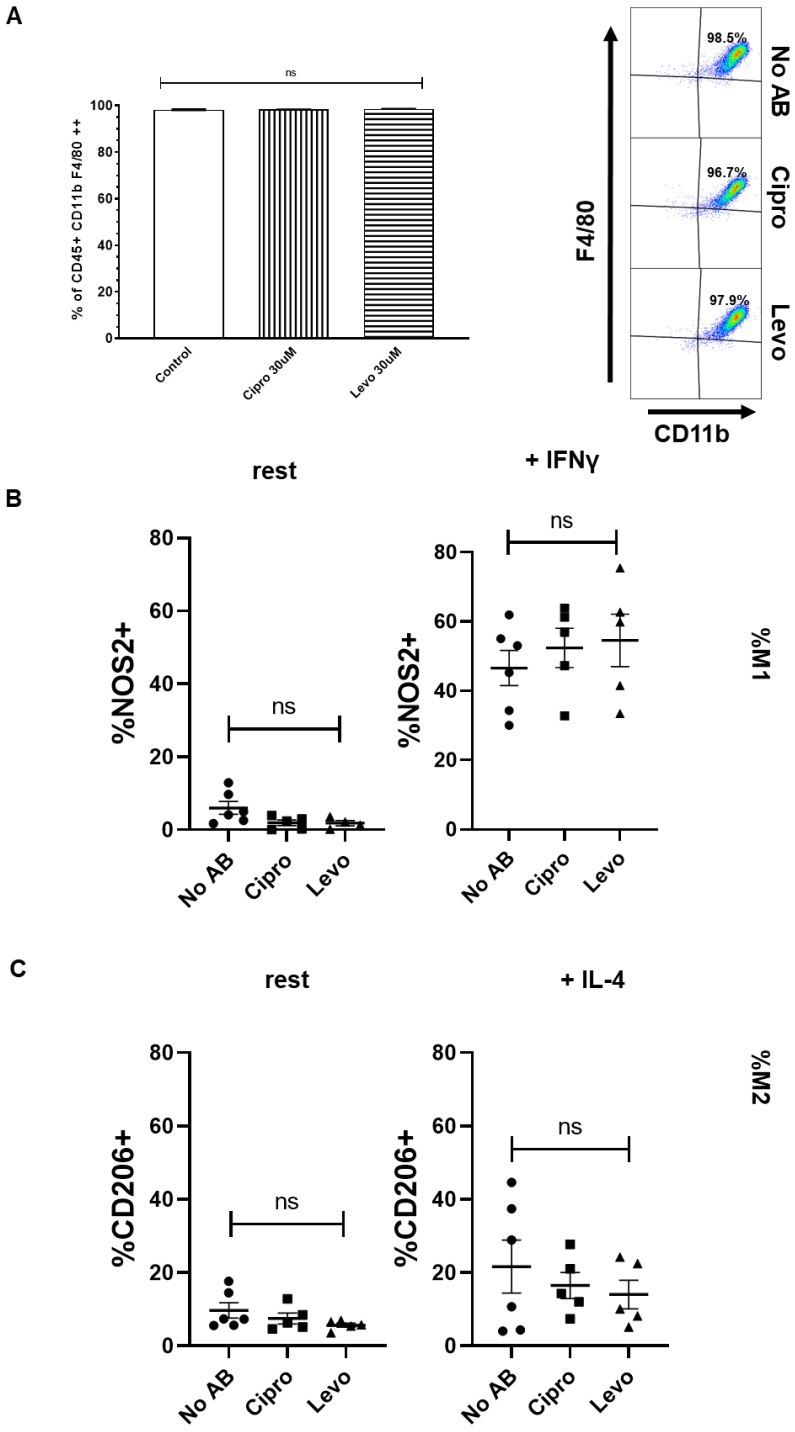


Figure 3.2 Fluoroquinolones do not alter mature macrophages to M1 or M2 polarisation. Bone marrow cells were isolated and grown into Bone Marrow-derived Macrophages (BMDMs) via the addition of 20ng/ml M-CSF for 7 days. BMDMs were then treated with 30µM fluoroquinolone antibiotics and polarised for M1/M2 with 20ng/ml IFNγ or 20ng/ml IL-4 for 24h. A) Macrophage purity (F4/80+ CD11b+) and representative flow cytometry plots of no antibiotic control and M1/M2 polarised macrophages with/without fluoroquinolone antibiotics. B) %NOS2+ BMDMs with and without 20ng/ml IFNγ M1 stimulation for control and ciprofloxacin or levofloxacin. C) %CD206+ BMDMs with and without 20ng/ml IL-4 M2 stimulation for control and ciprofloxacin or levofloxacin. *, P<0.05; **, P<0.01; ***, P<0.005; N.S., not significant using one way ANOVA followed by Tukey's multiple comparison test,(n=5-6) are from 3 independent experiments.

3.3 Levofloxacin drives developing BMDMs to be more responsive to M1 polarisation

Given that circulating monocytes are recruited from the bone marrow to specific sites of inflammation and mature to form monocyte-derived macrophages (Epelman et al., 2014), we next decided to focus on the effects fluoroquinolones could have on bone marrow cells if they were present during their development to macrophages. This was achieved by adding fluoroquinolones to cells immediately after their extraction and throughout their 7 day developing incubation and subsequent 1 day polarisation (Fig.3.1).

As previously seen, antibiotic free cultures of BMDMs developed into highly pure macrophage populations (>95%) (Fig.3.3A). The addition of ciprofloxacin or levofloxacin did not alter the potential for macrophage development with no significant difference as compared to the antibiotic free control cultures (Fig.3.3A). We next examined if fluoroquinolones were able to polarise developing macrophages to the M1/M2 phenotype alone, i.e. in the absence of IFNγ or IL-4. We found no significant change in NOS2 following either ciprofloxacin or levofloxacin treatment as compared to the no antibiotic controls (Fig. 3.3D).

However, when polarised to the M1 phenotype with IFNγ, levofloxacin treated cells showed significantly increased NOS2+ macrophages versus antibiotic free controls (Fig.3.3C and D). Ciprofloxacin treated BMDMs also showed an increased mean of M1 polarisation as compared to antibiotic free controls, though this did not reach significance (Fig.3.3C and D).

Given our strong NOS2 expression and significant results we next confirmed our antibody specificity via utilising a fluorescence minus one (FMO) panel lacking the NOS2 antibody (Fig. 3.3B), verifying our NOS2+ M1 cell staining.

Collectively, these data suggest levofloxacin may have a role in ‘priming’ bone marrow developing macrophage polarisation to a classical M1 phenotype in the presence of an existing stimulus.

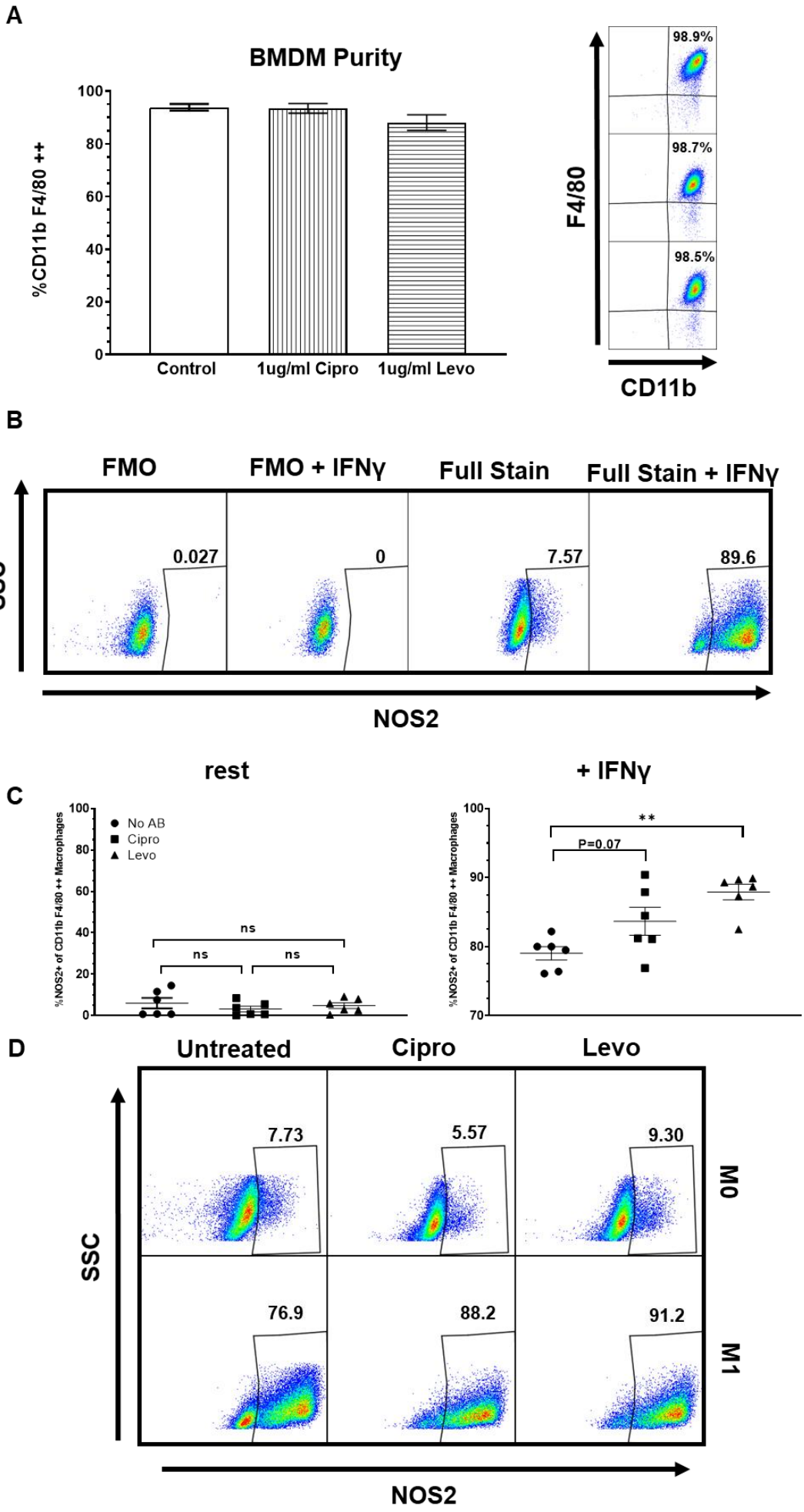

A
BMDM Purity
%CD11b F4/80 ++
100
80
60
40
20
0
Control
1ug/ml Cipro
1ug/ml Levo
98.9%
98.7%
98.5%
F4/80
CD11b
B
FMO
FMO + IFNγ
Full Stain
Full Stain + IFNγ
0.027
0
7.57
89.6
SSC
NOS2
C
rest
+ IFNγ
No AB
Cipro
Levo
%NOS2+ of CD11b F4/80 ++ Macrophages
ns
ns
ns
P=0.07
**
D
Untreated
Cipro
Levo
7.73
5.57
9.30
76.9
88.2
91.2
M0
M1
SSC
NOS2

Figure 3.3 Levofloxacin primes bone marrow derived macrophages for M1 polarisation. *Bone marrow cells were isolated and grown into Bone Marrow-derived Macrophages (BMDMs) via the addition of 20ng/ml M-CSF for 7 days with 30μM fluoroquinolone antibiotics and then polarised for M1/M2 with 20ng/ml IFNγ or 20ng/ml IL-4 for 24h. A) Macrophage purity (F4/80+ CD11b+) and representative flow cytometry plots of no antibiotic control and M1/M2 polarised macrophages with/without fluoroquinolone antibiotics. B) %NOS2+ BMDMs and C) representative flow cytometry plots with and without 20ng/ml IFNγ M1 stimulation for control and ciprofloxacin or levofloxacin. D) Verification of NOS2 staining in representative plots of levofloxacin treated BMDMs by FMO panel with and without IFNγ M1 stimulation. *, P<0.05; **, P<0.01; ***, P<0.005; N.S., not significant using one way ANOVA followed by Tukey's multiple comparison test (n=6 via 2 independent experiments).*

3.4 Fluoroquinolones do not alter cytokine production from developing BMDMs

Given the priming effect that levofloxacin had on developing BMDM M1/M2 phenotypical surface markers, we next assessed cytokine production from the cell supernatants flowing fluoroquinolone incubation via cytometric bead array. Supernatants were analysed for the presence of IL-13, TNF, IL-4, IL-5, IL-10, IL-9, IL-17a, and IFNγ. No detectable amounts of IL-5, IL-10, IL-9 or IL-17a were detected in any of the groups (data not shown).

IL-13 levels showed no alteration in BMDMs culture supernatants incubated with ciprofloxacin or levofloxacin as compared to no antibiotic control groups (Fig.3.4A). Interestingly the mean level of IL-13 was reduced in both the M1 and M2 polarised BMDM cultures in the ciprofloxacin and levofloxacin groups as compared to the non-antibiotic controls, however this did not reach significance in any groups (Fig.3.4A). Conversely, mean TNFα levels were higher in the fluoroquinolone treated groups as compared to the non-antibiotic control group, but again this did not reach significance (Fig. 3.4B).

Both IFNγ and IL-4 were only detected in the supernatants of cells that were stimulated to become M1 or M2 respectively, most likely due to the addition of these polarising cytokines (Fig. 3.4C and D), with no significant differences between fluoroquinolone treated groups and the non-antibiotic treated controls. Interestingly, the mean IL-4 levels were highest in the levofloxacin treated group, but as stated, this did not reach significance (Fig. 3.4D).

Collectively, beyond some trends, these data indicate that fluoroquinolones do not alter the cytokine output of BMDMs.

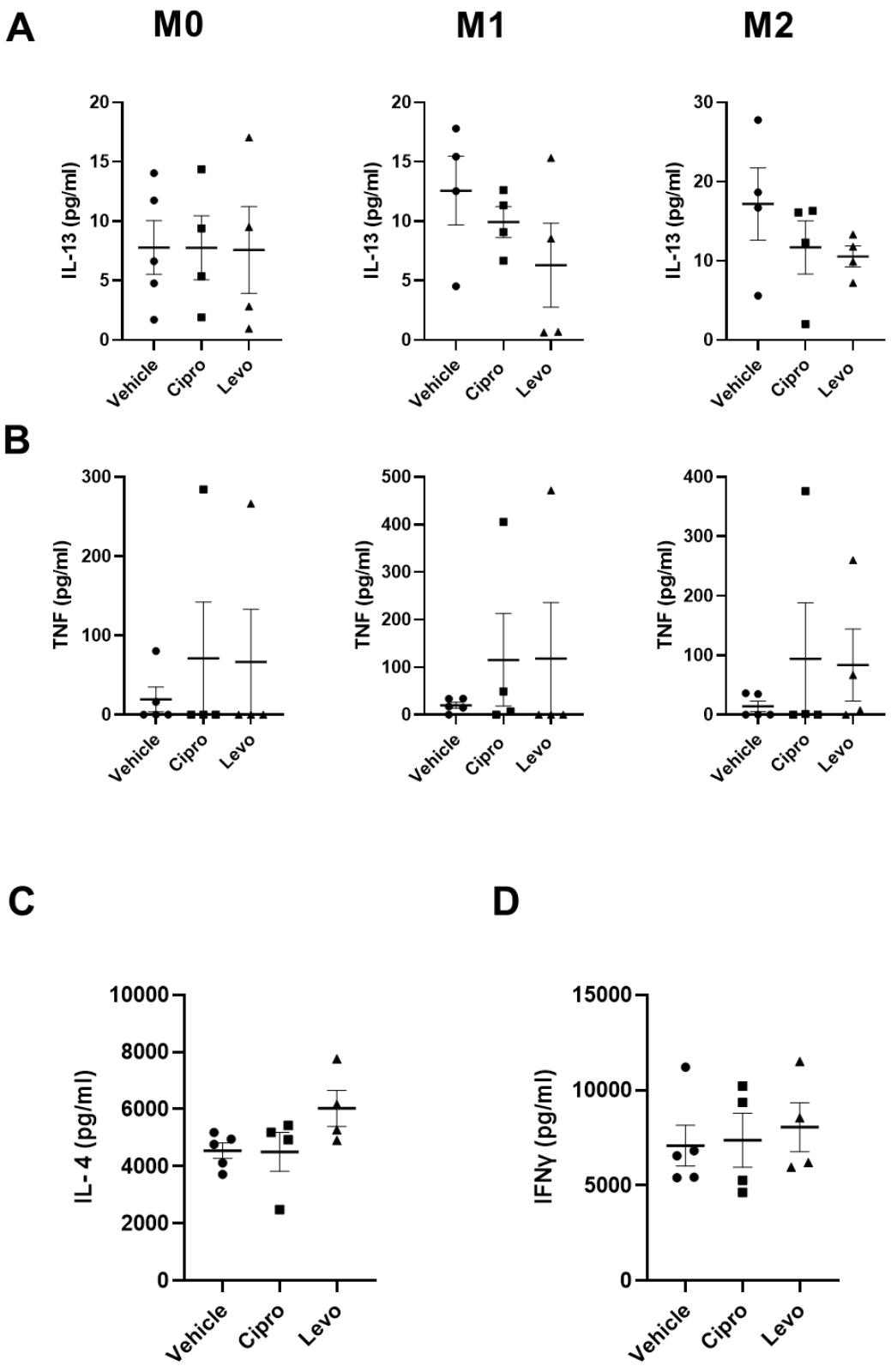


Figure 3.4. Fluoroquinolones do not alter cytokine output from BMDMs. *Bone marrow cells were isolated and grown into Bone Marrow-derived Macrophages via the addition of 20ng/ml M-CSF whilst incubated with 30μM Ciprofloxacin or Levofloxacin, and polarised with 20ng/ml IFNγ (M1) or 20ng/ml IL-4 (M2) for 24h after 7 days. (Purity >99% Gating strategy: CD45+, CD11b F4/80++). At day 8, supernatants were frozen and analysed for cytokines using a Cytometric Bead Array (BD). A) IL-13 production (pg/ml) by BMDMs with and without IFNγ or IL-4 polarisation. B) TNF production (pg/ml) by BMDMs with and without IFNγ or IL-4 polarisation. C) IL-4 levels (pg/ml) in BMDM media after IL-4 polarisation. D) IFNγ levels (pg/ml) in BMDM media after IFNγ polarisation. *, P<0.05; **, P<0.01; ***, P<0.005; N.S., not significant using one way ANOVA followed by Tukey's multiple comparison test (n=4-5 via 2 independent experiments).*

3.5 Levofloxacin increases phagocytosis in unpolarised (M0) and M2 BMDMs

Reduced phagocytic function has been shown as an indicator of both dysbiosis and metabolite driven macrophage modulation following antibiotic treatment (Yang et al., 2017, Lankelma et al., 2017). To determine if fluoroquinolone treatment of our *ex vivo* BMDM cultures occurred, we utilised a phagocytosis assay using latex beads coated with fluorescently labelled IgG. BMDMs were generated with fluoroquinolone treatment from day 0 and polarised as previously described. Initial examination demonstrated that after 30 minutes incubation with beads there was little phagocytosis, with a range of around 5% to 10% FITC+ in M0 conditions, with no significant difference between M1/2 polarised and M0 controls (Fig. 3.5A).

To gain more resolution, cells were incubated for 60 minutes and, as expected, there was a far greater level of phagocytosis in the region of 65%, with again with no significant difference between M1/2 and M0 controls (Fig. 3.5A). We therefore proceeded with the 60 minute incubation and observed with no cytokine polarisation (M0), although no differences were seen in ciprofloxacin treated BMDMs, levofloxacin caused a significant increase in phagocytosis vs non-antibiotic controls (Fig. 3.5C and D). We saw no significant alterations in phagocytosis following M1 polarisation in ciprofloxacin or levofloxacin treated groups versus non-antibiotic controls (Fig. 3.5C) Similarly to M0 results, under M2 polarising conditions, although ciprofloxacin did not cause a significant alteration, levofloxacin treatment significantly increased phagocytosis versus non-antibiotic controls (Fig.3.5C). Collectively, these data indicate that the 3rd generation levofloxacin increases phagocytic activity of BMDMs.

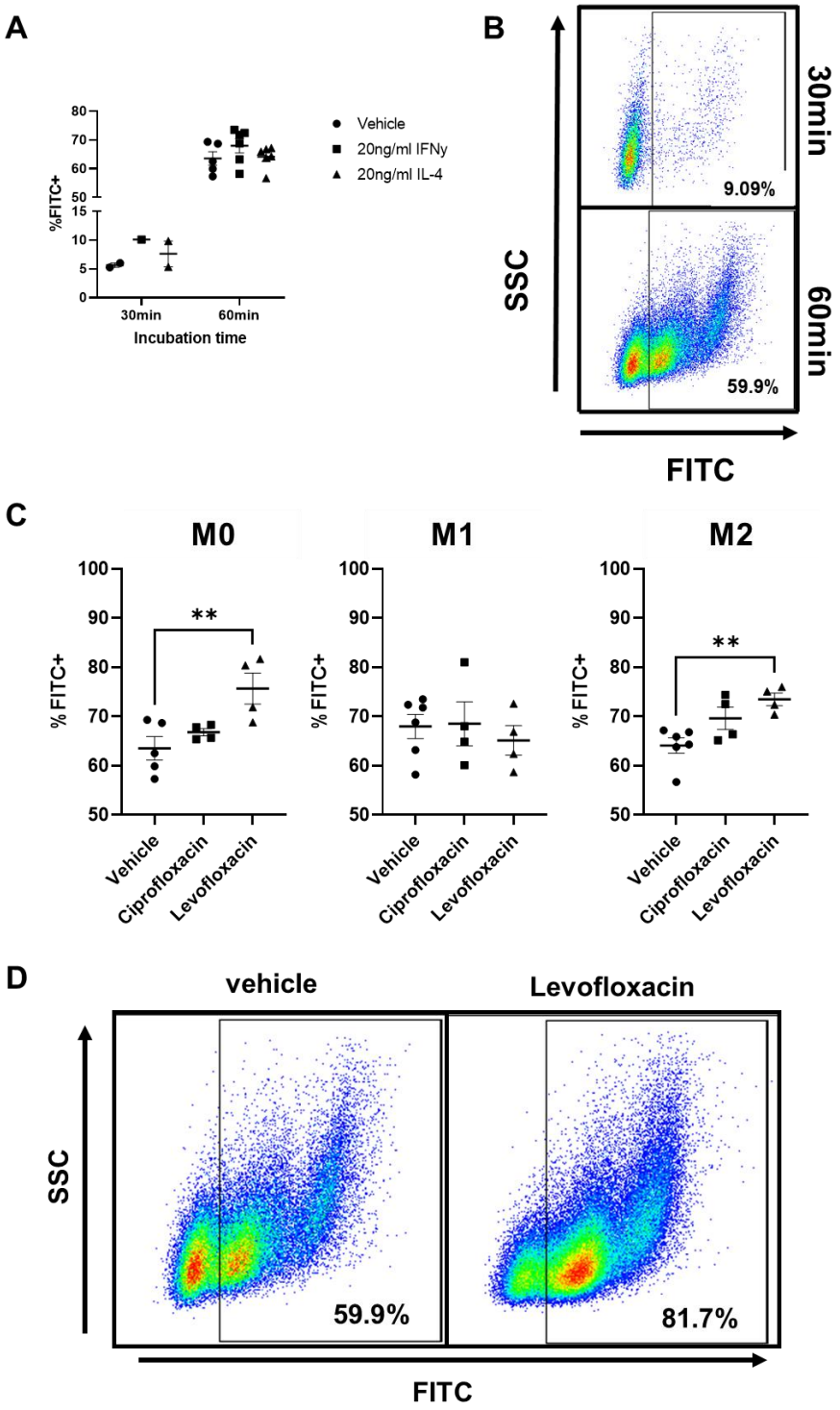


Figure 3.5.Levofloxacin Increases Phagocytosis in BMDMs after 60 minutes. *Bone marrow cells were isolated, and grown into Bone Marrow-derived Macrophages via the addition of 20ng/ml M-CSF whilst incubated with 30μM Ciprofloxacin or Levofloxacin, and polarised with 20ng/ml IFNγ (M1) or 20ng/ml IL-4 (M2) for 24h after 7 days. (Purity >99% Gating strategy: CD45+, CD11b F4/80++). After polarisation, cells were incubated with IgG-FITC labelled latex beads at 37°C, 5% CO_2. A) %FITC+ BMDMs M0 when polarised to M1 or M2, when incubated with IgG-FITC labelled beads for either 30 or 60 minutes B) Representative flow cytometry plots showing bead uptake after 30 and 60 minutes M0. C) %FITC+ BMDMs when grown in 30uM Ciprofloxacin or Levofloxacin, and polarised to M0, M1 or M2 and incubated with phagocytosis beads for 60 minutes. D) Representative flow cytometry plots showing bead uptake in Levofloxacin-treated BMDMs vs vehicle M0. *, P<0.05; **, P<0.01; ***, P<0.005; N.S., not significant using one way ANOVA followed by Tukey's multiple comparison test (n=2-6 via 3 independent experiments).*

3.6 Fluoroquinolone treatment does not induce cytotoxicity or alter pH in *ex vivo* BMDM culture conditions

In order to further understand the mechanisms by which levofloxacin was altering BMDM polarisation and phagocytosis, we first sought to rule out the possibility that fluoroquinolones were cytotoxic at the experimental concentrations used. To investigate this a Lactate Dehydrogenase (LDH) assay was performed. LDH is an enzyme released by damage to the plasma membrane, thus indicating a compound is having a cytotoxic effect on the cells present. Both Ciprofloxacin and Levofloxacin were titrated at concentrations ranging from 0.1µg/ml-100µg/ml (experimental concentrations 1-10µg/ml). As can be seen from the figure (figure 3.6A and B), neither Ciprofloxacin nor Levofloxacin caused any increases in LDH, and hence cytotoxicity, whatsoever at any of the concentrations tested, while forcibly lysed cells produced the expected increases in LDH acting as a positive control.

Certain antibiotics are known to be particularly acidic in *in-vivo* concentrations, namely Doxycycline Hyclate. Given macrophage polarisation can be influenced by pH (Wu et al., 2019), we wanted to determine if this was responsible in our *ex vivo* BMDM cultures. The pH of BMDM culture media was measured with and without fluoroquinolones, with no significant difference between pH observed in any of the conditions, and delivery times, as compared to non-antibiotic controls (Fig.3.7). Collectively, these data show neither ciprofloxacin nor levofloxacin affect BMDM cell survival or media pH, and that their effects on macrophage polarisation and phagocytosis are independent of cytotoxicity and pH.

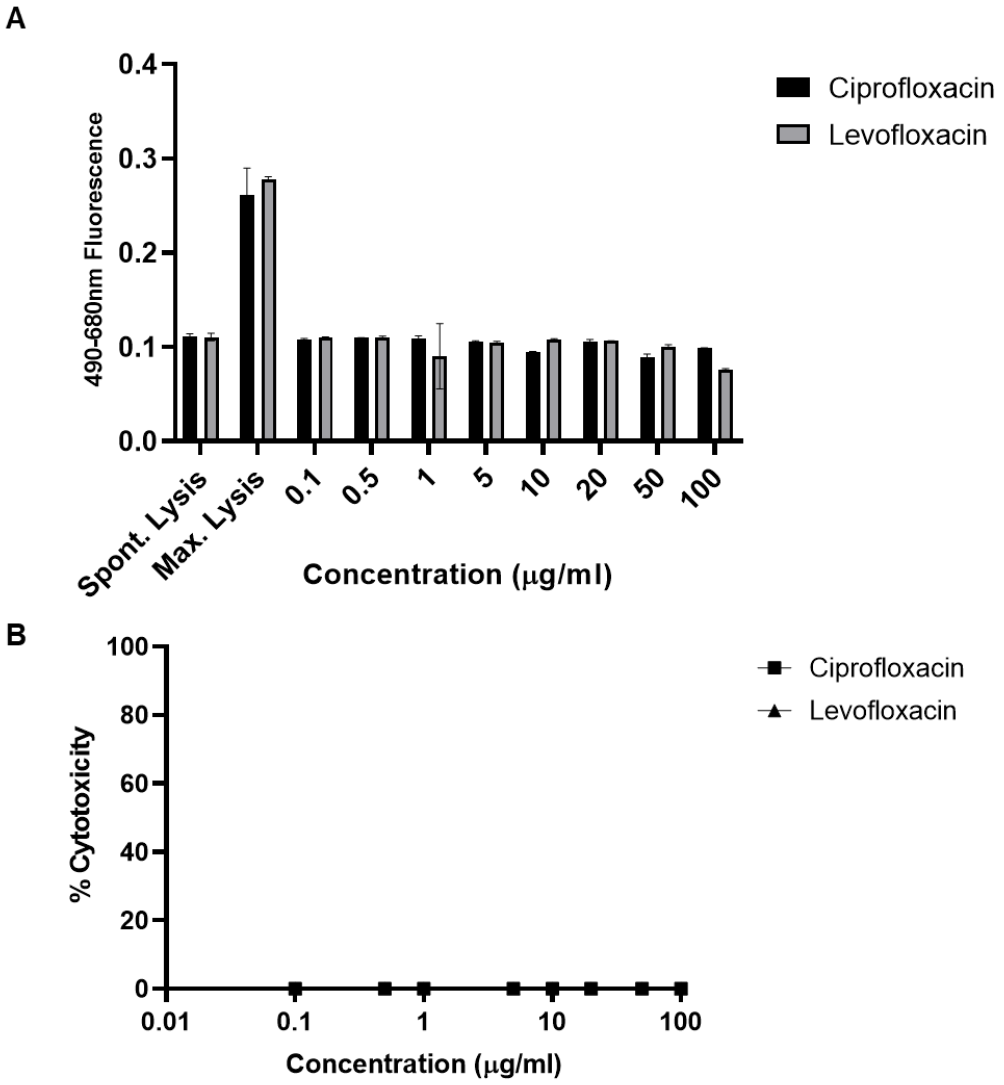


Figure 3.6 Fluoroquinolones do not cause cytotoxicity in BMDM cultures *Bone marrow cells were isolated and grown into Bone Marrow-derived Macrophages (BMDMs) via the addition of 20ng/ml M-CSF for 7 days They were treated with fluoroquinolones at a range of concentrations from 0.1-100μg/ml, and a Lactate Dehydrogenase assay performed. A) Fluorescence (480-690nm) of all conditions including positive and negative controls. B) Cytotoxicity of Ciprofloxacin and Levofloxacin on BMDMs at different concentrations. Both Ciprofloxacin and Levofloxacin are shown, with SEM error bars. *, P<0.05; **, P<0.01; ***, P<0.005; N.S., not significant using one way ANOVA followed by Tukey's multiple comparison test (n=3 via 3 independent experiments).*

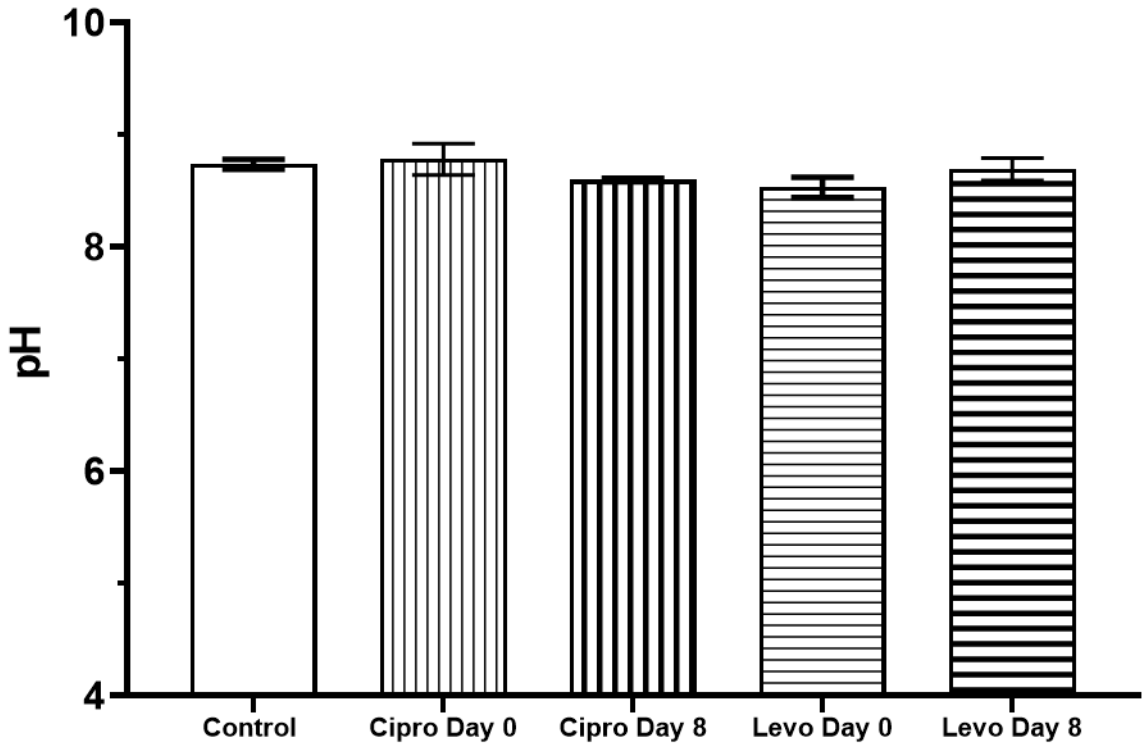


Figure 3.7.Fluoroquinolones do not influence BMDM culture media pH. *Bone marrow cells were isolated and grown into Bone Marrow-derived Macrophages (BMDMs) via the addition of 20ng/ml M-CSF for 7 days. 30μM Ciprofloxacin or Levofloxacin was added either from day 0, or to the media for 24hrs. Media was then tested for pH using a benchtop pH meter. *, P<0.05; **, P<0.01; ***, P<0.005; N.S., not significant using one way ANOVA followed by Tukey's multiple comparison test (n=2).*

3.7 Fluoroquinolone treatment produces mitochondrial hyperpolarisation

Mitochondrial damage has been postulated as a potential contributor to the various symptoms of FQAD for some time (Hangas et al., 2018, Kalghatgi et al., 2013, Kaur et al., 2016). However, research is yet to link fluoroquinolone induced mitochondrial alterations to any effects on immune cells. Interestingly, macrophage M1 polarisation is well established to be involve mitochondrial hyperpolarisation known to drive mitochondrial repurposing from ATP synthesis to ROS production (Mills et al., 2016). We therefore hypothesised if fluoroquinolone induced mitochondrial membrane potential alterations could be linked to our observations of increased M1 polarisation and phagocytosis.

To detect mitochondrial function in BMDMs, they were stained with MitoTrackers. BMDMs were grown as previously described, with either ciprofloxacin or levofloxacin added to bone marrow cells from day 0. After 7 days, they were artificially polarised to the M1 (classical) or M2 (Alternative) phenotype by adding 20ng/ml IFNγ or IL-4 (respectively) overnight. The following day they were stained MitoTracker Green FM was used to quantify overall mitochondrial mass, whereas MitoTracker Orange CMTMRos is membrane potential dependent, and used to quantify mitochondria activity.

In non-antibiotic controls, the Orange/Green ratio of MitoTrackers is around 1 in non-polarised M0 conditions, while M1 polarisation caused an increase in membrane potential, which was not observed in M2 polarising conditions. However, this did not reach significance (Fig.3.8A). When ciprofloxacin or levofloxacin were added, regardless of polarisation, the Orange/Green ratio increased significantly, to around 8-10 in some cases. This was dose-dependent, with Orange/Green ratio increasing when the fluoroquinolone dose was increased from 1 to 10µg/ml (Fig. 3.8A and B). These findings indicate both ciprofloxacin and levofloxacin cause mitochondrial hyperpolarisation in *ex vivo* developed macrophages *in vitro* and importantly is present in M0 conditions suggesting a priming of mitochondria for M1 macrophage polarisation.

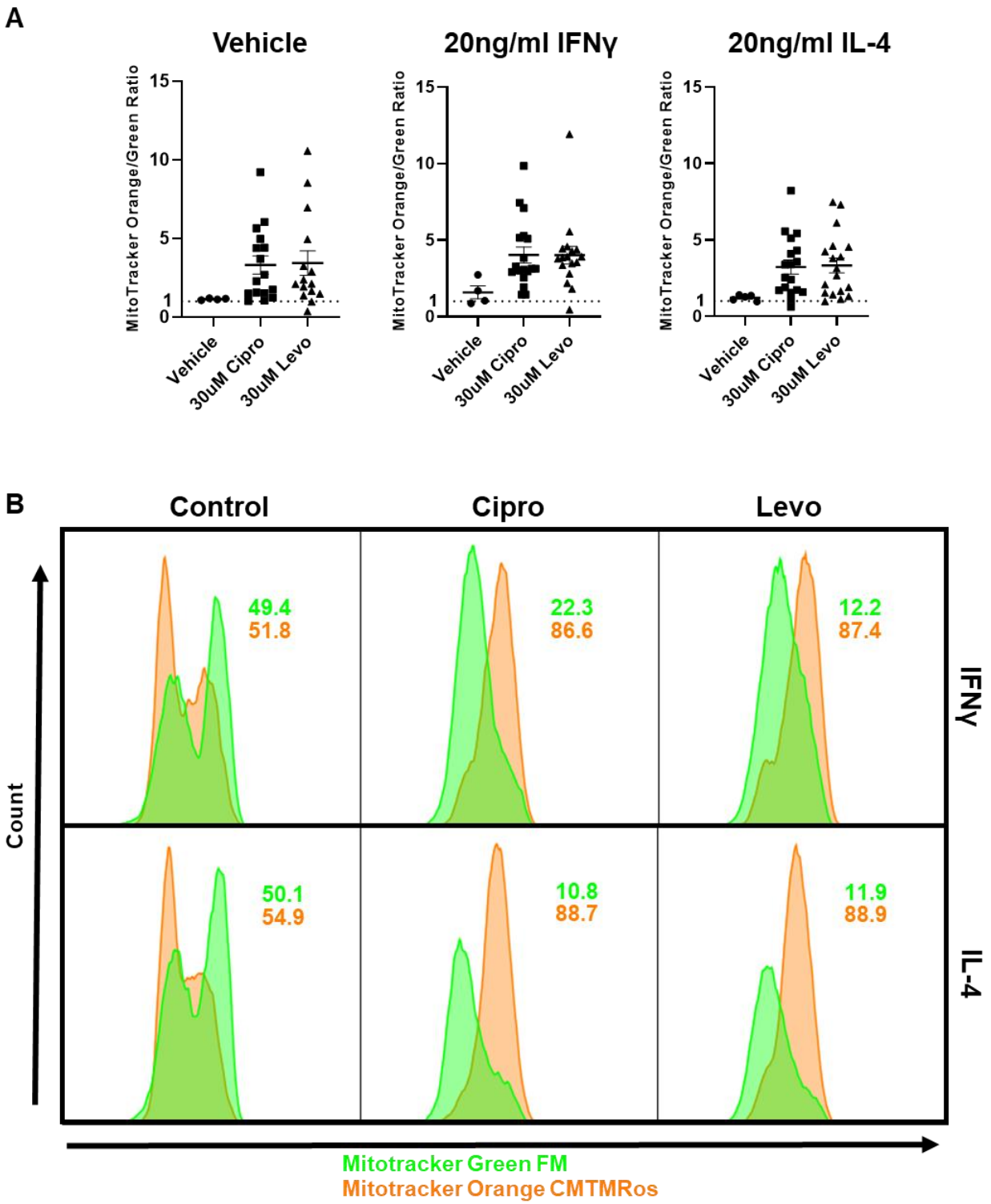


Figure 3.8 Fluoroquinolones cause hyperpolarisation in BMDM Mitochondria. *Bone marrow cells were isolated, and grown into Bone Marrow-derived Macrophages via the addition of 20ng/ml M-CSF whilst incubated with 30μM ciprofloxacin or levofloxacin, and polarised with 20ng/ml IFNγ (M1) or 20ng/ml IL-4 (M2) for 24h after 7 days (Purity >99%, gating strategy: CD45+, CD11b+, F4/80+). Samples were stained with MitoTracker Green FM (MTGreen) and MitoTracker Orange CMTMRos (MTOrange). A) Ratio of MTOrange+ to MTGreen+ BMDMs with/without fluoroquinolone treatment and M1/M2 polarisation. Line indicates an equal ratio. B) Representative flow cytometry histograms showing MTGreen and MTOrange staining overlaid, with numbers indicating % MTGreen+ and %MTOrange+. *, P<0.05; **, P<0.01; ***, P<0.005; N.S., not significant using one way ANOVA followed by Tukey's multiple comparison test (n=4-16 via 4 independent experiments).*

3.8 Discussion

In this chapter, the direct effects of the 2nd and 3rd generation fluoroquinolones ciprofloxacin and levofloxacin were investigated on macrophages in the BMDM system. Examining the effects of fluoroquinolones in an *in-vitro* macrophage based system provides a highly controlled, reductionist environment. This allows direct effects to be seen without the potential confounding factors, such as the microbiome or other immune cell types, which are known to affect macrophage function.

3.8.1 Although not affecting fully differentiated macrophages, levofloxacin primes developing macrophages to the M1 Macrophage phenotype.

One of the first questions addressed was whether fluoroquinolones were able to modulate mature macrophage polarisation, which in the context of clinical uses of fluoroquinolones, such as post-exposure prophylaxis in a Bioterror situation (FDA, 2009, FDA, 2017), was an important observation. It was found that when fluoroquinolones were introduced after 7 days of maturing macrophages, both ciprofloxacin and levofloxacin did not affect percentage purity or polarisation of BMDMs. There was no effect seen in non-antibiotic (M0) conditions or regardless of additional external M1/2 polarisation using IFNγ or IL-4 respectively. This was in stark contrast to the increased M1 polarisation observed when levofloxacin was added to bone marrow cells at the start of culture, along with M-CSF. Importantly, this increased in iNOS, our M1 marker, was not seen in M0 conditions, indicating no contamination of an individual antibiotic, with M1 polarising LPS for example. Overall, it could be that mature macrophages are less susceptible to the effects of fluoroquinolones, or simply that 24hrs is not enough time for the effects to take hold on mature macrophages.

Previous studies have however demonstrated short incubation times as altering macrophage polarisation with oxacillin stimulation of RAW 264.7 murine macrophages leading to significantly higher inducible nitric oxide synthase (iNOS). However, this was not observed on RAW cells alone and only present when pneumococci was also present; the bactericidal mechanism of delivering

cell wall components to the RAW macrophage cell line thought to be responsible for the increased M1 polarisation (Orman and English, 2000). Conversely, studies utilising short timed doses (30-90mins) of ofloxacin on murine peritoneal macrophages *ex vivo* increased reactive oxygen species while decreasing iNOS, with quinolone antibiotic tend to increase production of ROS (Dey and Bishayi, 2017). The bactericidal concentration of quinolones can lead to DNA fragmentation which results in production of superoxide from macrophages (Páez et al., 2008), again indicating the presence of bacteria is needed which was not present in our studies.

However, utilising isoniazid, rifampicin, pyrazinamide and ethambutol, the standard combination antibiotic treatment for *Mycobacterium tuberculosis*, Wang et al. observed that after 36 hours treatment the levels of COLEC12, HSPA1B and TREM1 transcripts were up-regulated in human pleural macrophages, whereas two IFNγ-mediated antiviral genes OAS3 and IFIT3 were down-regulated. This was consistent with their *in vivo* results that the antibiotic combination drove M2 polarisation (Wang et al., 2017). However, CCL18, a typical M2 marker, was down-regulated *in vitro* but up-regulated *in vivo* following antibiotics treatment (Wang et al., 2017). Nonetheless, this demonstrates macrophage polarisation alterations in the presence of macrophages in the absence of bacteria. Interestingly, in this study pleural macrophages before treatment had lower FSC/SSC flow cytometric parameters than those post, suggesting pleural macrophages before treatment tend to be in the intermediate status during monocytes to macrophage differentiation(Wang et al., 2017). This in combination with our studies, demonstrating levofloxacin induced effects only on developing and not mature macrophages, presents the possibility that antibiotics may be more influential on monocytes/intermediate monocytes than macrophages themselves.

The fluoroquinolone antibiotic moxifloxacin has been shown to have immunomodulatory activity through its capacity to increase the secretion of IL-1α and TNFα by human monocytes in the presence of LPS(Araujo et al., 2002a). In contrast another fluoroquinolone, trovafloxacin, significantly inhibited secretion of IL-1α, IL-1β, IL-6, IL-10, GM-CSF and TNFα by monocytes stimulated either with LPS (Khan et al., 1998). Both studies however, do

indicate that fluoroquinolones do have the potential to influence monocytes *in vitro* in a similar timescale to our own experiments. Further studies would therefore focus on *ex vivo* monocytes and intermediate monocytes in culture examining our previous parameters of polarisation.

3.8.2 Fluoroquinolones do not alter cytokine production but levofloxacin increases phagocytosis in BMDMs

There is little research published showing a similar effect to that seen here; antibiotic treatment leading to an increase in classical macrophage activation. As discussed previously, the primary drivers of M1 polarisation are proinflammatory cytokines and pathogen-associated molecular patterns. In comparison, alternatively activated macrophages, possess a greater variety of phenotypes and methods of activation, leading them to be subsequently classified as M2a, b c and d (Martinez and Gordon, 2014).

Therefore, the most expected mechanism by which antibiotics could increase M1 polarisation is by causing an increase of pro-inflammatory cytokines. Several studies have examined the effects of various antibiotics on cytokine production. Morikawa et al. (1996) looked at the effects of Fosfomycin and clarithromycin on cytokine production by human monocytes primed by LPS *in-vitro*. This is of particular relevance, as the pre-priming with LPS is similar to the IFNγ stimulation used here in chapter 3. Morikawa et al found that it was not so simple as antibiotics being pro or anti-inflammatory. Both antibiotics suppressed secretion of IL-1α, IL-1β, TNF and GM-CSF, all of which have a proinflammatory effect. In contrast, they increased secretion of IL-10, and Fosfomycin in particular also increased IL-6 secretion. This study implies a somewhat anti-inflammatory role for antibiotics, especially given the suppression of so many pro-inflammatory factors. However, the enhancement of IL-6 secretion by Fosfomycin shows that not all of these effects are anti-inflammatory. Furthermore, it also indicates how antibiotics cannot be grouped together has having the same effects. In this study, antibiotics of the same family had different effects, showing how even the most similarly prescribed antibiotics can have different immunomodulatory effects.

The effects seen by Morikawa et al, specifically a reduction in proinflammatory cytokine secretion, have also been seen in studies specifically focussing on fluoroquinolone antibiotics. Again, in LPS-primed human monocytes, Araujo et al. (2002b) found that moxifloxacin treatment caused a significant decrease in secretion of IL-1α in all patients, and secretion of TNF in 6 out of 10 patients examined. Though not significant, they found that moxifloxacin caused a reduction in IL-6, IL-10 and IL-12 secretion in contrast with Morikawa et al, further exemplifying the differences between antibiotics.

In murine peritoneal macrophages stimulated with LPS, Levofloxacin and Ciprofloxacin have been shown to have a similar effect, but again, differ slightly from one another (Ogino et al., 2009a). Ciprofloxacin inhibited secretion of IL-1β and TNF, whilst Levofloxacin only decrease IL-1β. Ogino et al also looked at the effects of fluoroquinolones on serum cytokine production *in vivo*. Similarly to the *in vitro* findings, Cipro significantly reduced serum TNF levels, and both Cipro and Levo decreased IL-1β levels (albeit not significantly). Interestingly, a reduced dose of Levo (10mg/kg) in fact caused a significant in serum TNF. The mechanism for this is unknown.

A further example of the anti-inflammatory effects of Cipro is shown by Kolios et al. (2006). They found that production of Nitric Oxide, a key product and marker of proinflammatory macrophages, is reduced in stimulated patient biopsies of colonic epithelium after Cipro treatment (measured by Griess reaction). This effect was replicated in a HT-29 colorectal adenocarcinoma cell line stimulated with IL-1α TNF-α and IFN-γ. Kolios et al also found that iNOS (inducible nitric oxide synthase) mRNA levels were reduced significantly by Cipro in both patient biopsies and HT-29 cells. This study did not find any differences in NO production in cells that had not been stimulated, similarly to the results found here.

This reduction in proinflammatory cytokines by Cipro has also been observed in a bacterial challenge model. In an experimental gram-negative pneumonia model, Cipro-treated animals exhibited inhibited TNF levels in their BAL fluid. Their BAL fluid also contained less LPS than mice treated with ceftadizime, a non-fluoroquinolone control antibiotic as well as saline-treated controls (Kawai

et al., 2006). It is likely, however, that in this study cytokine release was merely inhibited due to there being less infection present, rather than a direct effect of the antibiotic. In this thesis, no differences in macrophage cytokine release were observed after fluoroquinolone treatment. It is important to note though, that none of the studies mentioned measured cytokine release from bone marrow macrophages specifically, as examined here.

In all our experiments, we examined macrophage cytokine production in the supernatants of our BMDM cultures at the end of the 8 day incubation period via cytometric bead array. Surprisingly cytokine readouts were either greatly varied, in the case of IL-13 and TNF, while many samples gave a zero reading. An obvious future objective would be to repeat these measurements using ELISA, as CBA has been shown to be less sensitive at the low level production of cytokines. Interestingly, the means of IL-13 was reduced, while TNF-a was increased in both fluoroquinolone treated groups as compared to the non-antibiotic control, although this must be stated did not reach significance. Nevertheless, this does correlate with an increased M1 phenotype in our fluoroquinolone treated groups.

The effects of fluoroquinolones on TNF production have been measured in previous studies, namely Ogino et al. (2009a). In contrast to our M1 priming, they found that ciprofloxacin impaired TNF production by peritoneal macrophages stimulated with LPS, whereas levofloxacin had no effect. Moreover, both ciprofloxacin and levofloxacin impaired macrophage production of IL-1β (another proinflammatory cytokine). This may be due to the LPS treatment or macrophage source difference and it should also be noted that the concentration of fluoroquinolone used in this study was much higher (100ug/ml) than used in our studies. In addition, cells were incubated with fluoroquinolone for only one hour, compared to the 7 day incubation used here and that could represent an absence of maturing macrophage stimulation present in our studies. Indeed, importantly, no effects were seen on LPS naive cells in corroboration with our overall findings.

Cytokines were detected in the polarised groups, i.e. IFN-γ and IL-4, most likely due to residual polarising cytokines introduced in the methodology. Although no

differences were seen in IFN-γ levels, it is of interest that the level of IL-4 was higher in the levofloxacin treated groups, albeit not significantly. This suggests a reduction in uptake of IL-4, perhaps indicating lower levels of the IL4R in the levofloxacin treated group, again supporting the M1 polarisation with IL4Rbeing expressed on M2 macrophages at higher levels compared with M1 macrophages (Gabrilovich et al., 2012, Movahedi et al., 2010). However, this would need to be examined further and although a higher mean was present it was not significant.

Any effects fluoroquinolones have on macrophages would be of limited interest, if it were not determined whether these effects actually impacted the function of the cells. Furthermore, if there were no functional differences found in macrophages treated with fluoroquinolones, any differences in polarisation would be somewhat academic. For this reason, a phagocytosis assay was carried out to assess the competence of BMDMs after fluoroquinolone treatment.

Phagocytosis assays are commonly used to quantify impact on macrophage immune function, often with a reduction of phagocytosis as a marker of a treatment negatively affecting macrophage function (Lankelma et al., 2017). Yang et al. (2017) showed that in combination with causing reduced respiratory capacity, ciprofloxacin treatment lead to reduced phagocytic engulfing and pathogen killing in macrophages. In contrast, they found ampicillin treatment increased pathogen engulfment and killing Yang et al. (2017). These findings differ from those presented here, which found that the fluoroquinolone levofloxacin treatment in fact increased phagocytic capacity. There are key differences between the studies however. Firstly, we found levofloxacin increased phagocytosis, whereas ciprofloxacin (which Yang et al. studied) did not have an effect. Unfortunately, Yang et al. only presented phagocytosis as relative levels vs control, while absolute values were given here. Additionally, they measured phagocytosis of *E. coli* cells rather than IgG coated beads as used here limiting comparability. Furthermore, as well as phagocytic engulfing, Yang et al. measured pathogen survival and killing. This provides additional insight into the efficacy of any phagocytosis that occurs; if macrophages can engulf pathogens but are unable to kill them, then they could be incorrectly

deemed effective where their abilities are in fact greatly reduced. This is something that cannot be determined using latex beads. Therefore, while simulated pathogen engulfing was increased after levofloxacin treatment, the efficacy of said engulfing remains unknown and will be an interesting area of future work to be explored.

In the literature, classically activated M1 macrophages show high phagocytic activity unsurprisingly, given their primary function is to kill pathogens (Atri et al., 2018). M2 macrophages are less simple, given their heterogeneity in both phenotype and function. It has been shown that M2 macrophages activated by both IL-4, and a combination of the IL-4 and IL-13 show increased phagocytic ability vs M0 macrophages, due to an increased proteolytic capacity within the phagosome (Balce et al., 2011). Tarique et al. (2015) showed that while phagocytosis did occur in alternatively activated human macrophages (IL-13 and Il-4), it was not significantly greater than M0 undifferentiated macrophages. They did however, exhibit significantly increased levels of 'endocytosis' (uptake of dextran as opposed to *E.coli*) than both M0 and M1 macrophages. This is potentially due to the anti-inflammatory purposes of M2 macrophages' phagocytosis, such as the clearance of apoptotic cells. While in this case, M1 macrophages were shown to have a higher phagocytic ability than M2 macrophages, this is not found in all studies. This implies that the link between macrophage polarisation and phagocytic ability is very situation and pathology-specific.

For example, in HSV-1 infection, bone marrow-derived macrophages from M2 overexpressing (M2 OE) mice had significantly increased phagocytic activity than both WT mice and mice lacking the M2 phenotype (Jaggi et al., 2020). This difference occurred in both HSV infected and control cells.

Interestingly, this increase in phagocytosis did not translate into a significant difference in virus clearance or general pathology in M2 OE mice vs controls. These results were also shown *in-vitro*, with M2 OE peritoneal macrophages allowing increased viral replication vs control, both at rest and when polarised with IFNγ or IL-4. These results show a disconnect between phagocytosis and antiviral potency.

The lack of consensus between whether M1 or M2 macrophages can perhaps be explained by the fact that phagocytosis upregulation occurs via distinct mechanisms in the two types of macrophage (Canton, 2014). M1 macrophages show increased expression of specific phagocytosis markers on their surface after polarisation. These include FcγRI, FcγRII, and FcγRIII, which bind specifically to pathogenic and foreign objects opsonised with IgG (Mantovani et al., 2004, Beyer et al., 2012).

In contrast, one mechanism by which M2 macrophages upregulate phagocytosis is via 12/15-lipoxygenase expression. This enzyme is normally located in the cytosol of the macrophage until apoptotic cells are present. At which point, the enzyme is translocated to the surface, specifically to sites where apoptotic cells are bound (Miller et al., 2001).When on the surface of tissue resident M2 macrophages, 12/15-lipoxygenase serves to attract apoptotic cells towards them for phagocytosis. Interestingly, it also works in the opposite fashion, preventing uptake of apoptotic cells by proinflammatory $Ly6C^{hi}$ monocytes (Uderhardt et al., 2012). These two processes combine to create a 'sorting' mechanism whereby apoptotic cells are directed to the correct destination for processing.

The effects of antibiotics on macrophage phagocytic ability are of particular pertinence for obvious reasons- antibiotics are likely to be present in the system of someone with a bacterial infection, a situation in which phagocytosis is more important than normal. In this situation, phagocytosis by M1 macrophages is therefore key. The effects of various antibiotics on phagocytosis has therefore been studied for some time. One such early study is that by van den Broek (1989) who reviewed interactions between antimicrobials and phagocytes. They found that some antimicrobials have a negative effect on phagocytosis, with tetracyclines and bacitracin inhibiting phagocytosis by granulocytes and human monocytes respectively. This was found to occur due to calcium and magnesium ion binding by the antibiotics inhibiting the free movement of these ions across the cell membrane, and therefore formation of the phagosome. Interestingly they found that in contrast, pre-treatment with antibiotics tended to increase phagocytosis. For example, when *S. aureus* was pre-treated with sub-MIC amounts of chloramphenicol, erythromycin or rifampicin, phagocytosis by

macrophages was enhanced. Similar effects were seen in *S. aureus* with penicillin G, and in *K. pneumoniae* with clindamycin. This effect was not universal however, with phagocytic killing not enhanced when serum was not present in the system. This pre-treatment effect shows that at sub-MIC concentrations, antibiotics still work to weaken the structure and defences of many bacteria. It should be noted that the results in this paper measured phagocytic killing of pathogenic bacteria as opposed to uptake of fluorescent beads as used in this thesis.

This pre-treatment boost in phagocytosis was also observed specifically in fluoroquinolones. When multiple species of bacteria such as *S. aureus* and *L. pneumophila* were pre-treated with sub-MIC amounts of fluoroquinolones such as Ciprofloxacin, Ofloxacin and Norfloxacin, phagocytosis was increased (Desnottes, 1987).

More recently, the effects of fluoroquinolones on phagocytosis have been not so clear-cut, with studies giving the opposite results to each other. Tawfik et al. (1990) found that Ciprofloxacin, pefloxacin, norfloxacin, and ofloxacin all suppressed phagocytic activity, whilst having no effect on other cell-mediated immune functions. Similarly, Wenisch et al. (1995) studied the same antibiotics as Tawfik et al, with the addition of fleroxacin, via flow cytometry. They also found that incubation with these fluoroquinolones led to significantly reduced phagocytosis of fluorescently labelled *E.coli* by human blood phagocytes. This reduction has also been seen in macrophages treated with fluoroquinolones, this time including Levofloxacin (Azuma et al., 2001).

Conversely, Cacchillo and Walters (2002) found that Ciprofloxacin accumulated in human polymorphonuclear leukocytes (PMLs), and that when this occurred, phagocytic killing of *Actinobacillus actinomycetemcomitans* was significantly improved vs control.

The fact that research is in such disagreement in this area indicates that alteration of phagocytosis by fluoroquinolones (and antibiotics as a whole) is not only drug-dependent, but both pathogen and immune cell-dependent as well.

It should be noted that the papers discussed here used a variety of methods to measure phagocytosis, compared to that used here. In addition, they used different sources of macrophage, with therefore different characteristics than those used here. This therefore makes comparison of results not as simple as if the methods had been consistent. One key standout finding was that in an infection setting, pre-incubation with antibiotics (including fluoroquinolones) led to improved phagocytosis once a pathogen was introduced. Whilst it was not possible to replicate this using the IgG FITC beads used here, it does raise an important point of prophylactic treatment.

3.8.3 Fluoroquinolones are not Cytotoxic, nor alter pH at the concentrations used *in vitro*

The next step was to ascertain the cause of these effects on macrophages. The first step was to rule out the possibility that the drugs were in fact cytotoxic to BMDMs at the concentrations used. Whilst the cytotoxicity of fluoroquinolones against BMDMs has not been published, there has been research into their cytotoxicity against other cell lines, namely human corneal keratinocytes (HCK), endothelial (HCEN) and epithelial cells (HCEP). It was found that cytotoxicity is concentration and time-dependent. Ciprofloxacin showed cytotoxicity against both HCKs and HCENs at concentrations from 1mg/ml-10ng/ml and incubation times from 15min-4 hrs. Levofloxacin also showed cytotoxicity, albeit to a lesser extent. Cytotoxicity was only seen against HCKs and HCENs at 1mg/ml at all incubation times, and from 1hr -4hrs at concentrations between 100µg/ml and 1µg/ml (Bezwada et al., 2008). Tsai et al. (2010) found that against HCEPs, fluoroquinolone solutions were largely cytotoxic because of the preservatives they contain. They also found ciprofloxacin was cytotoxic after 1hr, but Levofloxacin was not.

To determine if Cipro and Levo were cytotoxic against BMDMs, a Lactate Dehydrogenase (LDH) assay was carried out, which measures LDH released by cells in response to membrane damage. At concentrations from 0.1-100µg/ml of both Cipro and Levo, no cytotoxicity was detected, and could therefore be ruled out. This contrasts the studies mentioned above, though this is likely due to a combination of a different cell type, as well as a shorter

incubation time as dictated by the manufacturer's instructions for the assay. However, examining live/dead staining in our BMDM flow cytometry samples did not indicate any difference; making cytotoxicity unlikely to be responsible for the observed polarisation effects.

Another possible cause was that fluoroquinolone treatment had altered the pH of the BMDM growth environment. Literature has shown that an altered environmental pH has the ability to modulate macrophage polarisation *in vitro* (Wu et al., 2019). Reduced media pH (6.6) caused RAW 264.7 cell line macrophages to polarise towards an M2 phenotype, showing upregulated CD206 gene expression and arginase-1 secretion. In contrast, increased pH led to the opposite occurring, with increased secretion of nitric oxide synthase (NOS2) and TNFα gene expression amongst others in line with the M1 phenotype. This was also seen in BMDMs, with cell culture medium pH affecting cells stimulated with either IFNγ/LPS or IL-4. At pH 6.8, BMDMs stimulated with IFNγ/LPS showed reduced expression of pro-inflammatory markers such as NOS2 and CCL2, whilst IL-4-stimulated BMDMs displayed enhanced expression of CD206 and arg1, amongst other anti-inflammatory markers. In this case, this was linked to macrophages taking a tumour-promoting phenotype (El-Kenawi et al., 2019). A mechanism for M2 polarisation in extracellular acidosis was not identified by Wu et al. or El-Kenawi et al., although Wu et al did speculate that ovarian cancer G-protein-coupled receptor 1 (OGR1) was involved.

OGR1 is in fact a family of G protein-coupled receptors, including amongst others OGR1, G protein-coupled receptor 4 (GPR4), G2A (G2 accumulation), and T cell death-associated gene 8 (TDAG8). OGR1 family receptors are proton, and therefore low pH, sensing receptors, becoming fully active at pH 6.8 (Ludwig et al., 2003, Seuwen et al., 2006). The activity of these receptors in macrophages has been linked to inflammation, albeit, interestingly, in different ways. TDAG8 was shown to be partially responsible for low pH-induced inhibition of proinflammatory cytokine release by peritoneal macrophages. At a pH of 6.8, LPS-induced TNFα release by peritoneal macrophages is significantly reduced vs control (pH 7.8). This effect was removed in macrophages from TDAG8$^{Tp/Tp}$ mice. This was specific to the TDAG8 receptor,

with OGR1$^{geo/geo}$ macrophages unaffected. This also partially occurred in macrophages treated with TDAG8-siRNA, and did not occur in G2A-siRNA treated cells, again showing specificity to TDAG8 (Mogi et al., 2009).

This shows that if an antibiotic alters the extracellular pH of BMDMs, it has the ability to modulate their pro or anti-inflammatory characteristics. To determine whether fluoroquinolone treatment affected extracellular pH *in-vitro,* media was removed from BMDM culture after an 8 day experiment with or without antibiotics, and was tested using a benchtop pH meter. Additionally, to determine the whether the presence of cells had an effect, antibiotics were added to control media immediately prior to testing.

No significant difference in pH was found when antibiotics were added to the media, at either day 0 or day 8. Whilst there was no difference between conditions, measured pH was considerably higher than expected (pH~8-9 compared to control pH of 7.4 seen in the literature). This may indicate possible inaccuracy of the measurement method, as alkalosis of the media to that extent is known to inhibit cell growth significantly (Mackenzie et al., 1961), which did not occur in that experiment. Further experiments in this area are certainly required, both by repeating the pH measurement method used here, as well as by other methods. One possibility would be to measure mRNA levels of TDAG8, or OGR1 in BMDMs with and without antibiotic treatment, to see if pH-dependant receptors were activated, even if no change was detected by currently available methods.

Indeed, one possibility would be to use more precise measurement methods, or include intracellular pH measurement using carboxy-SNARF-1 (Wieder et al., 1993) or a similar dye, and flow cytometry. Measuring pH of specific cells would allow a more accurate link to be established between a cell's pH and other factors, such as expression of various activation and polarisation markers. Whilst this would give an advantage in terms of data acquired, it would not be as useful with respect to the existing literature. The studies of OGR1 family receptors only involved manipulation of extracellular pH via cell culture medium, and so measurement of internal pH would not be relevant. In addition, the internal pH of cells widely varies at different locations in the cell. Whilst the

cytosol is typically fairly neutral (pH 7.2), the mitochondria are alkali (pH 8), and organelles such as endosomes and lysosomes being varying degrees of acidity, depending on their stage (Casey et al., 2010).

3.8.4 Fluoroquinolones Cause Mitochondrial Hyperpolarisation in BMDMs

Though unconfirmed, several studies have linked FQAD symptoms in patients to mitochondrial damage, likely due to mitochondria's evolutionary semblance to prokaryotic cells (Kalghatgi et al., 2013, Hangas et al., 2018, Kaur et al., 2016). In addition, mitochondria are intrinsically linked with macrophages and their function. When macrophages polarise from M0 to M1 or M2, their metabolism shifts (Ramond et al., 2019). M1 macrophages, specialised for pathogen killing, alter their metabolism to limit oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO), both mitochondrial processes. They in turn switch towards a glycolysis- heavy metabolism, in order to rapidly produce ATP to fuel intensive pathogen killing (Garedew et al., 2010). This is thought to be at least partially caused by LPS-induced upregulation of the hypoxia-inducible factor 1 α (HIF-1α) gene, which enhances glycolysis (Cramer et al., 2003, Nishi et al., 2008). In contrast, M2 macrophages display enhanced OXPHOS and FAO. This is thought to be caused by pro M2 cytokines IL-4, IL-10 and IL-13, promoting the STAT6 transcription complex and subsequently activating peroxisome proliferator activating receptors (PPARs). This has the effect of increasing the oxidation rate of OXPHOS and FAO (Vats et al., 2006).

It therefore stands to reason that any effects of fluoroquinolones on macrophage polarisation may well be caused by altered mitochondrial function. Bedaquiline induced a significant metabolic reprogramming of human monocyte-derived resting macrophages, without altering cell viability (Giraud-Gatineau et al., 2020). The gene set upregulated by bedaquiline was significantly enriched for genes associated with lysosome, phagocytic vesicle membrane, vacuolar lumen, hydrolase activity and lipid homeostasis (Giraud-Gatineau et al., 2020). Although mitochondrial functions were not seen to alter significantly by the measured parameters in this study, conflicting reports suggest that bedaquiline can inhibit the mitochondrial ATPase (Fiorillo et al.,

2016, Haagsma et al., 2009). Moreover, diarylquinoline specifically inhibits a subunit of the bacterial adenosine triphosphate (ATP) synthase, decreasing intracellular ATP levels (Andries et al., 2005), but does have affinity for human ATP synthase (Haagsma et al., 2009), although 20,000 times less. Therefore, it remains possible that antibiotics may alter macrophage function via subtle effects on mitochondria function.

We used MitoTrackers to quantify both total and active mitochondrial mass in BMDMs with the ration indicating mitochondrial function. M0 BMDMs demonstrated a ratio of around 1, which increased upon M1 stimulation. Interestingly, when fluoroquinolones were added, the ratio increased above 1 by some margin, regardless of polarisation state. This is indicative of mitochondrial hyperpolarisation, a component of the apoptotic pathway that constitutes dysfunction when occurring in excess or spontaneously. This phenomenon is known to exist in T cells of systemic lupus erythematosus (SLE) patients (Gergely et al., 2002, Perl et al., 2004), as well as those of type 1 diabetes (Chen et al., 2017). Moreover, glycolytic ATP production facilitates a mitochondrial hyperpolarisation that is required for the pro-inflammatory effects of LPS on BMDMs (Mills et al., 2016). Indeed, during inflammatory activation ‘glycolytically competent cells’ such as macrophages use significant amounts of the glycolytically generated ATP to drive mitochondrial hyperpolarisation and thereby prevent apoptosis(Garedew et al., 2017). Indeed, simply targeting the mitochondrial protein LETM1 domain–containing protein 1 (LETMD1), also known as HCCR-1, results in mitochondrial hyperpolarisation followed by ROS expression as well as increased phagocytosis in monocyte and macrophage human cell lines (Lim et al., 2020) demonstrating that mitochondrial hyperpolarisation alone can drive macrophage alterations.

The ideal way to determine mitochondrial damage in fluoroquinolone-treated BMDMs would be to use an Agilent seahorse analyser. However, due to equipment limitations this is not currently possible. Such experiments would allow more detailed analysis of OXPHOS and glycolysis rates, and if/how they are affected by fluoroquinolone treatment.

3.8.5 Conclusion

This chapter focussed around the treatment of *ex-vivo* bone marrow-derived macrophages with ciprofloxacin and levofloxacin, and the effects this caused. Several conclusions can be drawn from the findings.

-Levofloxacin causes an increased potential for M1 macrophage polarisation in developing BMDMs.

-Levofloxacin does not alter mature macrophage polarisation

-Fluoroquinolone treatment is not cytotoxic to BMDMs, and does not alter media pH

-Ciprofloxacin and Levofloxacin cause mitochondrial hyperpolarisation in BMDMs

Chapter 4: Investigating immune modulation of fluoroquinolones *in vivo*

4.1 Introduction

Utilising the BMDM *in vitro* system my previous chapter demonstrated that fluoroquinolones Ciprofloxacin and Levofloxacin cause mitochondrial hyperpolarisation in BMDMs and that Levofloxacin causes a significant increased potential for M1 macrophage polarisation in developing BMDMs. BMDMs are a very reductionist method, which can be an advantage, offering highly controlled, sterile conditions. It does however exclude many factors present in a living system. These include the microbiome, endocrine and neuronal systems as well as the interconnected nature of the immune system itself. It was therefore important to apply the findings to *in vivo* experiments. Indeed, Lankelma et al. (2017) showed that broad spectrum antibiotic-induced dysbiosis impairs the ability of alveolar macrophages to phagocytose pathogens *in vivo*.

It is widely known that different strains of wild type laboratory mice differ physiologically, particularly with respect to their immune systems. Because of this, it was important to take the strain into account when carrying out *in vivo* investigations so as to be more reflective of a mixed human population. In order to achieve this, C57BL/6 and BALB/c mice were treated with the 100mg/kg Ciprofloxacin or Levofloxacin twice daily for two weeks via oral gavage. They were then monitored for a further 7 day ‘washout period’. This dosing technique allowed for precise dose-control per animal, ensuring every animal received the same amount of antibiotic, which cannot be ensured when antibiotics are added to drinking water.

A wide variety of samples were taken during these experiments in order to maximise the data that could be obtained from them (Fig. 4.1).

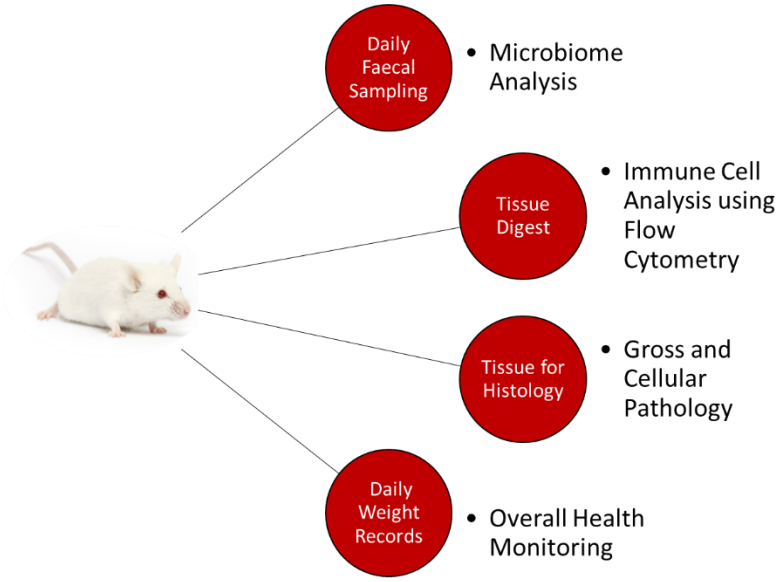


Figure 4.1. In vivo Experimental Design

Schematic showing the variety of samples taken during in vivo experiments. C57BL/6 and BALB/c mice were treated with 100mg/kg Ciprofloxacin or Levofloxacin twice daily for two weeks via oral gavage. Mice were weighed daily to monitor their health and tissues were harvested at day 7, 14 and 21 following a washout no antibiotic period. Spleen, lung, large intestine, and mesenteric lymph node were processed for analysis via flow cytometry and histology, while faecal samples were taken daily in order to track any fluoroquinolone-induced dysbiosis.

The main objectives of the work in this chapter were:

I. **To investigate the effect of the fluoroquinolones ciprofloxacin and levofloxacin on macrophage M1/M2 polarisation *in vivo***
II. **To investigate the immunomodulatory effect of the fluoroquinolones ciprofloxacin and levofloxacin on innate immune cells *in vivo***
III. **To investigate the intestinal dysbiosis resulting from the fluoroquinolones ciprofloxacin and levofloxacin**
IV. **To investigate potential alterations in weight during fluoroquinolone ciprofloxacin and levofloxacin treatment**

4.2 Fluoroquinolone treatment increases C57BL/6 M1 lung macrophage polarisation *in vivo*

One of the key findings in BMDMs was that with IFNγ stimulation, M1 macrophage polarisation is increased with fluoroquinolone treatment, particularly levofloxacin. We therefore examined macrophage polarisation on the C57BL/6 background, to mirror our BMDM read out strain source. C57BL/6 mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days to mimic the human dose.

To give an overall readout of the peripheral state of macrophage polarisation we first examined splenic macrophages and saw no difference in NOS2 M1 populations in either of the ciprofloxacin or levofloxacin treated groups as compared to PBS vehicle controls (Fig.4.2). This was also the case when examining CD206+ M2 populations, with neither fluoroquinolone treatments significantly altering baseline levels of PBS vehicle treated controls (Fig.4.2).

Given the strong intestinal resident microflora, we next analysed macrophage populations in the gut draining mLN where CD64 macrophages reside in low numbers at the steady state while during inflammation recruited monocytes differentiate into inflammatory macrophages (Tamoutounour et al., 2012). mLN macrophage populations had no difference in NOS2 M1 in following either of the ciprofloxacin or levofloxacin treatments as compared to PBS vehicle controls (Fig.4.2). This was also the case when examining CD206 M2 populations, with neither fluoroquinolone treatments significantly altering baseline levels of PBS vehicle treated controls (Fig.4.2).

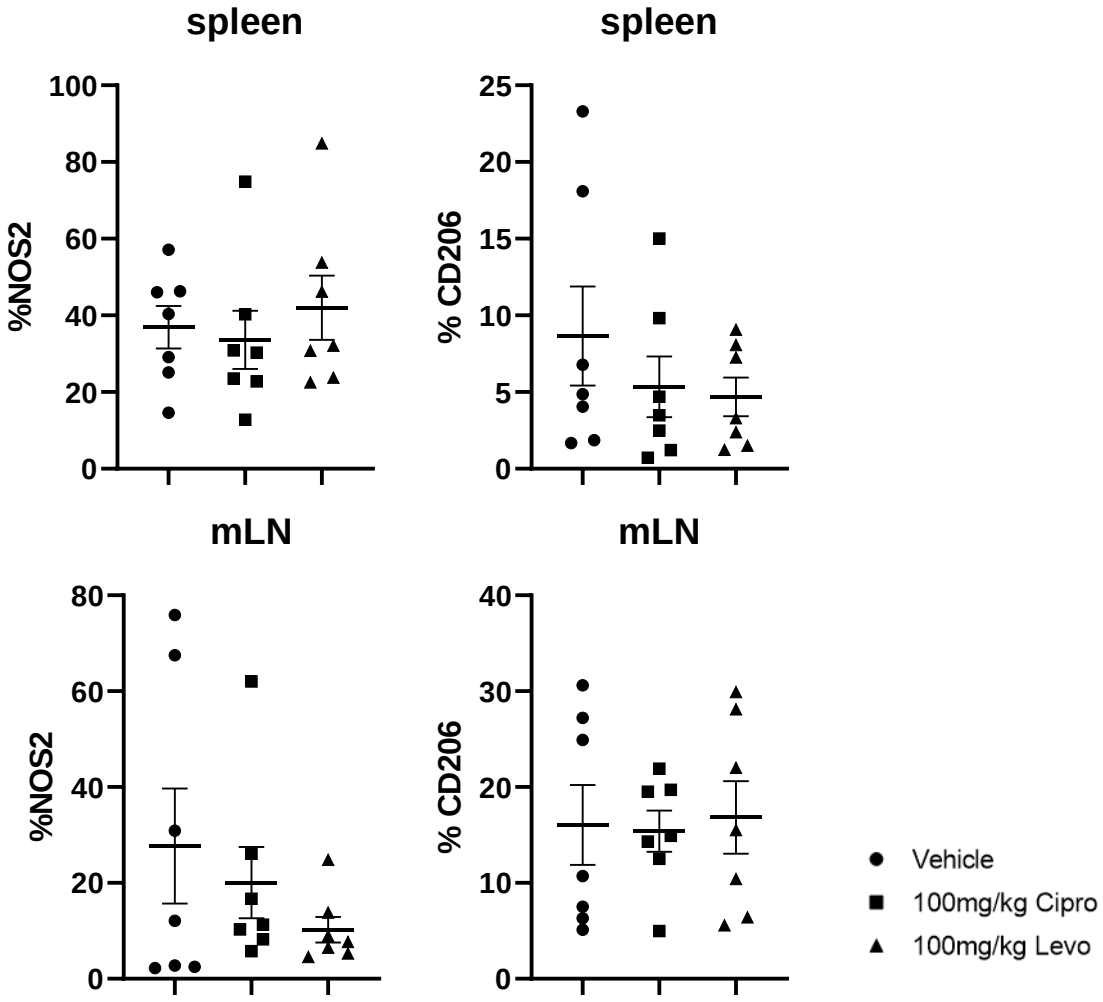


Figure 4.2: Fluoroquinolones do not modulate splenic or macrophage polarisation in *vivo. C57BL/6 mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=7/group). Spleens/mLN were harvested at 14 days. Significance tested using one-way ANOVA followed by Sidak's multiple comparisons test.*

The immunomodulatory effects of fluoroquinolones are particularly important in the lung. They are the site at which immune function is most pertinent in the situation in which fluoroquinolones are prescribed, severe respiratory tract infections. As a result, any modulation of the lung immune and microbiome environment could have a severe impact on the outcome of infection. Utilising the lower expression of CD11b in the CD45+/CD64+ macrophage population we were able to broadly distinguish between alveolar macrophages and the remaining interstitial macrophage, monocyte/macrophage population (Misharin et al., 2013a) (Fig.4.3). Examining the expression of NOS2 and CD206 in the tissue-resident alveolar macrophage population we saw no alteration in M1 or M2 populations in either ciprofloxacin or levofloxacin treated groups as

compared to PBS vehicle treated controls (Fig. 4.3). However, analysis of the short-lived and monocyte derived interstitial and monocyte/macrophage populations (Misharin et al., 2013a) demonstrated a significant increase in M1 NOS2 expression in the ciprofloxacin treated animals as compared to the PBS vehicle controls (Fig.4.3). Although this pattern was also seen in the levofloxacin treated group and there was no significant difference between fluoroquinolone treated groups, this did not reach significance as compared to the PBS vehicle treated group ($p=0.06$). The M2 CD206 marker had a trend for a reduced mean in both the ciprofloxacin and levofloxacin treatments, but this did not reach significance as compared to the PBS vehicle controls in the interstitial macrophage/monocyte population (Fig. 4.3).

Collectively, these data indicate that the lung is a specific site of macrophage modulation following fluoroquinolone treatment, driving a pro-inflammatory M1 phenotype specifically within the short-lived, monocyte derived macrophage population.

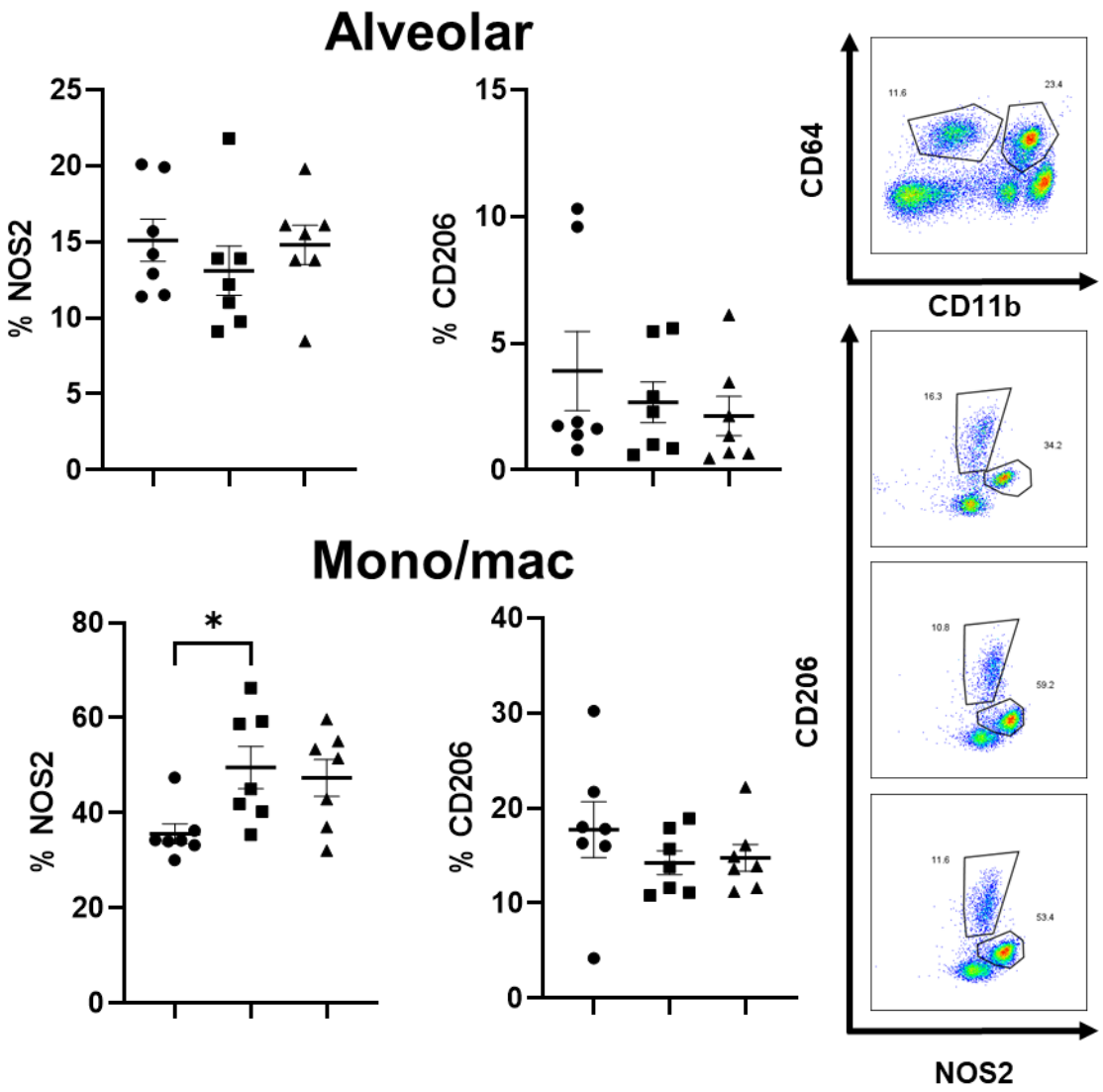


Figure 4.3: 14 day Cipro treatment significantly increases C57BL/6 M1 IM polarisation. *C57BL/6 mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=7/group). Lungs were harvested at 14 days. Proportion of Alveolar Macrophages and Monocyte-Macrophages in the lung expressing NOS2 or CD206. Significance tested using one-way ANOVA followed by Sidak's multiple comparisons test.*

4.3 Fluoroquinolone treatment increases BALB/c M1 lung macrophage polarisation *in vivo*

We next assessed the effect of fluoroquinolone treatment on macrophage populations in the BALB/c mouse, which as well as being a more prone Th2 background strain, is known to have far more inert M1 macrophages to the stimulus of IFN-gamma-plus LPS for the production of NO as compared to the C57BL/6 background (Santos et al., 2006). We examined time-points across the course of treatment as well as a run-off period of 7 days analysing the mLN and Lung populations, albeit with limited animals and the F4/80 general macrophage marker in the panel.

Within the mLN, we saw no significant alteration in M1 NOS2 or M2 CD206 populations following 7 day treatment of either ciprofloxacin or levofloxacin as compared to PBS vehicle controls (Fig.4.4). After 14 days there was a strong trend for increased M1 NOS 2 expression particularly in the ciprofloxacin treated group, but this did not reach significance as compared to the PBS vehicle treated controls (Fig.4.4). Interestingly, we also saw a strongly reduced mean of M2 CD206 in both fluoroquinolone treated groups, but again this did not reach significance as compared to the PBS treated control group (Fig.4.4). Finally, these trends were absent following the run-off period in all fluoroquinolone treated groups as compared to the PBS treated controls (Fig.4.4).

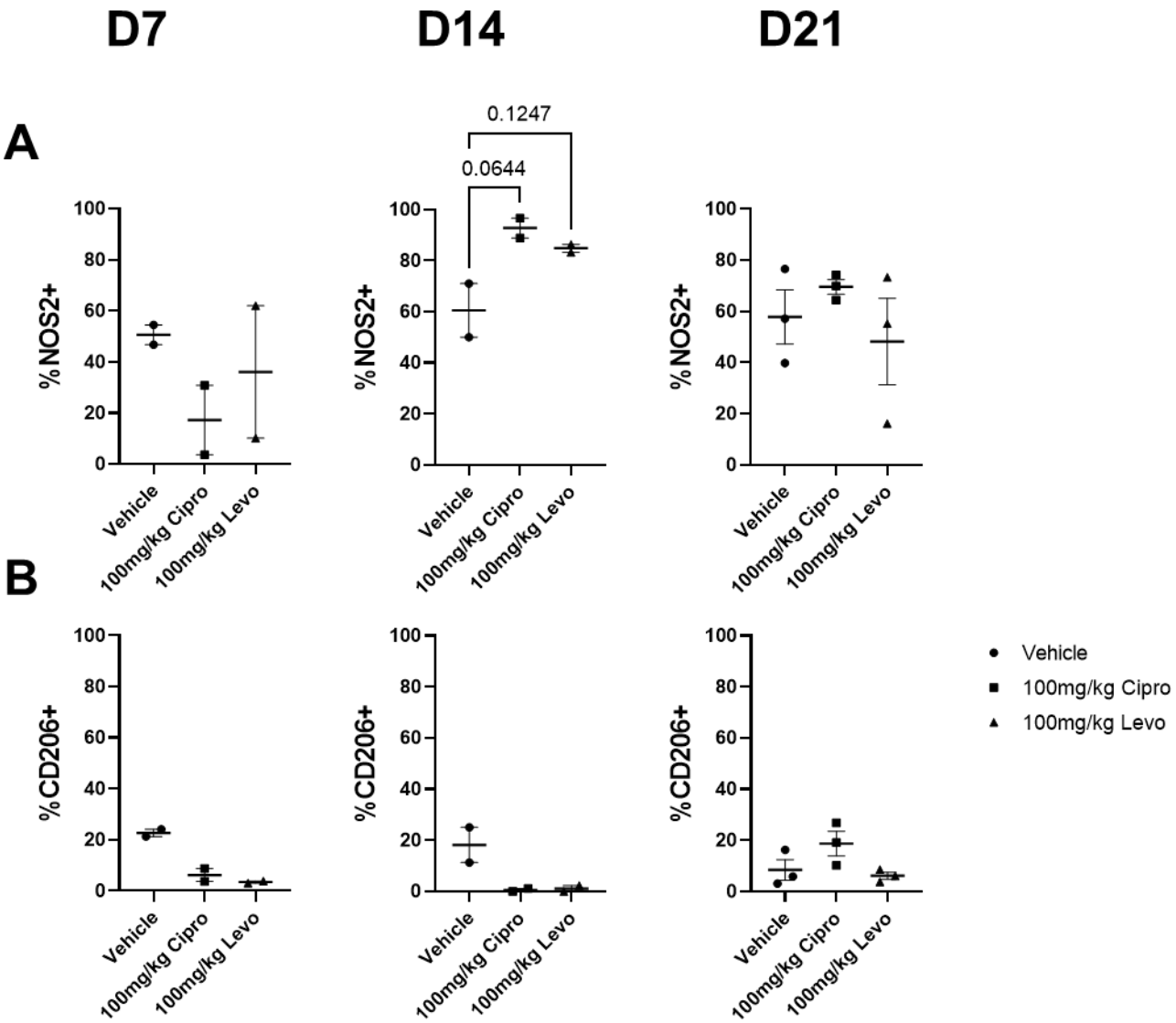


Figure 4.4: **Fluoroquinolones do not alter MLN macrophage polarity in BALB/c mice across the time course of treatment.** *Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily for 14 days, with a 7 day treatment-free 'washout period'. Mesenteric Lymph Nodes were harvested every 7 days. A: %NOS2+ (M1) of MLN macrophages after 7 days, 14 days and 14 days of treatment with 7 day runoff period. B: %CD206+ (M2) of MLN macrophages after 7 days, 14 days and 14 days of treatment with 7 day runoff period. Significance tested using one-way ANOVA followed by Dunnet's multiple comparisons test (n=2-3).*

We next attempted to isolate the long-lived resident alveolar macrophages, this time via performing a Bronchoalveolar Lavage (BAL). We saw no significant alteration in M1 NOS2 or CD206 M2 populations in either of the ciprofloxacin or levofloxacin treated groups as compared to the PBS vehicle group following 7 days of treatment (Fig.4.5). At 14 days there was an increased mean of M1 NOS2 macrophages in the ciprofloxacin treated group, but again we saw no significant alteration in M1 NOS2 or CD206 M2 populations in either of the

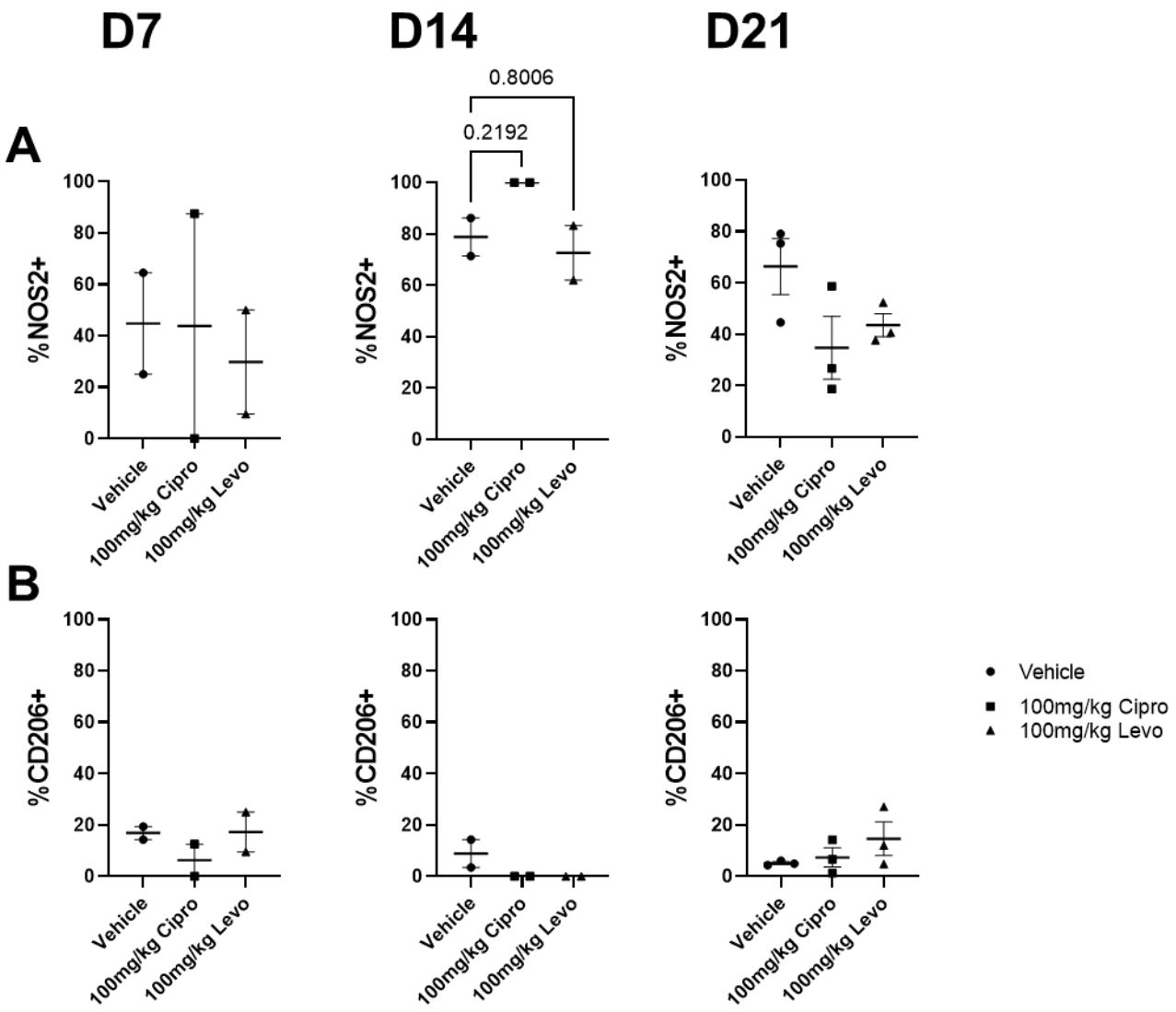


***Figure 4.5: BALB/c BAL macrophage polarisation is not significantly affected by Fluoroquinolones**. Wild type BALB/c mice were treated by oral gavage with either dH_2OVehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily for 14 days, with a 7 day treatment-free 'washout period'. BAL fluid was taken every 7 days. A: %NOS2+ (M1) of BAL macrophages after 7 days, 14 days and 14 days of treatment with 7 day runoff period. B: %CD206+ (M2) of BAL macrophages after 7 days, 14 days and 14 days of treatment with 7 day runoff period. Significance tested using one-way ANOVA followed by Dunnet's multiple comparisons test (n=2-3).*

fluoroquinolone treated groups as compared to the PBS vehicle group (Fig.4.5), nor following the 7 day run off period (Fig. 4.5).

Interestingly, on examining the remaining monocyte/macrophage populations following crude BAL removal of alveolar macrophages a similar pattern was observed in the BALB/c lung. There was no difference in M1 or M2 polarisation after 7 days of treatment in either fluoroquinolone treated group as compared to PBS vehicle controls (Fig.4.6). However, there was again a strong M1 NOS2 polarisation after 14days treatment, while M2 CD206 polarisation had significantly decreased after both ciprofloxacin and levofloxacin treatment as compared to PBS vehicle controls (Fig.4.6). After the 7-day runoff period, populations had equalised again, with no differences between fluoroquinolone treated groups and PBS vehicle controls (Fig.4.6).

Collectively, these data again indicate that the lung is a specific site of macrophage modulation following fluoroquinolone treatment, driving a pro-inflammatory M1 phenotype specifically within the short-lived, monocyte derived macrophage population, independent of immune strain background

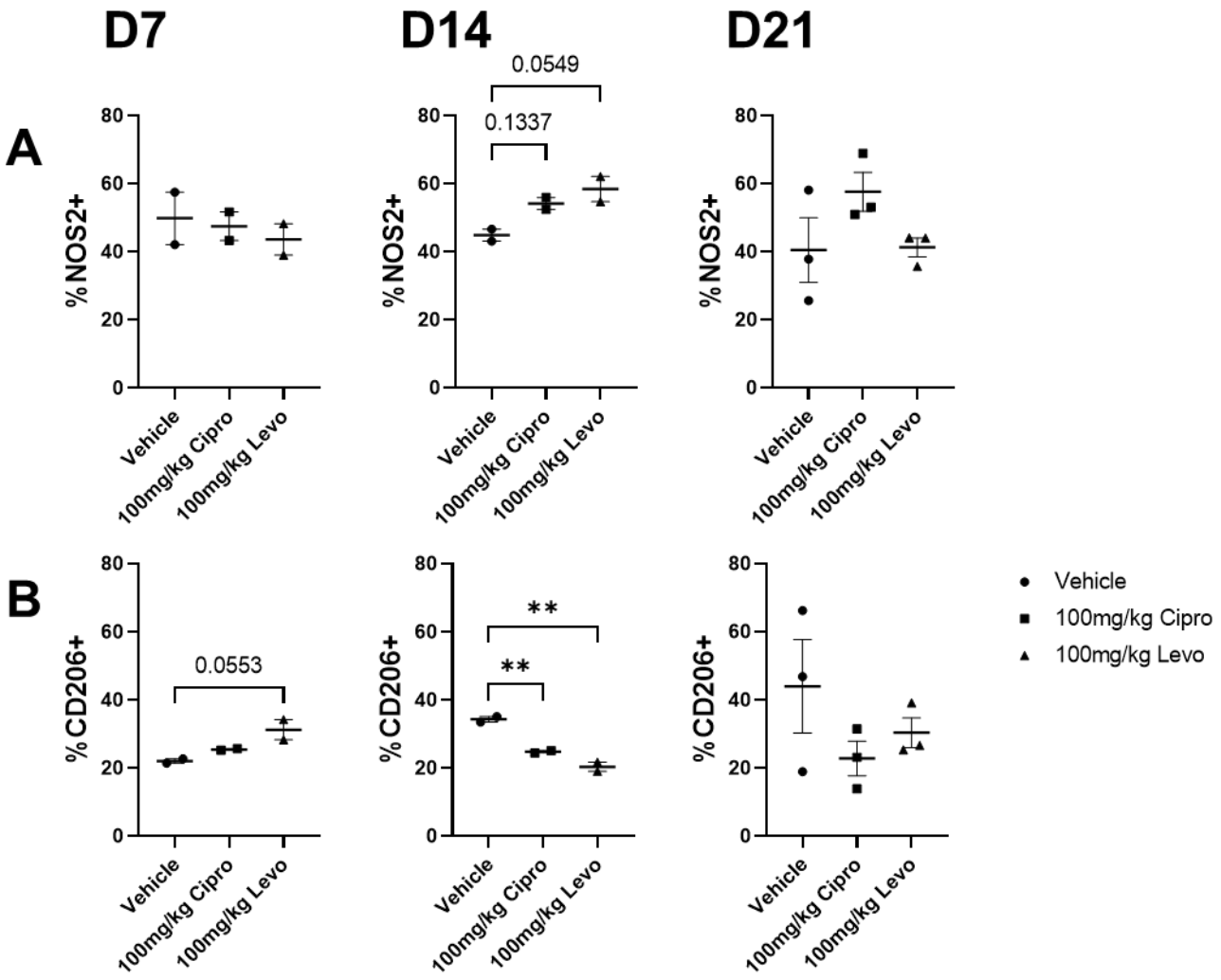


Figure 4.6: **Fluoroquinolones drive M1 lung macrophage polarity in BALB/c mice across the time course of treatment.** *Wild type BALB/c mice were treated by oral gavage with either Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. Lungs were harvested every 7 days. A: %NOS2+ (M1) of lung macrophages after 7 days, 14 days and 14 days of treatment with 7 day runoff period. B: %CD206+ (M2) of lung macrophages after 7 days, 14 days and 14 days of treatment with 7 day runoff period. Significance tested using one-way ANOVA followed by Dunnet's multiple comparisons test.*

4.4 Fluoroquinolone treatment does not alter immune cell populations in the BALB/c spleen

To further ascertain whether fluoroquinolone treatment affected the overall phenotype of circulating immune cells *in vivo*, we utilised a broad flow cytometry panel in BALB/c mice, analysing multiple organs and barrier sites across the timecourse of treatment. Spleens were harvested at the 7, 14 and 21 day wash out timepoints. We first examined cellularity of the spleen across the time course and observed that fluoroquinolone treatment did not cause any significant differences in cell number (fig 4.7). At day 14, Levo-treated spleens were more consistently high in their cellularity than the spread shown in vehicle and Cipro-treated spleens. This did not however translate to a statistically significant difference.

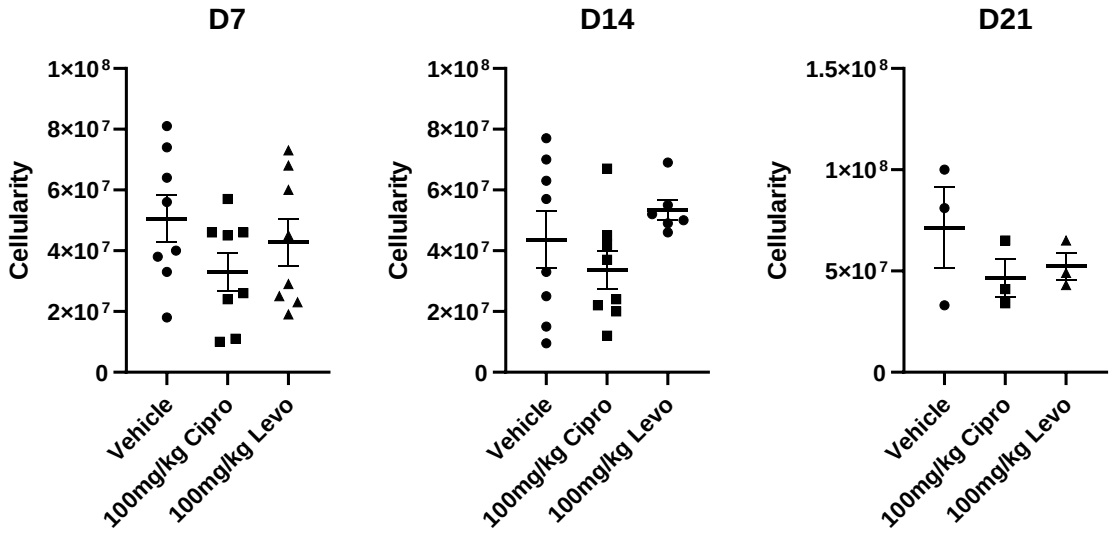


Figure 4.7: Spleen cellularity after Fluoroquinolone treatment. Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. Spleens were harvested every 7 days. Cellularity of samples on day 7, 14 and 21 was counted after disaggregation and red blood cell lysis.

The myeloid phenotype was next analysed using a broad innate immune flow cytometry panel as previously described. After 7 days of fluoroquinolone treatment, there was a trend for increased CD45+ cells in the spleen in both ciprofloxacin and levofloxacin treated animals as compare to the vehicle treated controls, but this did not reach significance. (Fig. 4.8A). Examining the myeloid population, we saw no significant alteration in neutrophils, monocytes, eosinophils or NK cells following 7 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.8B).

As we have seen changes in macrophage polarisation *in vivo* following fluoroquinolone treatment we next examined macrophages and dendritic cell populations but observed no significant difference following 7 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.8C).

Finally, although the cells were stained with a myeloid-focused panel, populations of B and T cells were also quantified, although limited conclusions should be drawn from this as they were determined by negative gating. Despite these caveats, we saw similar percentage populations across all 3 independent experiments, albeit no significant difference following 7 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.8D).

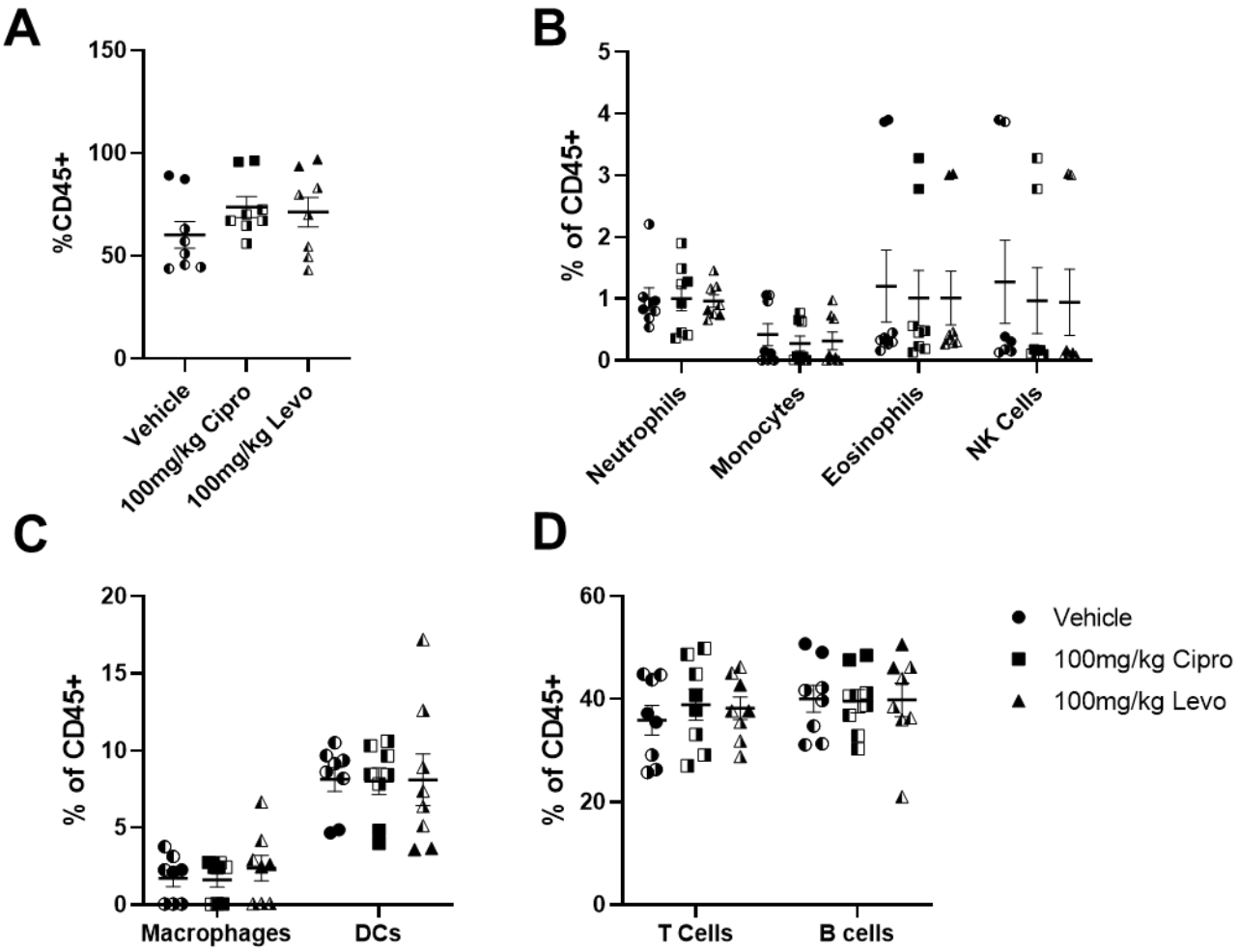


Figure 4.8: Fluoroquinolones do not alter spleen immune cell subsets following 7 day treatment. *Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. Spleens were harvested every 7 days. A: Percentage of CD45+ cells in the spleen after 7 days of fluoroquinolone treatment. B: Percentage (of total CD45+ spleen cells) of Neutrophils, Monocytes, Eosinophils, and NK cells after 7 days of fluoroquinolone treatment. C: Percentage (of total CD45+ spleen cells) of Macrophages and Dendritic Cells after 7 days of fluoroquinolone treatment. D: Percentage (of total CD45+ spleen cells) of B and T Cells after 7 days of fluoroquinolone treatment. (n=8-9). Significance tested independently for each experiment one or two-way ANOVA followed by Dunnet's or Sidak's multiple comparisons test respectively. Differentially shaded points represent different independent experiments.*

We next examined the immune populations of the spleen following the full 14 day course of fluoroquinolone dosing. After 14 days of fluoroquinolone treatment there was comparable CD45+ cells in the spleen in both ciprofloxacin and levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.9A). Examining the myeloid population, we saw a strong trend for an increase in neutrophils following levofloxacin treatment, but this did not quite reach significance as compared to the vehicle treated controls. As such, no significant alteration in neutrophils as well as monocytes, eosinophils or NK cells occurred following 14 days of either ciprofloxacin and levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.9B).

We next examined antigen presenting macrophages and dendritic cell populations but observed no significant difference following 14 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.9C).

Despite the negative gating, we again saw similar percentage populations of T and B-cells across all 3 independent experiments, albeit again no significant difference following 14 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.9D).

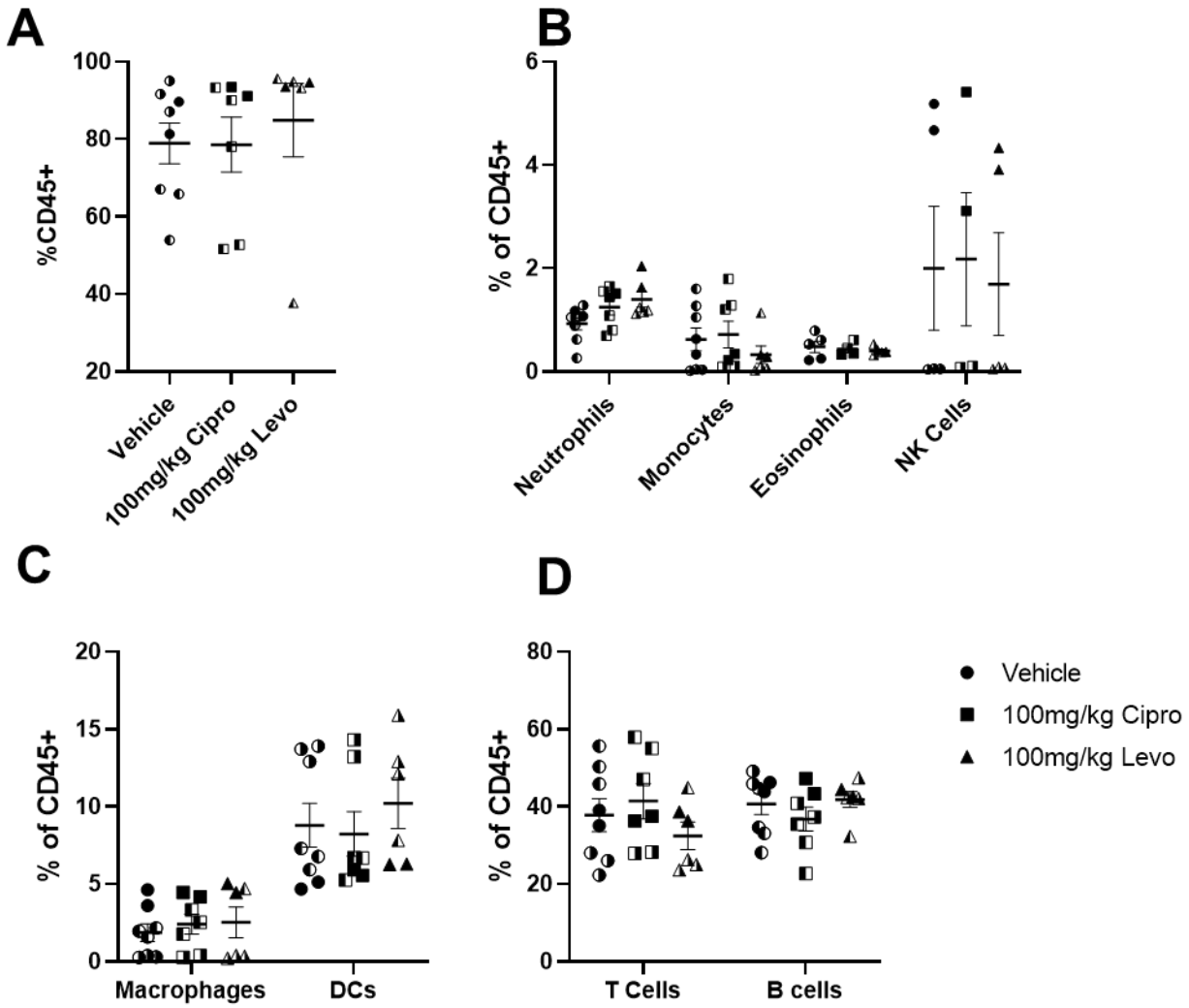


Figure 4.9: **Fluoroquinolones do not alter spleen immune cell subsets following 14 day treatment**
Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. Spleens were harvested every 7 days. A: Percentage of CD45+ cells in the spleen after 14 days of fluoroquinolone treatment. B: Percentage (of total CD45+ spleen cells) of Neutrophils, Monocytes, Eosinophils, and NK cells after 14 days of fluoroquinolone treatment. C: Percentage (of total CD45+ spleen cells) of Macrophages and Dendritic Cells after 14 days of fluoroquinolone treatment. D: Percentage (of total CD45+ spleen cells) of B and T Cells after 14 days of fluoroquinolone treatment. (n=8-9). Significance tested independently for each experiment one or two-way ANOVA followed by Dunnet's or Sidak's multiple comparisons test respectively. Differentially shaded points represent different independent experiments.

Finally, we examined splenic populations following a 7 day washout period after the 14 day fluoroquinolone treatment. After 7 days washout of fluoroquinolone treatment there was no significant change in the CD45+ population in either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.10A). Examining the myeloid population, we saw no significant alteration in neutrophils, monocytes, eosinophils or NK cells following 7 day washout period in either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.10B).

We observed no significant difference in macrophage or dendritic cell populations following 7 day washout in either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.10C).

Finally, we saw similar T and B-cell percentage populations across all 3 independent experiments following 7 day washout in either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.10D).

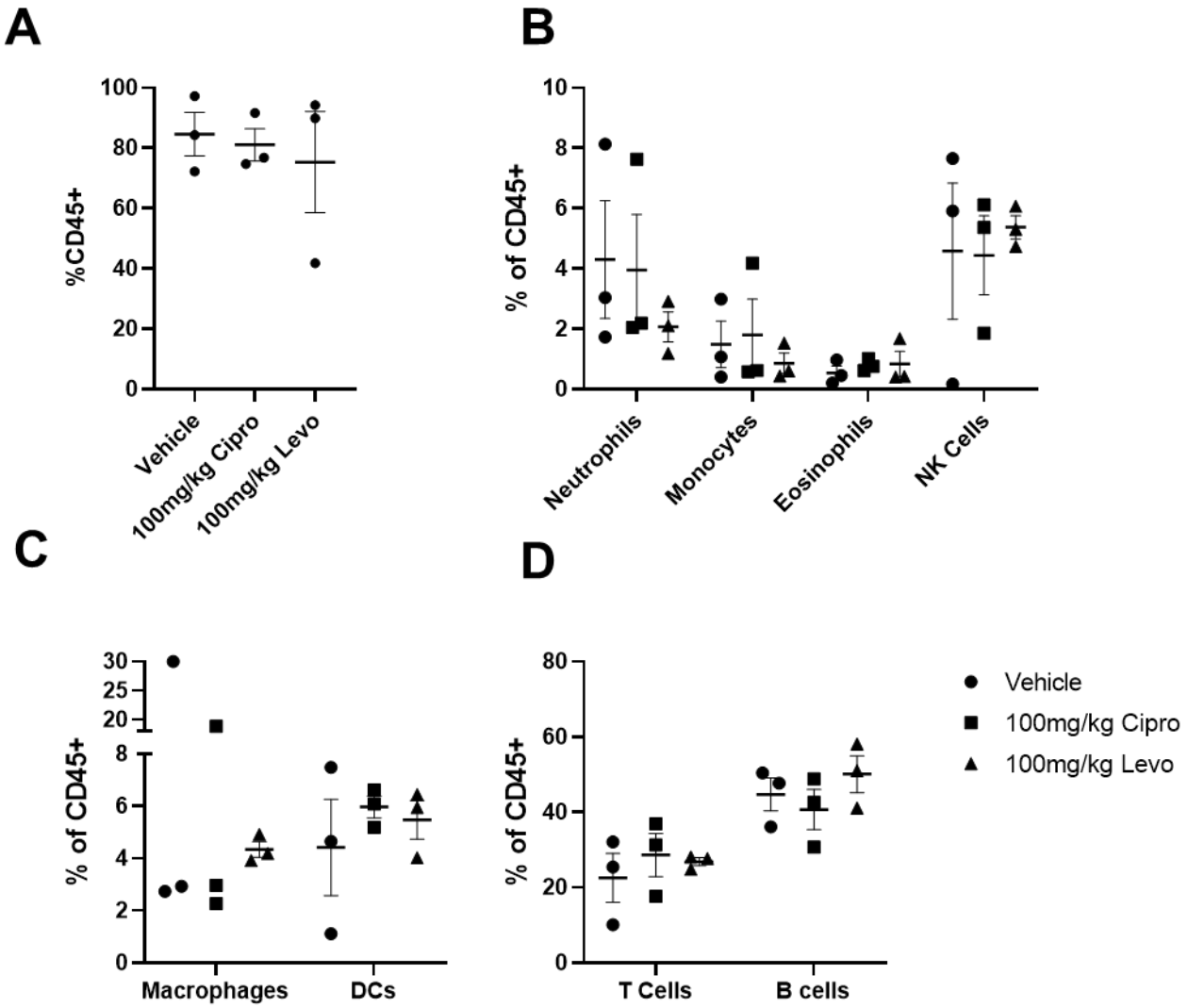


Figure 4.10: Fluoroquinolones do not alter spleen immune cell subsets following 14 day treatment and washout period. *Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. Spleens were harvested every 7 days. A: Percentage of CD45+ cells in the spleen after 14 days of fluoroquinolone treatment and 7 day runoff. B: Percentage (of total CD45+ spleen cells) of Neutrophils, Monocytes, Eosinophils, and NK cells after 14 days of fluoroquinolone treatment and 7 day runoff. C: Percentage (of total CD45+ spleen cells) of Macrophages and Dendritic Cells after 14 days of fluoroquinolone treatment and 7 day runoff. D: Percentage (of total CD45+ spleen cells) of B and T Cells after 14 days of fluoroquinolone treatment and 7 day runoff. n=3. Significance tested using one or two-way ANOVA followed by Dunnet's or Sidak's multiple comparisons test respectively.*

Spleen cells were next stimulated overnight with cell stimulation cocktail (with Golgi stop) in order to determine cytokine production ability of the CD45+ leukocyte population. No significant differences in IFN-g or TNF-a were seen in the ciprofloxacin or levofloxacin treated groups over the 14 day timecourse of treatment or following the 7 day wash out period (day 21) as compared to the vehicle treated controls (Fig. 4.11A and B).

Collectively, these data indicate that both ciprofloxacin and levofloxacin treatment have minimal impact on spleen immune cell population percentages across the time course of our experimental analysis.

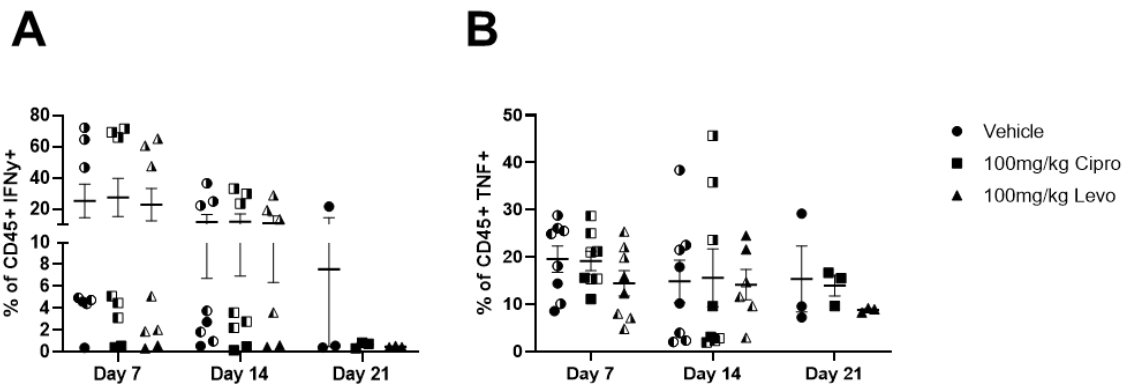


Figure 4.11: Fluoroquinolones do not alter spleen immune cell inflammatory cytokine production. *Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. Spleens were harvested every 7 days. Spleens were incubated overnight and stimulated with Cell Stimulation Cocktail with Golgi Stop. A: Percentage of IFNy+ cells (of total CD45+) in the spleen after 7, 14 and 21 days. B: Percentage of TNF+ cells (of total CD45+) in the spleen after 7, 14 and 21 days. (n=8-9) Significance tested independently for each experiment by two-way ANOVA followed by Sidak's multiple comparisons test.*

4.5 Fluoroquinolone treatment does not alter immune cell populations in the Gut- Associated lymphoid tissue (LI and MLN), but populations do alter following a 7 day wash out period in the mLN.

As well as the spleen, both the large intestine and mesenteric lymph node were harvested from animals every 7 days. These are particularly important tissues to analyse in this experiment, as they have the most direct connection with the site of drug administration. Immune cell analysis of the LI lamina propria (LILP) allows any direct ‘on site’ cellular changes to be detected, whereas the MLN shows any immune cells draining from the intestine into the lymphatic system.

We first examined cellularity of the gut-associated tissues across the time course and observed no significant differences (fig 4.12).

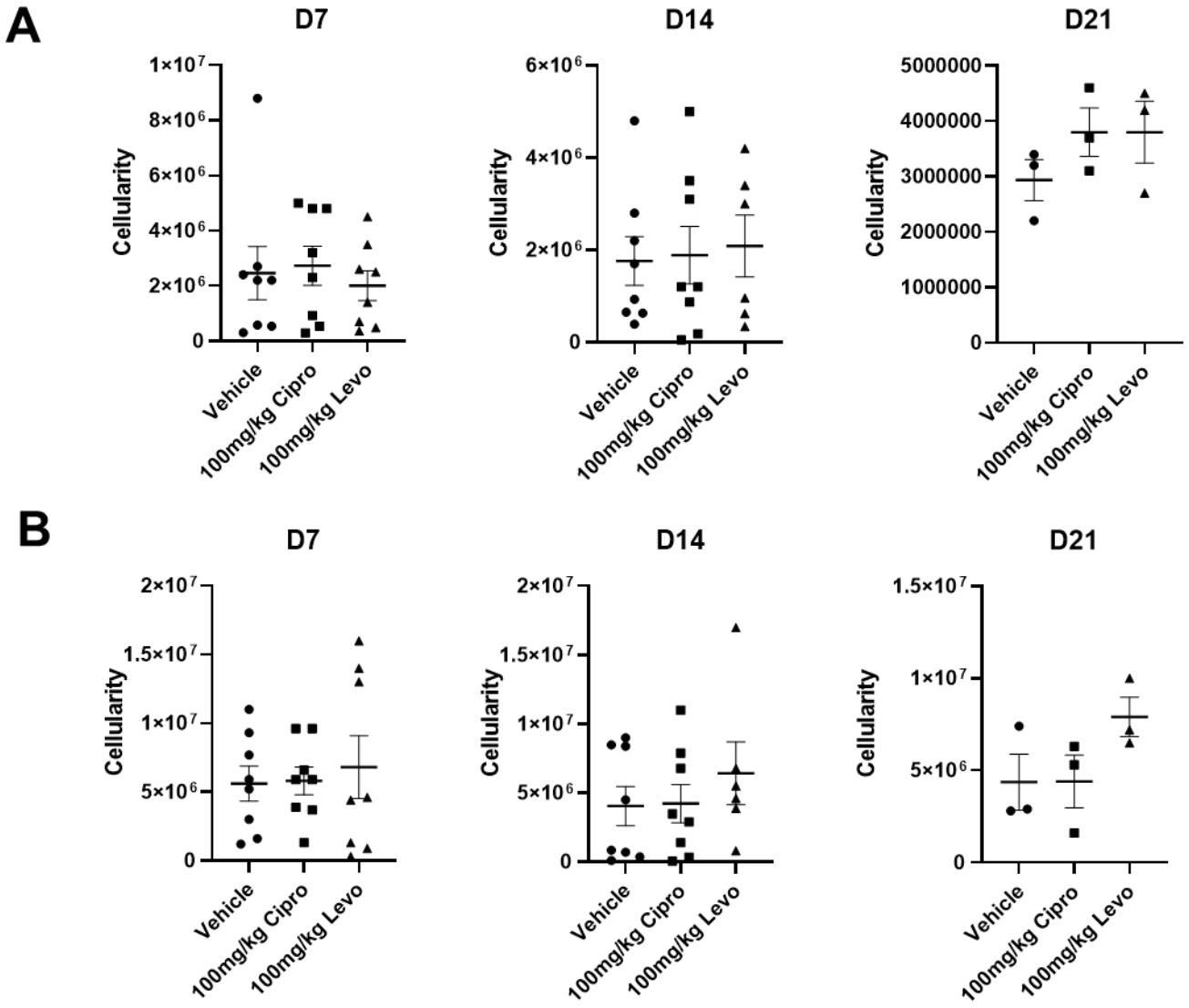


Figure 4.12: GALT cellularity after Fluoroquinolone treatment *Wild type BALB/c mice were treated by oral gavage with either Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. Large intestines and mesenteric lymph nodes were harvested every 7 days. A: Cellularity of Large intestine samples on day 7, 14 and 21 after lamina propria digestion. B: Cellularity of mesenteric lymph node samples on day 7, 14 and 21 after disaggregation.*

The myeloid phenotype was next analysed using the same broad innate immune flow cytometry panel. After 7 days of fluoroquinolone treatment there was no change in CD45+ cells in the LILP in both ciprofloxacin and levofloxacin treated animals as compared to the vehicle treated controls. (Fig. 4.13A). Examining the myeloid population, we saw no significant alteration in neutrophils, monocytes or eosinophils following 7 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.13B).

We saw no alteration in macrophages populations in the LILP following 7 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls. Despite no significant difference in DC populations following 7 days of either ciprofloxacin and levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.13C), it could be seen that DC populations had reduced means of around half in both fluoroquinolone treated groups.

Finally, we saw similar percentage populations of T and B-cells across all 3 independent experiments, albeit no significant difference following 7 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.13D).

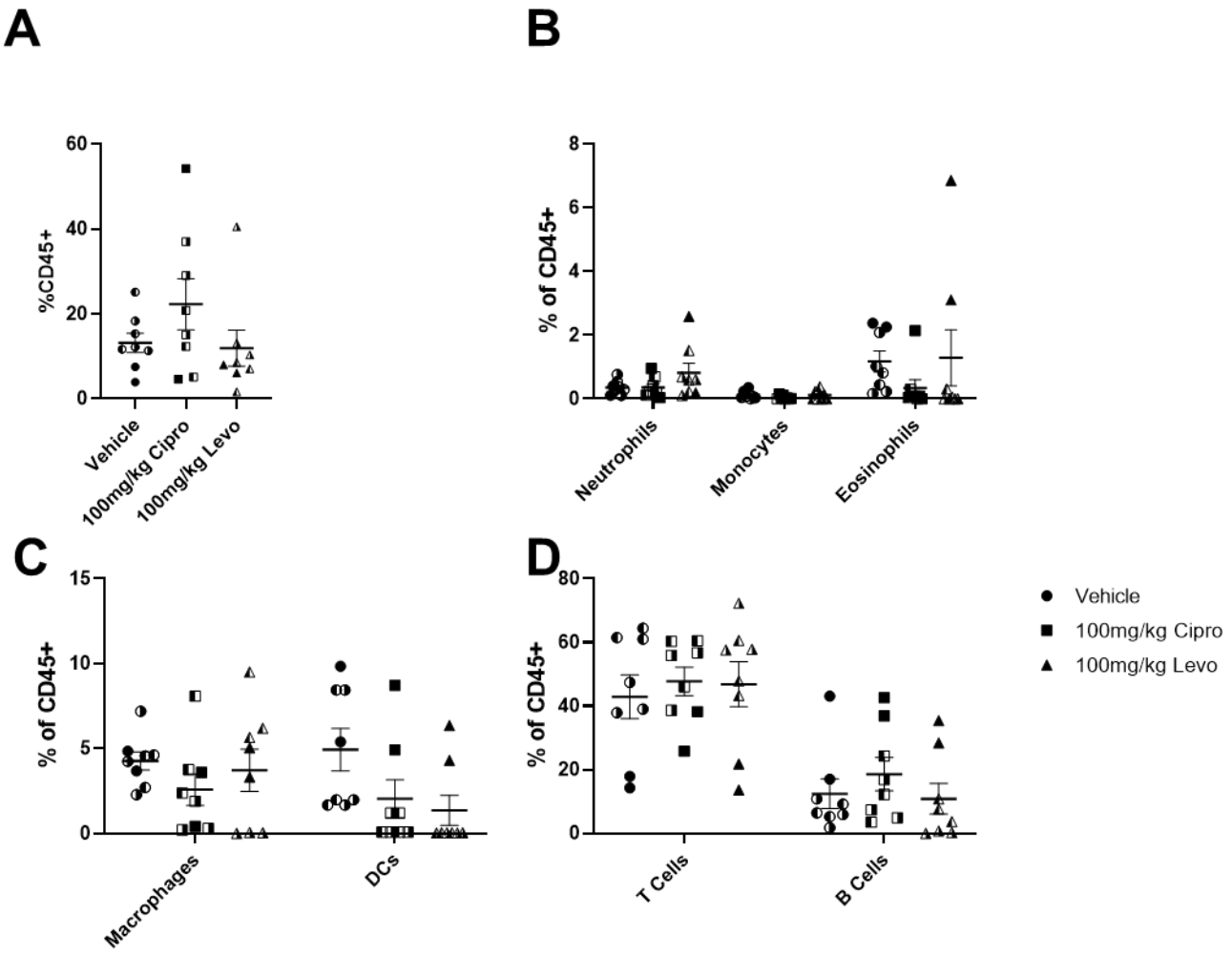


Figure 4.13: Fluoroquinolones do not alter LILP immune cell subsets following 7 day treatment. *Wild type BALB/c mice were treated by oral gavage with either* dH_2O *Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. Large intestines (LI) were harvested every 7 days. A: Percentage of CD45+ cells in the LI after 7 days of fluoroquinolone treatment. B: Percentage (of total CD45+ LI cells) of Neutrophils, Ly6c MHCII++ Monocytes, and Eosinophils after 7 days of fluoroquinolone treatment. C: Percentage (of total CD45+ LI cells) of Macrophages and Dendritic Cells after 7 days of fluoroquinolone treatment. D: Percentage (of total CD45+ LI cells) of B and T Cells after 7 days of fluoroquinolone treatment. (n=8-9). Significance tested independently for each experiment one or two-way ANOVA followed by Dunnet's or Sidak's multiple comparisons test respectively. Differentially shaded points represent different independent experiments.*

We next examined the immune populations of the LILP following the full 14 day course of fluoroquinolone dosing. After 14 days of fluoroquinolone treatment there was comparable CD45+ cells in the LILP in both ciprofloxacin and levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.14A). Examining the myeloid population, we saw no significant alteration in neutrophils, monocytes or eosinophils following 14 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.14B).

We next examined antigen presenting macrophages and dendritic cell populations but observed no significant difference in the LILP following 14 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.14C).

We again saw similar LILP percentage populations of T and B-cells across all 3 independent experiments, albeit again no significant difference following 14 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.14D).

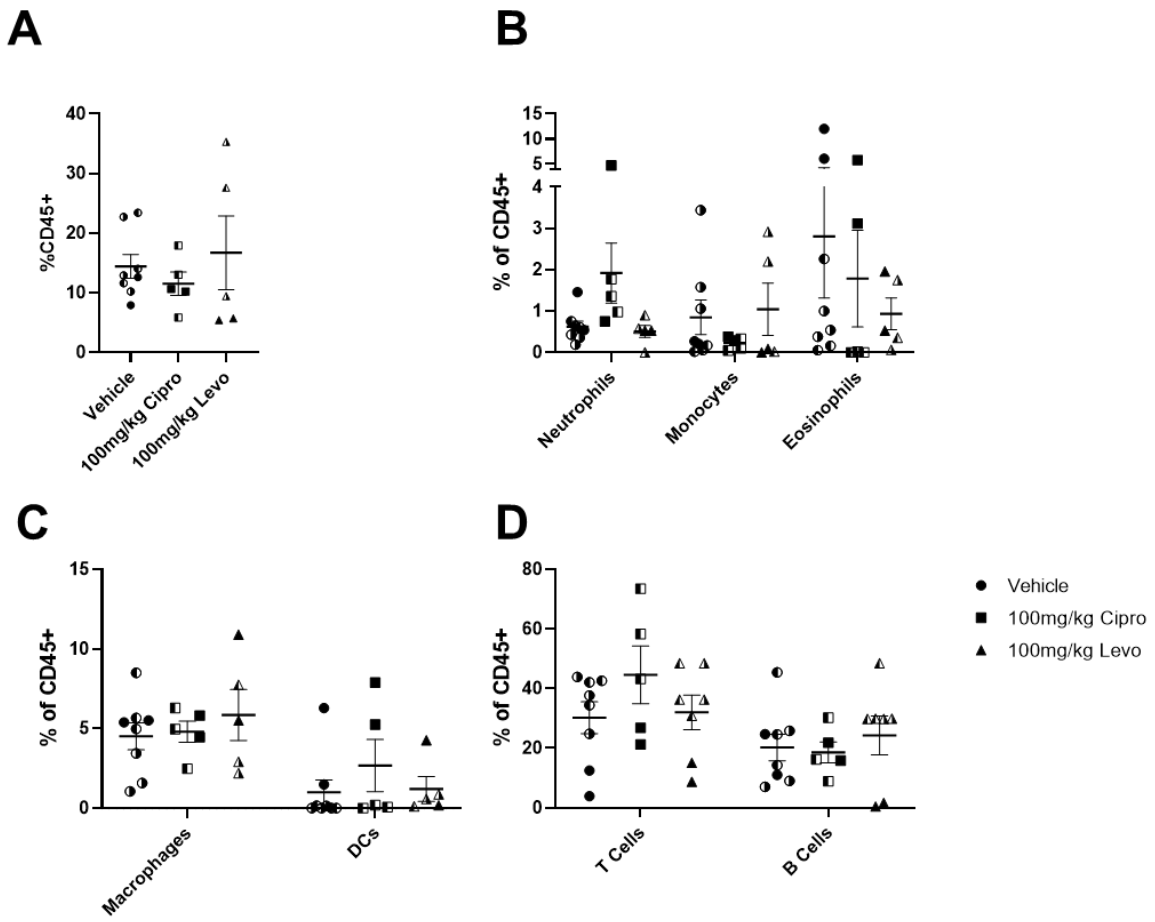


Figure 4.14: Fluoroquinolones do not alter LILP immune cell subsets following 14 day treatment. *Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. Large intestines (LI) were harvested every 7 days. A: Percentage of CD45+ cells in the LI after 7 days of fluoroquinolone treatment. B: Percentage (of total CD45+ LI cells) of Neutrophils, Ly6c MHCII++ Monocytes, and Eosinophils after 14 days of fluoroquinolone treatment. C: Percentage (of total CD45+ LI cells) of Macrophages and Dendritic Cells after 14 days of fluoroquinolone treatment. D: Percentage (of total CD45+ LI cells) of B and T Cells after 14 days of fluoroquinolone treatment. (n=8-9). Significance tested independently for each experiment one or two-way ANOVA followed by Dunnet's or Sidak's multiple comparisons test respectively. Differentially shaded points represent different independent experiments.*

We next examined the immune populations of the LILP following the 7 day wash out period after the full 14 day course of fluoroquinolone dosing. There was comparable CD45+ cells in the LILP in both ciprofloxacin and levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.15A). Examining the myeloid population, we saw no significant alteration in neutrophils, monocytes or eosinophils following the wash out period in either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.15B).

We next examined antigen presenting macrophages and dendritic cell populations but observed no significant difference in the LILP following the wash out period in either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.15C). We again saw similar LILP percentage populations of T and B-cells across all 3 independent experiments, albeit again no significant difference following the wash out period of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.15D).

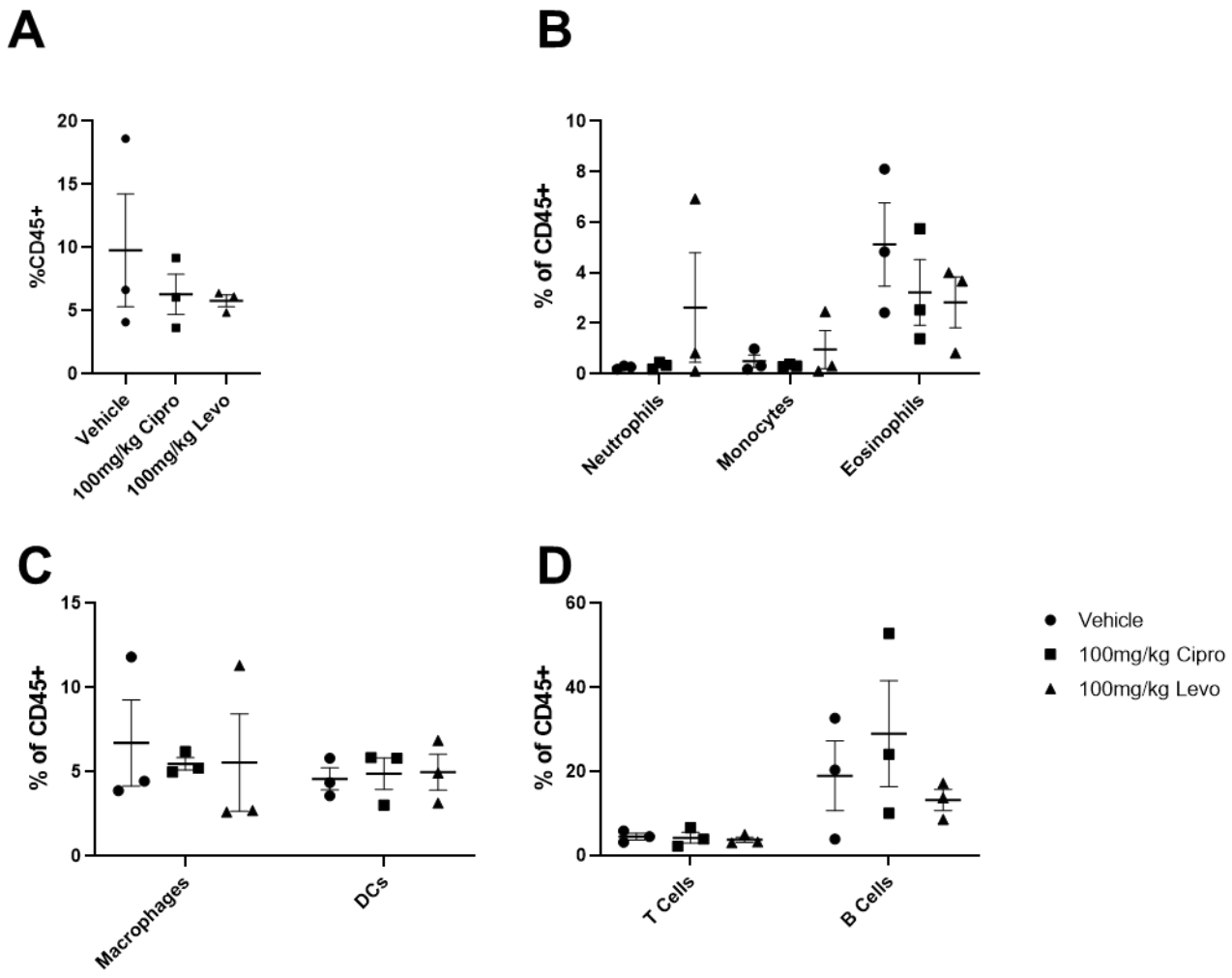


Figure 4.15 : Fluoroquinolones do not alter LILP immune cell subsets following 14 day treatment and washout period *Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=3/group) for 14 days, with a 7 day treatment-free 'washout period'. Large intestines (LI) were harvested every 7 days. A: Percentage of CD45+ cells in the LI after 14 days of fluoroquinolone treatment and 7 day runoff. B: Percentage (of total CD45+ LI cells) of Neutrophils, Ly6c MHCII++ Monocytes, and Eosinophils after 14 days of fluoroquinolone treatment and 7 day runoff. C: Percentage (of total CD45+ LI cells) of Macrophages and Dendritic Cells after 14 days of fluoroquinolone treatment and 7 day runoff. D: Percentage (of total CD45+ LI cells) of B and T Cells after 14 days of fluoroquinolone treatment and 7 day runoff. n=3. Significance tested using one or two-way ANOVA followed by Dunnet's or Sidak's multiple comparisons test respectively.*

We next analysed the gut draining mLN to further assess the immune response at the sight of antibiotic delivery. After 7 days of fluoroquinolone treatment there was no change in CD45+ cells in the mLN in both ciprofloxacin and levofloxacin treated animals as compared to the vehicle treated controls. (Fig. 4.16A). Examining the myeloid population, we saw no significant alteration in neutrophils, monocytes or eosinophils following 7 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.16B).

We saw a trend for increased percentage macrophages and DCs in the mLN of levofloxacin treated animals, but overall there was no alteration in macrophages or DC populations in the mLN following 7 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.16C).

Finally, we saw similar mLN percentage populations of T and B-cells across all 3 independent experiments, with no significant difference following 7 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.16D).

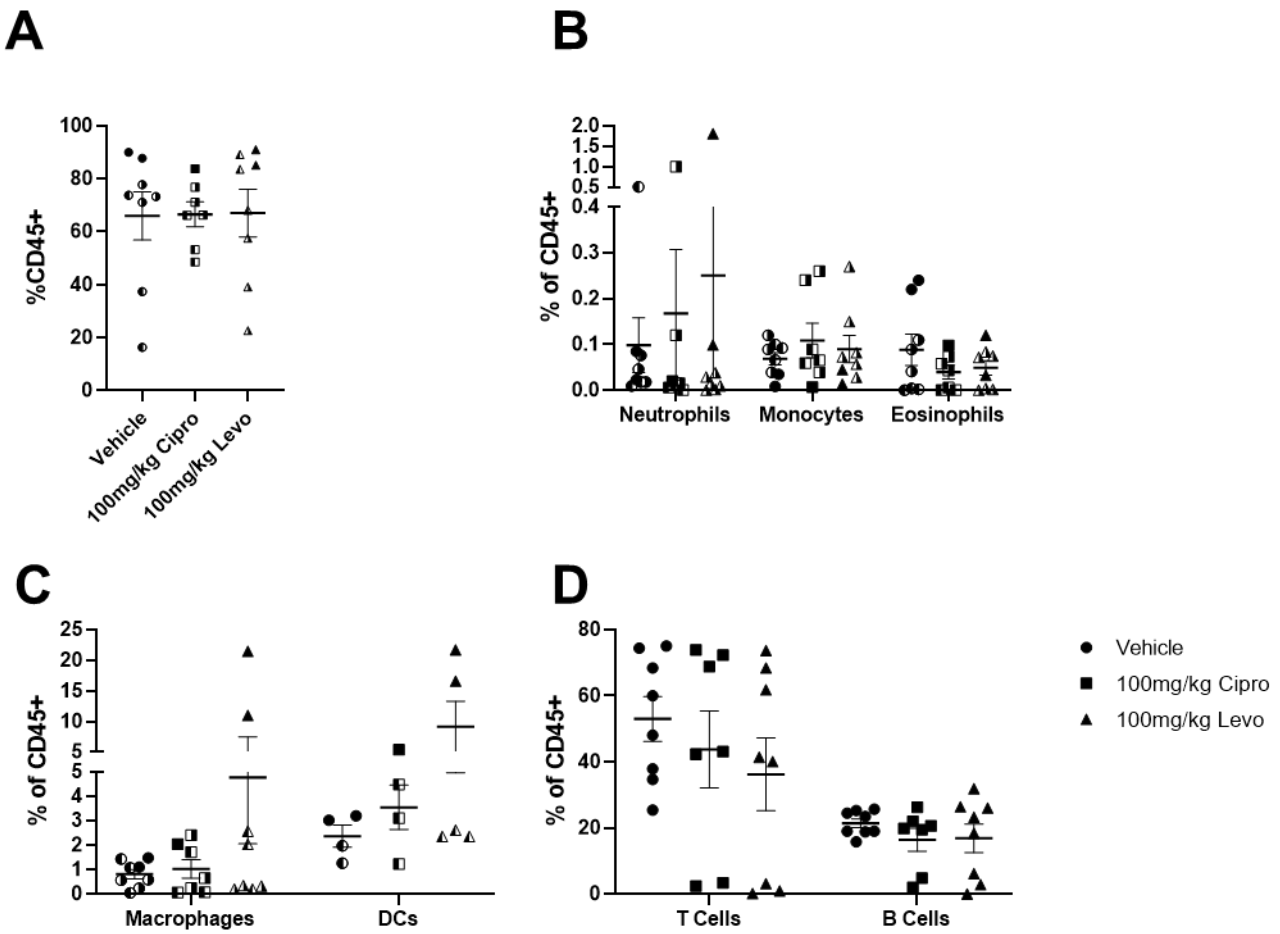


Figure 4.16 : Fluoroquinolones do not alter mLN immune cell subsets following 7 day treatment. *Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. Mesenteric lymph nodes (MLN) were harvested every 7 days. A: Percentage of CD45+ cells in the MLN after 7 days of fluoroquinolone treatment. B: Percentage (of total CD45+ MLN cells) of Neutrophils, Monocytes, and Eosinophils after 7 days of fluoroquinolone treatment. C: Percentage (of total CD45+ MLN cells) of Macrophages and Dendritic Cells after 7 days of fluoroquinolone treatment. D: Percentage (of total CD45+ MLN cells) of B and T Cells after 7 days of fluoroquinolone treatment. (n=8-9). Significance tested independently for each experiment one or two-way ANOVA followed by Dunnet's or Sidak's multiple comparisons test respectively. Differentially shaded points represent different independent experiments.*

We next examined the immune populations of the mLN following the full 14 day course of fluoroquinolone dosing. After 14 days of fluoroquinolone treatment there was comparable CD45+ cells in the mLN in both ciprofloxacin and levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.17A). Examining the myeloid population, we saw no significant alteration in neutrophils, monocytes or eosinophils following 14 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.17B).

We next examined antigen presenting macrophages and dendritic cell populations but observed no significant difference in the mLN following 14 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.17C). We again saw similar mLN percentage populations of T and B-cells across all 3 independent experiments. Although a slight trend for increased T-cells was observed in the fluoroquinolone groups, again no significant difference following 14 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls was seen for the T and B-cell populations of the mLN (Fig. 4.17D).

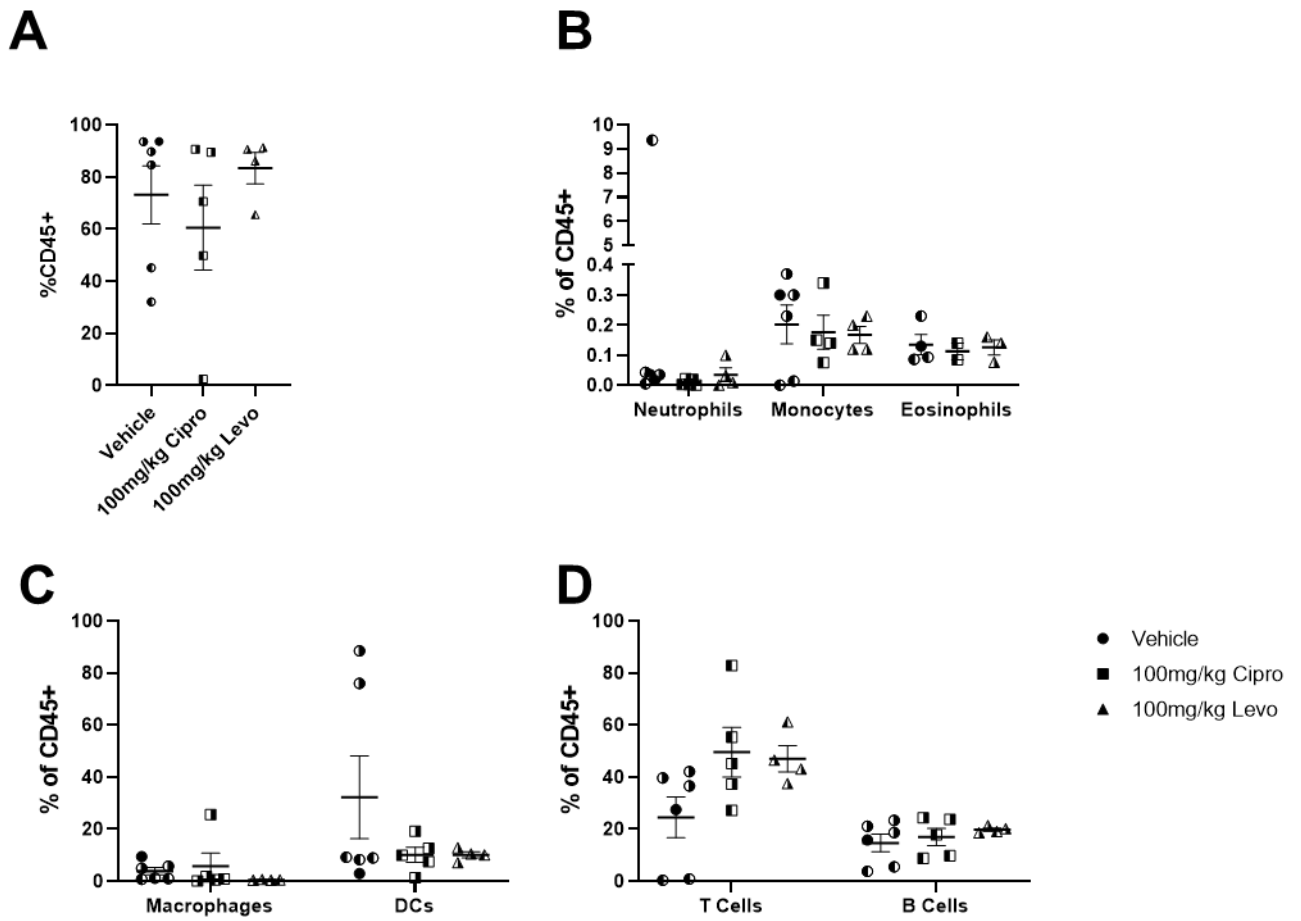


Figure 4.17: Fluoroquinolones do not alter mLN immune cell subsets following 14 day treatment. *Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. Mesenteric lymph nodes (MLN) were harvested every 7 days. A: Percentage of CD45+ cells in the MLN after 7 days of fluoroquinolone treatment. B: Percentage (of total CD45+ MLN cells) of Neutrophils, Monocytes, and Eosinophils after 14 days of fluoroquinolone treatment. C: Percentage (of total CD45+ MLN cells) of Macrophages and Dendritic Cells after 14 days of fluoroquinolone treatment. D: Percentage (of total CD45+ MLN cells) of B and T Cells after 14 days of fluoroquinolone treatment. (n=8-9). Significance tested tested independently for each experiment one or two-way ANOVA followed by Dunnet's or Sidak's multiple comparisons test respectively. Differentially shaded points represent different independent experiments.*

We next examined the immune populations of the LILP following the 7 day wash out period after the full 14 day course of fluoroquinolone dosing. There was comparable CD45+ cells in the mLN in both ciprofloxacin and levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.18A), although some of the treated animals had surprisingly low CD45 populations for the mLN.

Examining the myeloid population of levofloxacin treated animals, we saw significant increases in neutrophils, eosinophils and NK cells as compared to the vehicle treated controls (Fig. 4.18B). Examining the ciprofloxacin treated group we saw no significant difference in neutrophils and eosinophils as compared to vehicle treated controls, but we did see a significant increase in NK cells (Fig. 4.18B). We saw no alteration in monocytes in the wash out period in either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.18B).

We next examined antigen presenting dendritic cell populations but observed no significant difference in the mLN following the wash out period in either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.18C). We saw similar mLN populations of macrophages in the vehicle controls and levofloxacin treated groups, but a significant increase following ciprofloxacin treatment after the wash our period as compared to the vehicle controls (Fig. 4.18C).

The percentage populations of T and B-cells had no significant difference following the wash out period in the levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.18D). The ciprofloxacin treated groups had greatly reduced T and B-cell populations reaching significance for the T-cell population compared to the vehicle treated controls (Fig. 4.18D).

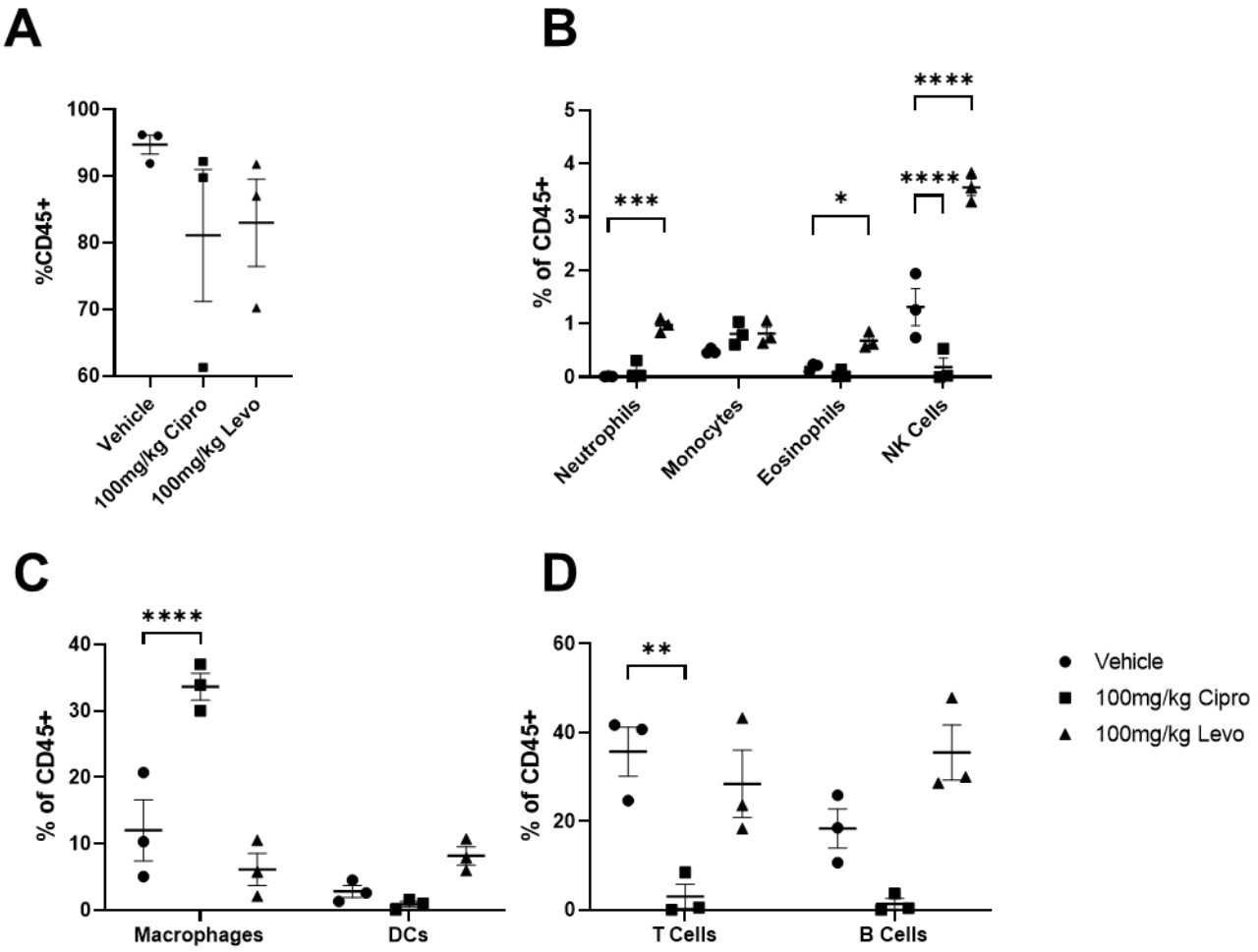


***Figure 4.18*: Fluoroquinolones differentially alter multiple immune cell subsets following 14 day treatment and washout period** *Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. Mesenteric lymph nodes (MLN) were harvested every 7 days. A: Percentage of CD45+ cells in the MLN after 14 days of fluoroquinolone treatment and 7 day runoff. B: Percentage (of total CD45+ MLN cells) of Neutrophils, Monocytes, and Eosinophils after 14 days of fluoroquinolone treatment and 7 day runoff. C: Percentage (of total CD45+ MLN cells) of Macrophages and Dendritic Cells after 14 days of fluoroquinolone treatment and 7 day runoff. D: Percentage (of total CD45+ MLN cells) of B and T Cells after 14 days of fluoroquinolone treatment and 7 day runoff. n=3. Significance tested using one or two-way ANOVA followed by Dunnet's or Sidak's multiple comparisons test respectively.*

LILP and MLN cells were next stimulated overnight with cell stimulation cocktail (with Golgi stop) in order to determine cytokine production ability of the CD45+ leukocyte population. No significant differences in IFNγ or TNFα were seen in the ciprofloxacin or levofloxacin treated groups over the 14 day timecourse of treatment or following the 7 day wash out period (day 21) as compared to the vehicle treated controls in either of the LILP or MLN experiments (Fig. 4.19A and B).

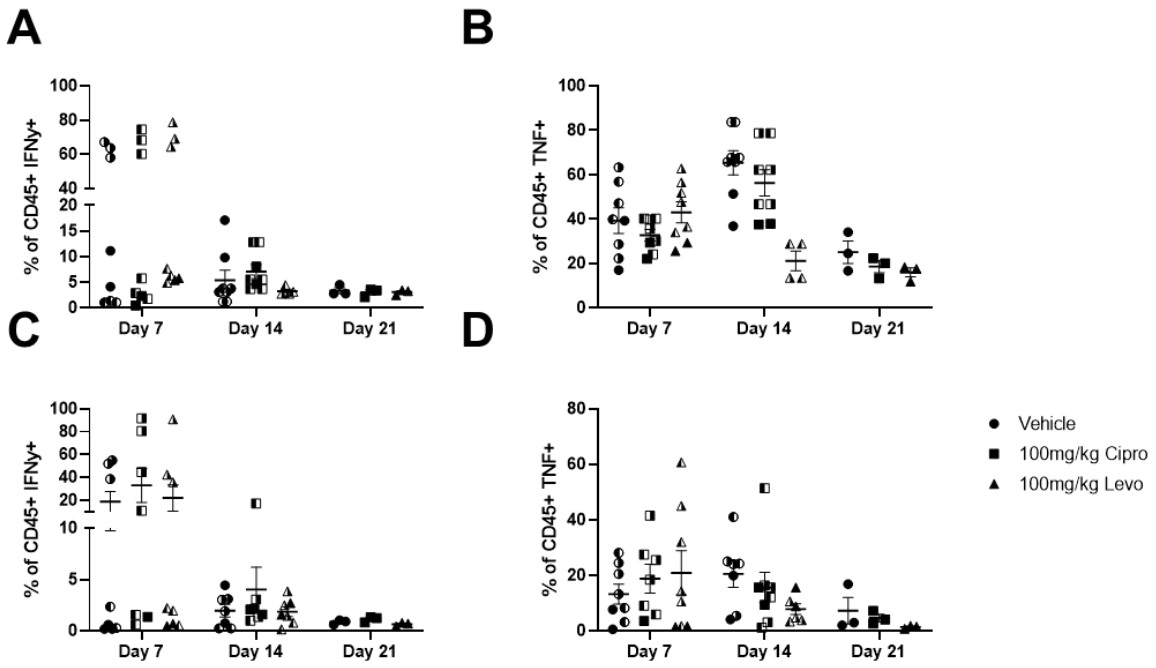


Figure 4.19: Fluoroquinolones do not alter GALT immune cell inflammatory cytokine production. *Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. MLNs and LIs were harvested every 7 days. Cells were incubated overnight and stimulated with Cell Stimulation Cocktail with Golgi Stop A: Percentage of IFNγ+ cells (of total CD45+) in the LI after 7, 14 and 21 days. B: Percentage of TNF+ cells (of total CD45+) in the LI after 7, 14 and 21 days. C: Percentage of IFNγ+ cells (of total CD45+) in the MLN after 7, 14 and 21 days. D: Percentage of TNF+ cells (of total CD45+) in the MLN after 7, 14 and 21 days. . (n=8-9d7-14, n=3 d21). Significance tested independently for each experiment one or two-way ANOVA followed by Dunnet's or Sidak's multiple comparisons test respectively. Differentially shaded points represent different independent experiments.*

Collectively, these data indicate that both ciprofloxacin and levofloxacin treatment have minimal impact on LILP immune cell population percentages across the time course of our experimental analysis, but the wash out period produces multiple alterations in populations in the mLN which are distinct in ciprofloxacin and levofloxacin.

4.5 Fluoroquinolone treatment does not alter immune cell populations in the BALB/c lung, but levofloxacin alters lung tissue histologically

We next repeated the flow cytometry panels on lung tissue and Bronchoalveolar lavage (BAL) from animals every 7 days. These are particularly important niches as any immunomodulatory effects fluoroquinolones have on lung cells are of particular importance. Fluoroquinolones are prescribed to treat a variety of severe respiratory tract infections, and as such, any negative effects on the lung immune system could have serious consequences, while positive would be in addition to direct bactericidal modulation.

We first examined cellularity of the lung and BAL across the time course and observed no significant differences in either BAL or lung cellularity after fluoroquinolone treatment (Fig. 4.20).

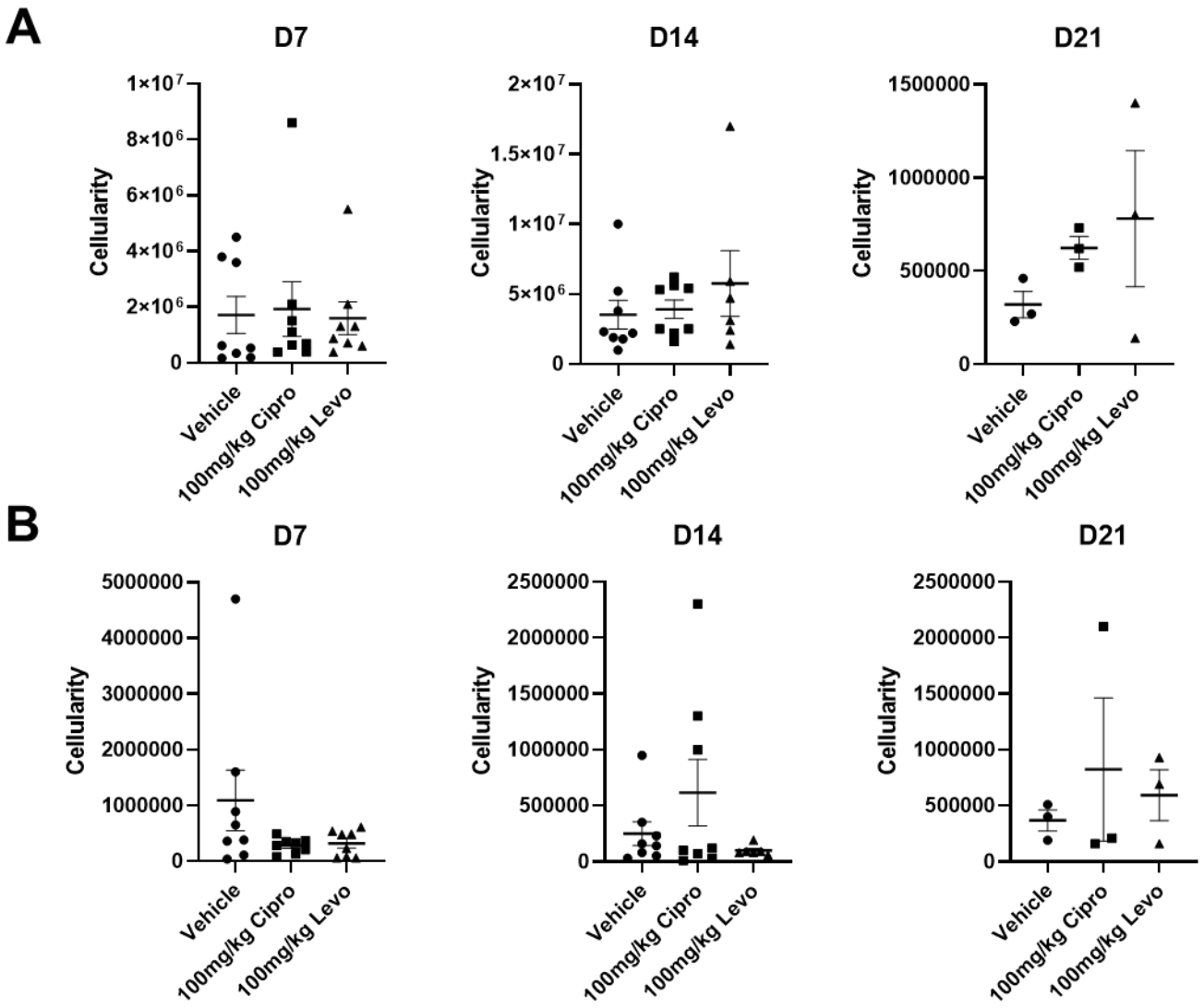


Figure 4.20: Lung and BAL cellularity after Fluoroquinolone treatment*. Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. Bronchoalveolar lavage was performed and Lungs were harvested every 7 days. A: Cellularity of lung samples on day 7, 14 and 21 after lung digest and red blood cell lysis. B: Cellularity of Bronchoalveolar lavage samples on day 7, 14 and 21.*

We next analysed the lung tissue to further assess the immune response at the key site of bacterial infection. After 7 days of fluoroquinolone treatment there was no change in CD45+ cells in the lung in both ciprofloxacin and levofloxacin treated animals as compared to the vehicle treated controls. (Fig. 4.21A). Examining the myeloid population, we saw no significant alteration in neutrophils, monocytes, NK cells or eosinophils following 7 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.21B).

Both macrophages and DC populations in the lung of both ciprofloxacin and levofloxacin treated animals were not altered following 7 days of either antibiotic as compared to the vehicle treated controls (Fig. 4.21C).

Finally, we saw similar lung percentage populations of T and B-cells with no significant difference following 7 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.21D).

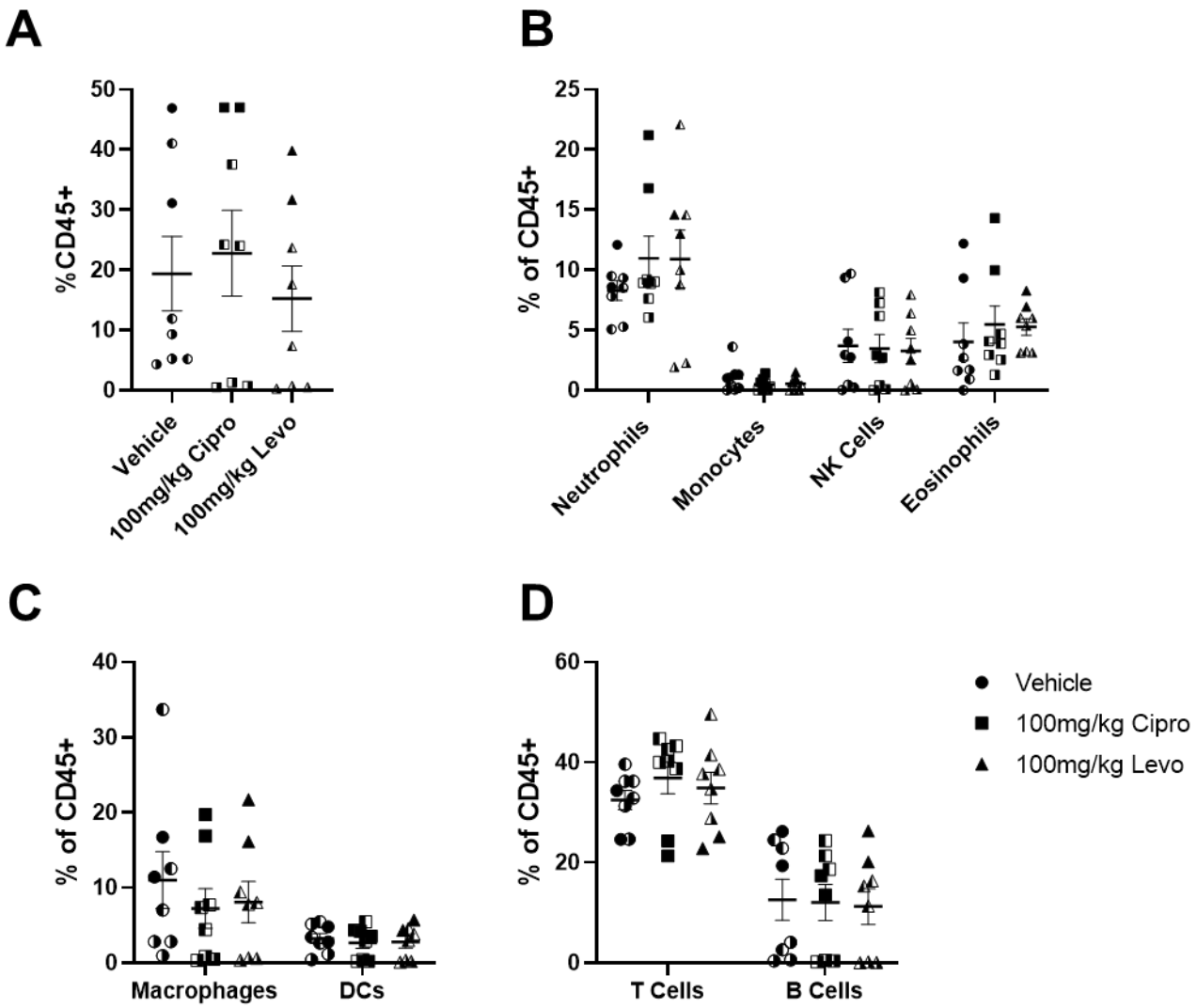


Figure 4.21: Fluoroquinolones do not alter Lung immune cell subsets following 7 day treatment Wild type BALB/c mice were treated by oral gavage with either *dH_2O* Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. Lungs were harvested every 7 days. A: Percentage of CD45+ cells in the lung after 7 days of fluoroquinolone treatment. B: Percentage (of total CD45+ Lung cells) of Neutrophils, Monocytes, and Eosinophils after 7 days of fluoroquinolone treatment. C: Percentage (of total CD45+ Lung cells) of Macrophages and Dendritic Cells after 7 days of fluoroquinolone treatment. D: Percentage (of total CD45+ Lung cells) of B and T Cells after 7 days of fluoroquinolone treatment. (n=8-9). Significance tested independently for each experiment one or two-way ANOVA followed by Dunnet's or Sidak's multiple comparisons test respectively. Differentially shaded points represent different independent experiments.

We next examined the immune populations of the lung following the full 14 day course of fluoroquinolone dosing. After 14 days of fluoroquinolone treatment, despite a trend for increased population in the levofloxacin treated group, there was comparable CD45+ cells in the lung in both ciprofloxacin and levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.22A).

Examining the myeloid population, we saw no significant alteration in monocytes, NK cells or eosinophils following 14 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls. Although again not significant as compared to vehicle treated controls, we did see higher averages of neutrophils in both antibiotic treated groups (Fig.4.22B).

Both macrophages and DC populations in the lung of both ciprofloxacin and levofloxacin treated animals were not altered following 14 days of either antibiotic as compared to the vehicle treated controls, although a broad variation between experiments was observed (Fig. 4.22C).

Finally, we saw similar lung percentage populations of T and B-cells with no significant difference following 14 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.22D).

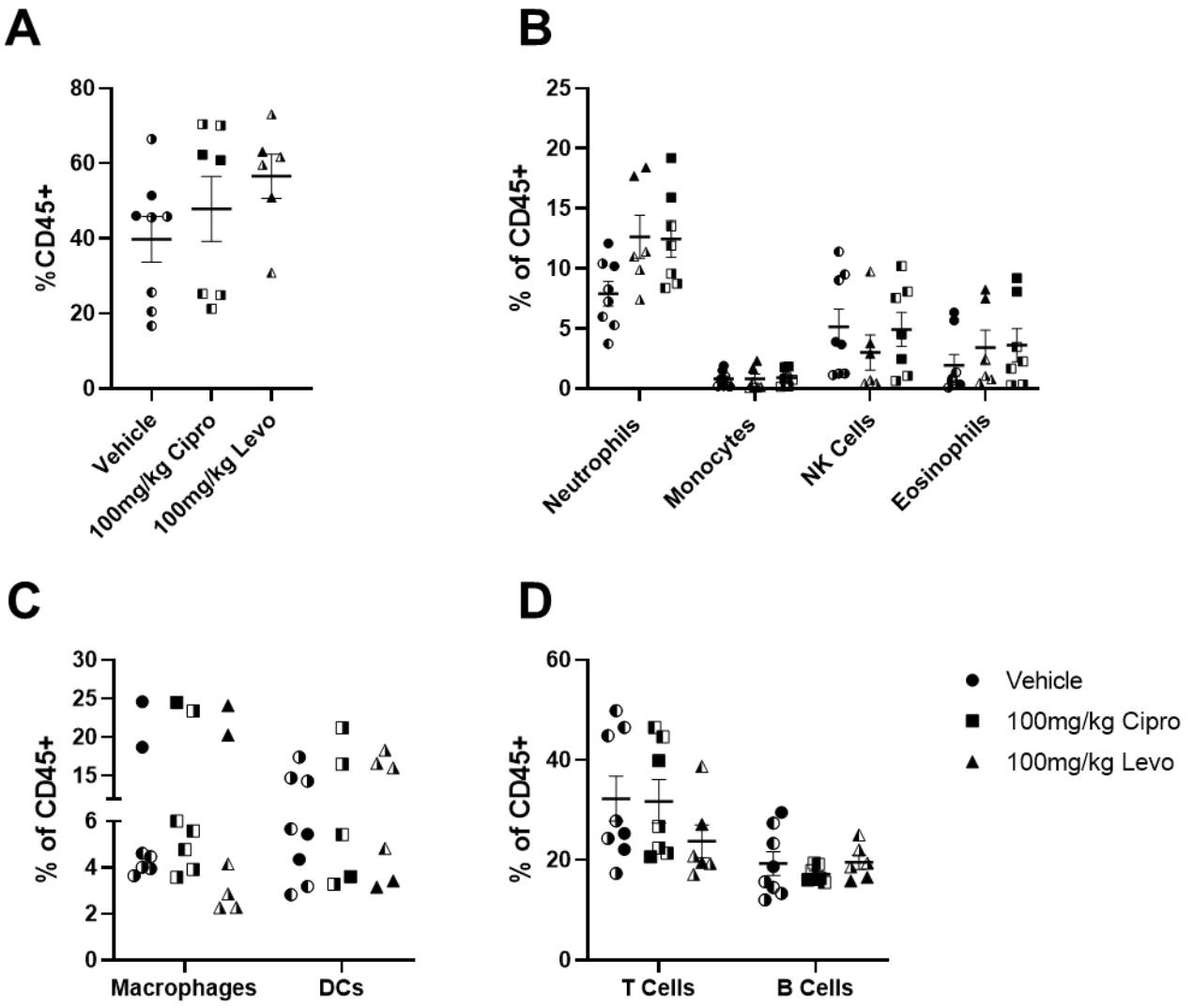


Figure 4.22: **Fluoroquinolones do not alter Lung immune cell subsets following 14 day treatment** *Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. Lungs were harvested every 7 days. A: Percentage of CD45+ cells in the lung after 14 days of fluoroquinolone treatment. B: Percentage (of total CD45+ Lung cells) of Neutrophils, Monocytes, and Eosinophils after 14 days of fluoroquinolone treatment. C: Percentage (of total CD45+ Lung cells) of Macrophages and Dendritic Cells after 14 days of fluoroquinolone treatment. D: Percentage (of total CD45+ Lung cells) of B and T Cells after 14 days of fluoroquinolone treatment. (n=8-9). Significance tested independently for each experiment one or two-way ANOVA followed by Dunnet's or Sidak's multiple comparisons test respectively. Differentially shaded points represent different independent experiments.*

We next examined the immune populations of the Lung following the 7 day wash out period after the full 14 day course of fluoroquinolone dosing. There was comparable CD45+ cells in the lung in both ciprofloxacin and levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.23A).

Examining the lung myeloid population, we saw no significant alteration in neutrophils, monocytes or eosinophils following the wash out period in either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.23B). Although again a trend for increased neutrophils was again observed in both antibiotic groups, albeit non-significant.

We next examined antigen presenting macrophages and dendritic cell populations but observed no significant difference in the Lung following the wash out period in either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.23C). The percentage populations of T and B-cells had no significant difference following the wash out period in the levofloxacin and ciprofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.23D).

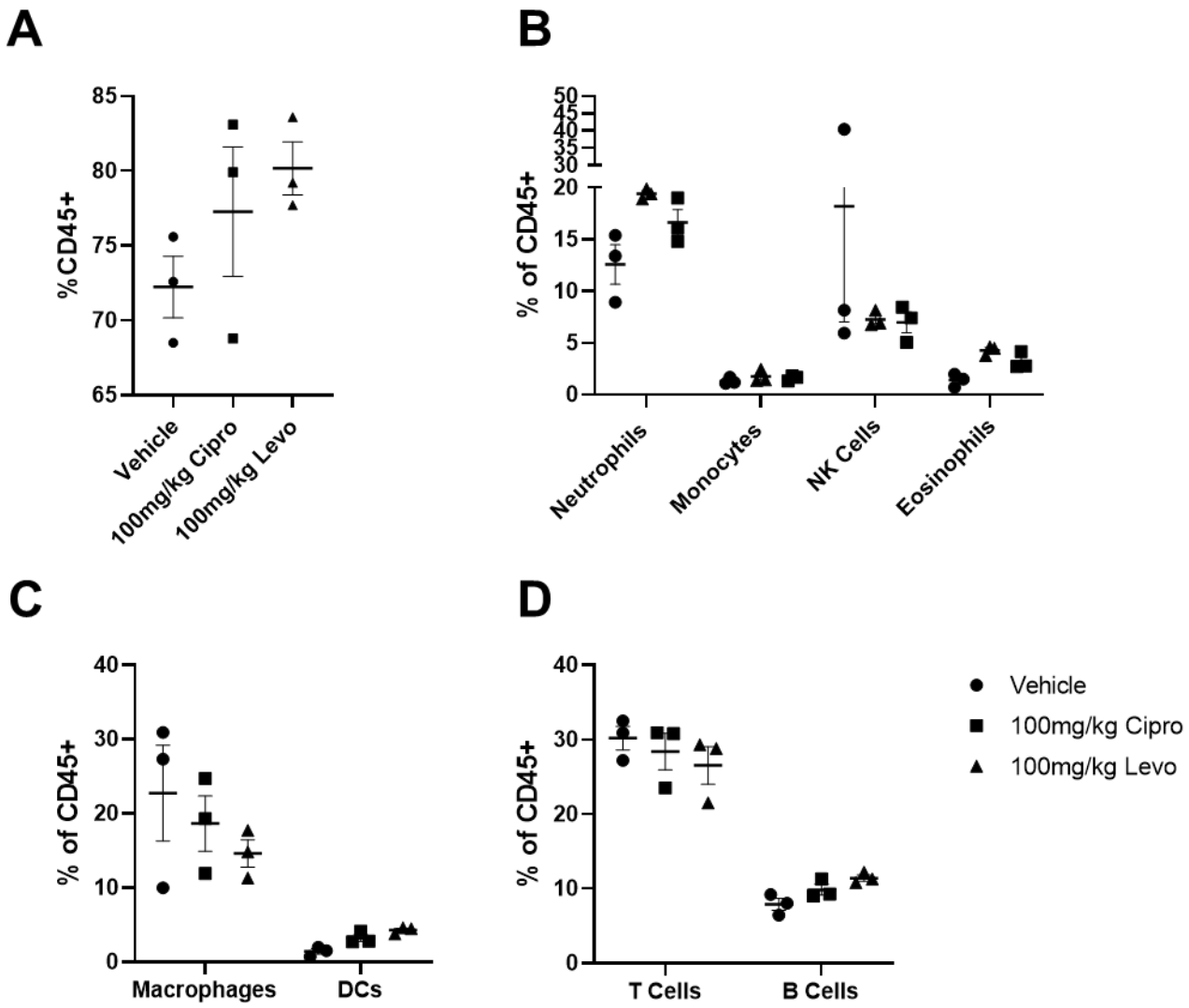


Figure 4.23: **Fluoroquinolones do not alter lung immune cell subsets following 14 day treatment and washout period** *Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. Lungs were harvested every 7 days. A: Percentage of CD45+ cells in the lung after 14 days of fluoroquinolone treatment and 7 day runoff. B: Percentage (of total CD45+ Lung cells) of Neutrophils, Monocytes, and Eosinophils after 21 days of fluoroquinolone treatment and 7 day runoff. C: Percentage (of total CD45+ Lung cells) of Macrophages and Dendritic Cells after 21 days of fluoroquinolone treatment and 7 day runoff. D: Percentage (of total CD45+ Lung cells) of B and T Cells after 21 days of fluoroquinolone treatment and 7 day runoff. n=3. Significance tested using one or two-way ANOVA followed by Dunnet's or Sidak's multiple comparisons test respectively.*

We next analysed the BAL to further assess the immune response at the niche of bacterial infection. After 7 days of fluoroquinolone treatment, despite an increased mean in both antibiotic treated groups, there was no change in CD45+ cells in the lung in both ciprofloxacin and levofloxacin treated animals as compared to the vehicle treated controls. (Fig. 4.24A). Examining the myeloid population, we saw no significant alteration in neutrophils, monocytes, NK cells or eosinophils following 7 days of either ciprofloxacin and levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.24B), although a trend for increased neutrophils was again present in both fluoroquinolone treated groups.

Both macrophages and DC populations in the BAL of both ciprofloxacin and levofloxacin treated animals, although increasing in mean, were not significantly altered following 7 days of either antibiotic as compared to the vehicle treated controls (Fig. 4.24C).

Finally, we saw reduced BAL percentage populations of T and B-cells but this did not reach significance following 7 days of either ciprofloxacin and levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.24D).

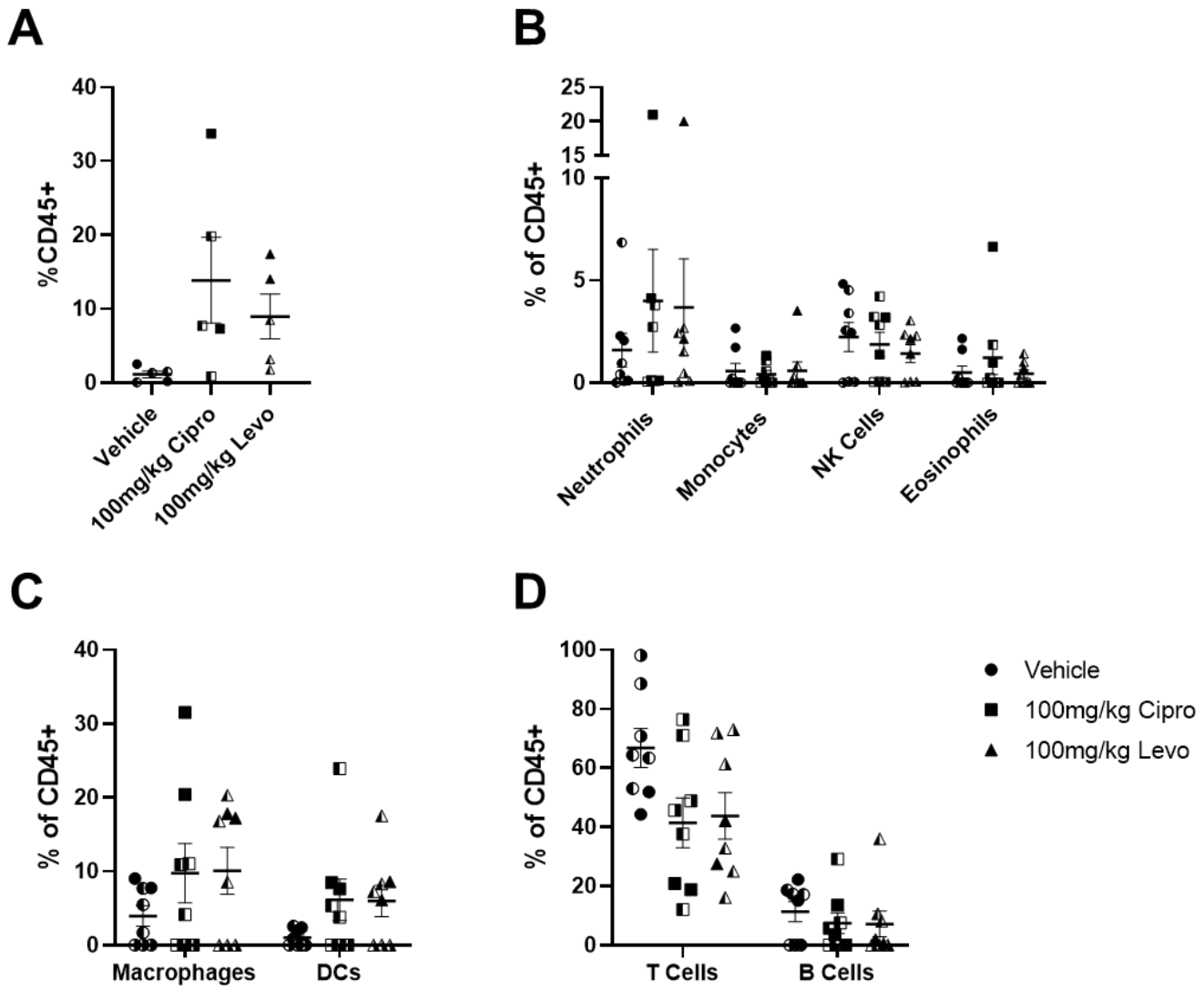


Figure 4.24: **Fluoroquinolones do not alter BAL immune cell subsets following 7 day treatment** *Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. Bronchoalveolar lavage (BAL) was performed post-mortem every 7 days. A: Percentage of CD45+ cells in the BAL fluid after 7 days of fluoroquinolone treatment. B: Percentage (of total CD45+ BAL cells) of Neutrophils, Monocytes, and Eosinophils after 7 days of fluoroquinolone treatment. C: Percentage (of total CD45+ BAL cells) of Macrophages and Dendritic Cells after 7 days of fluoroquinolone treatment. D: Percentage (of total CD45+ BAL cells) of B and T Cells after 7 days of fluoroquinolone treatment. (n=8-9). Significance tested independently for each experiment one or two-way ANOVA followed by Dunnet's or Sidak's multiple comparisons test respectively. Differentially shaded points represent different independent experiments.*

We next examined the BAL immune populations following the full 14 day course of fluoroquinolone dosing. After 14 days of fluoroquinolone treatment, despite a trend for decreased population in the ciprofloxacin treated group, there was comparable CD45+ cells in the BAL in both ciprofloxacin and levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.25A). Examining the myeloid population, we saw no significant alteration in neutrophils, monocytes, NK cells or eosinophils following 14 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls. Both macrophages and DC populations in the BAL of both ciprofloxacin and levofloxacin treated animals were not altered following 14 days of either antibiotic as compared to the vehicle treated controls, although a broad variation between experiments was observed (Fig. 4.25).

Finally, we saw similar BAL percentage populations of T and B-cells with no significant difference following 14 days of either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.25D).

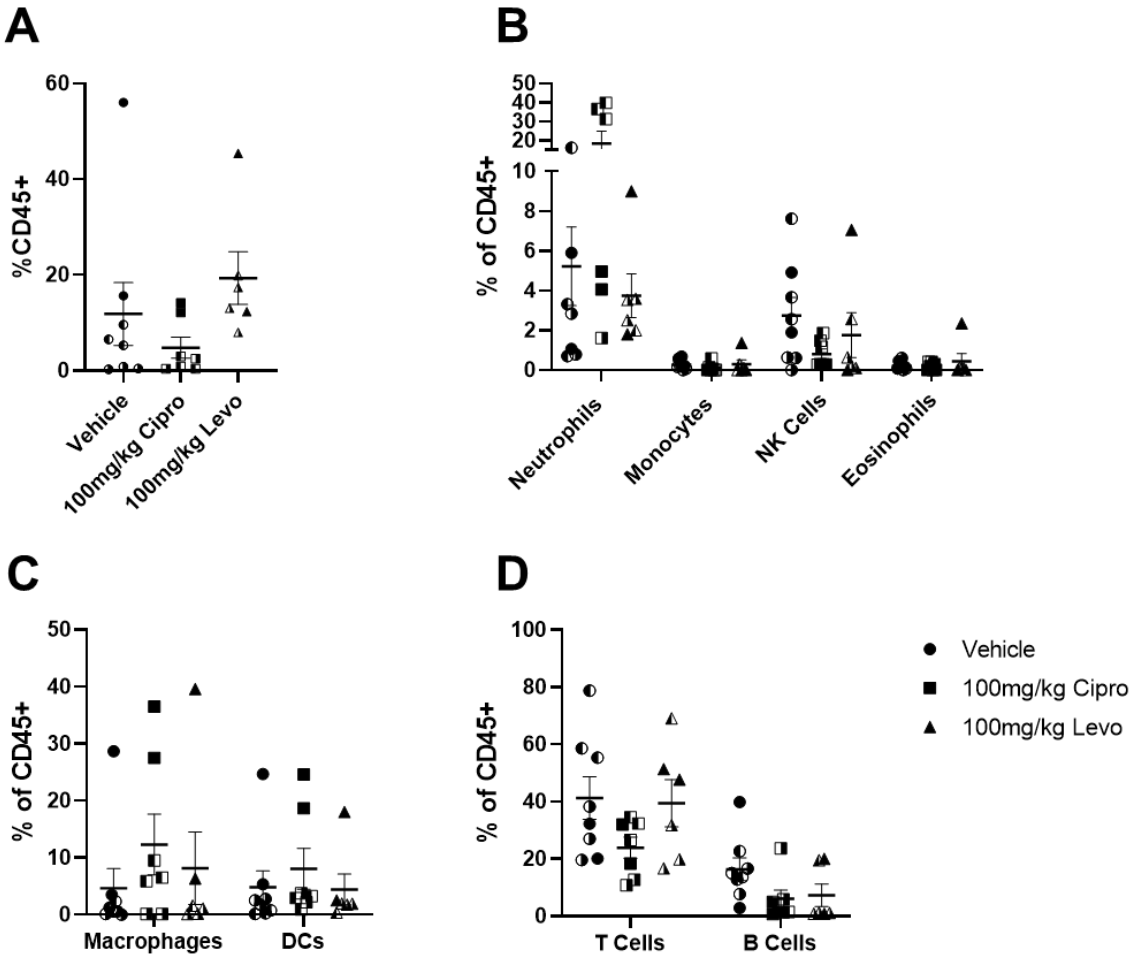


Figure 4.25: Fluoroquinolones do not alter BAL immune cell subsets following 14 day treatment *Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. Bronchoalveolar lavage (BAL) was performed post-mortem every 7 days. A: Percentage of CD45+ cells in the BAL fluid after 14 days of fluoroquinolone treatment. B: Percentage (of total CD45+ BAL cells) of Neutrophils, Monocytes, and Eosinophils after 14 days of fluoroquinolone treatment. C: Percentage (of total CD45+ BAL cells) of Macrophages and Dendritic Cells after 14 days of fluoroquinolone treatment. D: Percentage (of total CD45+ BAL cells) of B and T Cells after 14 days of fluoroquinolone treatment. (n=8-9). Significance tested tested independently for each experiment one or two-way ANOVA followed by Dunnet's or Sidak's multiple comparisons test respectively. Differentially shaded points represent different independent experiments.*

We next examined BAL immune populations following the 7 day wash out period after the full 14 day course of fluoroquinolone dosing. There was comparable CD45+ cells in the BAL in both ciprofloxacin and levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.26A).

Examining the lung myeloid population, despite varied percentages we saw no significant alteration in neutrophils, as well as monocytes, NK cells or eosinophils following the wash out period in either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.26B).

We next examined antigen presenting macrophages and dendritic cell populations but observed no significant difference in the BAL following the wash out period in either ciprofloxacin or levofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.26C). The percentage populations of T and B-cells had no significant difference following the wash out period in the levofloxacin and ciprofloxacin treated animals as compared to the vehicle treated controls (Fig. 4.26D).

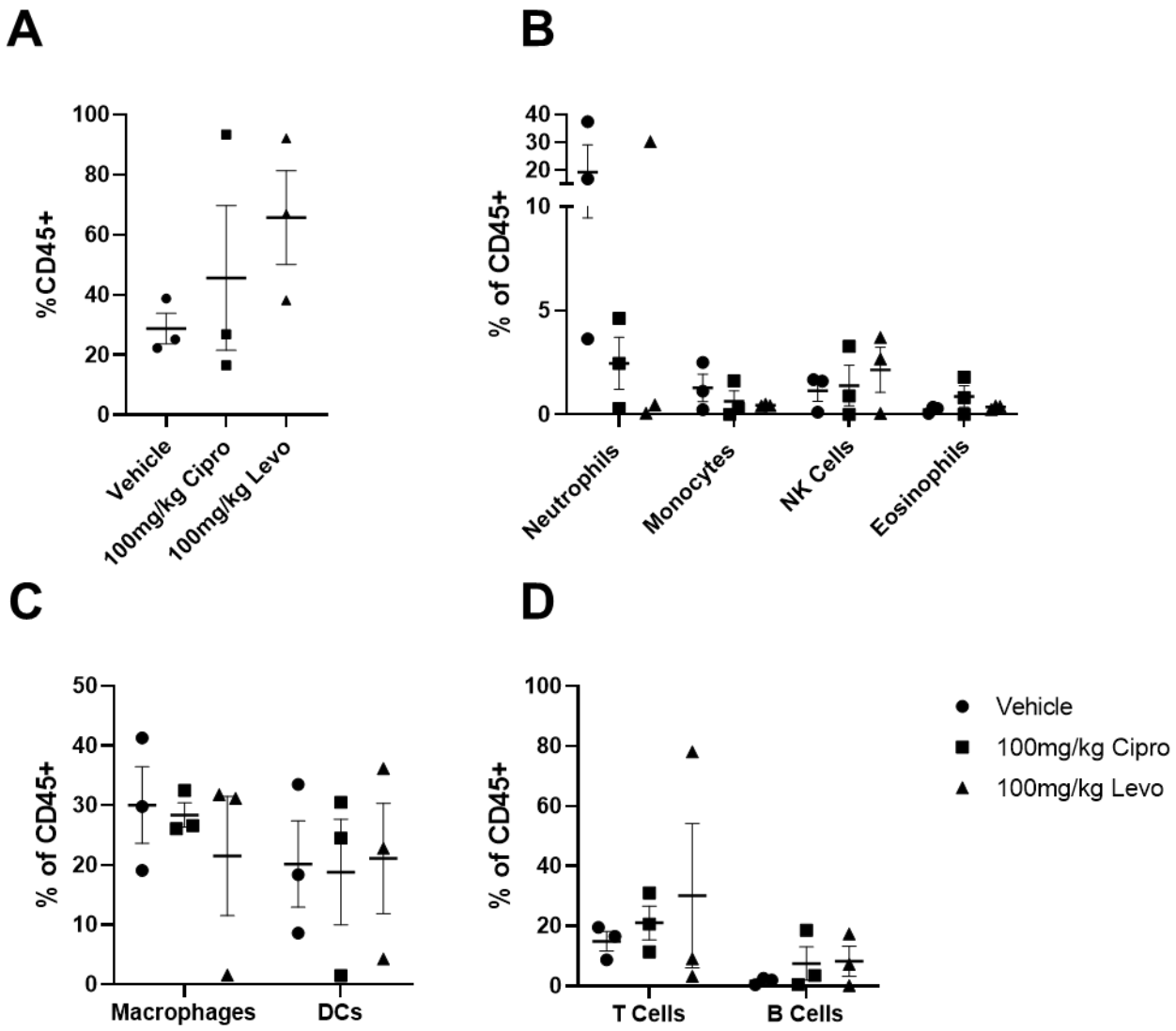


Figure 4.26: **Fluoroquinolones do not alter BAL immune cell subsets following 14 day treatment and washout period** *Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. '. Bronchoalveolar lavage (BAL) was performed post-mortem every 7 days. A: Percentage of CD45+ cells in the BAL fluid after 21 days of fluoroquinolone treatment and 7 day runoff. B: Percentage (of total CD45+ BAL cells) of Neutrophils, Monocytes, and Eosinophils after 21 days of fluoroquinolone treatment and 7 day runoff. C: Percentage (of total CD45+ BAL cells) of Macrophages and Dendritic Cells after 21 days of fluoroquinolone treatment and 7 day runoff. D: Percentage (of total CD45+ BAL cells) of B and T Cells after 21 days of fluoroquinolone treatment and 7 day runoff. n=3. Significance tested using one or two-way ANOVA followed by Dunnet's or Sidak's multiple comparisons test respectively.*

Lung cells were next stimulated overnight with cell stimulation cocktail (with Golgi stop) in order to determine cytokine production ability of the CD45+ leukocyte population. No significant differences in IFNγ or TNFα were seen in the ciprofloxacin or levofloxacin treated groups over the 14 day timecourse of treatment or following the 7 day wash out period (day 21) as compared to the vehicle treated controls (Fig. 4.27).

Finally, we looked at lung histology to assess interstitial space via imageJ analysis. We observed no significant change in the levofloxacin treated group following the 14 day timecourse of the antibiotic. (Fig.4.28). However, ciprofloxacin treatment resulted in a significant increase in lung interstitial space as compared to vehicle dosed controls (Fig.4.28).

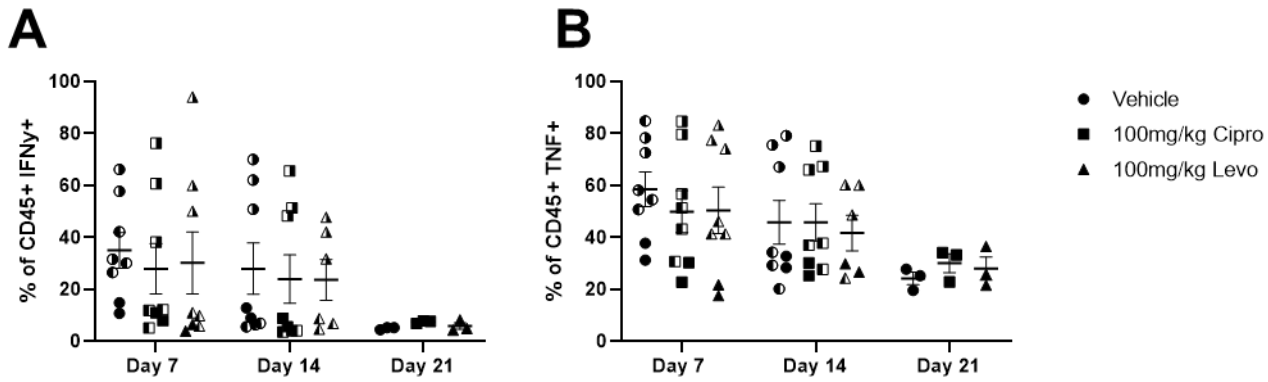


Figure 4.27: **Fluoroquinolones do not alter Lung immune cell inflammatory cytokine production.** *Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. Lungs were harvested every 7 days. Cells were incubated overnight and stimulated with Cell Stimulation Cocktail with Golgi Stop. A: Percentage of IFNγ+ cells (of total CD45+) in the Lung after 7, 14 and 21 days. B: Percentage of TNF+ cells (of total CD45+) in the lung after 7, 14 and 21 days. Representative graphs shown due to inter-experimental variability (n=8). Significance tested independently for each experiment using two-way ANOVA followed by Sidak's multiple comparisons test. Only shown if occurs in all experiments.*

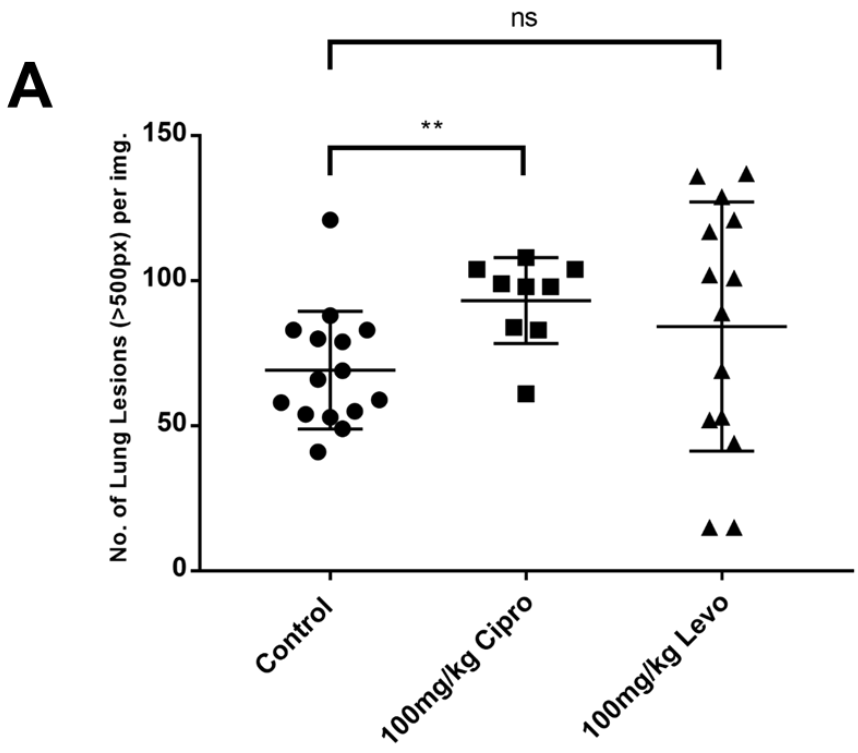


Figure 4.28: Lung lesion number is increased after Cipro treatment*. Number of tissue lesions (>500px) in lung sections of BALB/c mice dosed twice daily with 100mg/kg of Ciprofloxacin, Levofloxacin or water mock dose via gavage for 14 days. Significance tested via T Test.*

Collectively, these data indicate that both ciprofloxacin and levofloxacin treatment have minimal impact on lung and BAL immune cell population percentages across the time course of our experimental analysis, but ciprofloxacin treatment may benefit lung capacity.

4.6 Fluoroquinolones produce distinct intestinal dysbiotic phenotypes *in vivo*

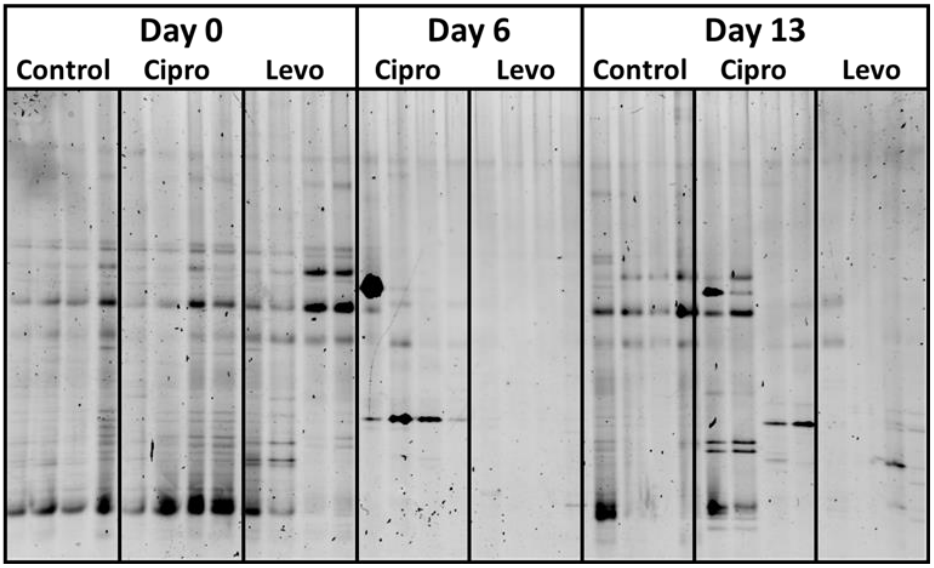
My initial results suggest that fluoroquinolones have direct modulation of developing macrophages *in vitro*, yet antibiotics can also cause immunomodulation via dysbiosis Lankelma et al. (2017). To investigate the nature and extent of dysbiosis caused by our fluoroquinolone dosing regimen, faecal samples were taken from every animal daily (with the exception of d7 and d14 cull days). Bacterial 16s DNA was extracted from faecal samples, amplified via PCR, and purified. Denaturing Gradient Gel Electrophoresis (DGGE) was then performed on individual samples, allowing the microbiome of each faecal sample to be visualised, analysed, and compared. Faecal samples were matched within groups between days in order to compare between the microbiome of the same animal throughout treatment.

Due to lane limits, the first DGGE was performed on samples from day 0, 6, and 13 of two independent experiments to as closely represent 1 and 2 weeks of treatment as possible (Fig.4.29). The results of this were clear. After 6 days of treatment, bands of bacterial DNA were greatly depleted, in both number and their intensity. This indicated that major dysbiosis had occurred after one week of antibiotic dosing (Fig.4.29A). Clustering comparison demonstrated the difference was significant in both ciprofloxacin and levofloxacin treated animals at day 6 vs their own day 0 samples (Fig.4.29B and C). Visually, the dysbiosis appeared more severe in levofloxacin treated animals as compared to ciprofloxacin at 6 days of treatment, with a greater visible loss of DGGE bands to the extent that bands were almost absent. Comparison of the two treated groups at day 6 demonstrated that the microbiome had been significantly altered versus that of the same animals prior to treatment. In addition, the microbiomes of Cipro-treated animals were significantly different from those of Levo-treated animals.

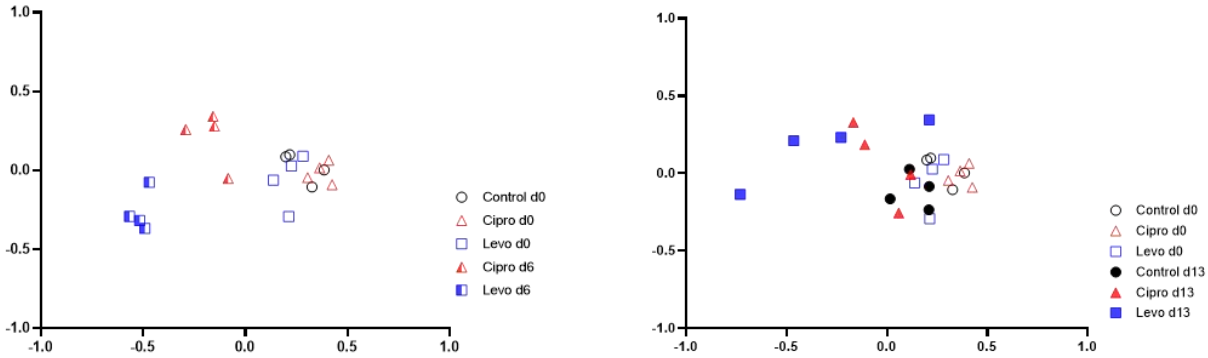
After two weeks, despite a considerable amount of recolonization being observed on the gel via additional banding (Fig.4.29A), statistically both fluoroquinolone treatments were still significantly different from their equivalent day 0 samples (Fig.4.29B and C). Recolonisation banding was again visually

more prominent in ciprofloxacin treated animals, however, they were not significantly different from their day 6 samples, although levofloxacin treated day 13 samples were at p=0.057. Interestingly, persisting bands after day 6 and day 13 were much stronger than at day 0, indicating a potentially opportunistic species that was able to take advantage of a lack of competition caused by antibiotic treatment. Vehicle-treated samples did not change significantly after 13 days indicating alterations were due to antibiotic treatments as opposed to time/gavage stress. Collectively, these data indicate a strong dysbiosis and microbiome depletion in fluoroquinolone treated animals at both time points of day 6 and 13. Moreover, distinct patterns were visible between the ciprofloxacin and levofloxacin at the time points examined, which were statistically significant at day 6.

A



B



C

	Veh D0	Cipro D0	Levo D0	Cipro D6	Levo D6	Veh D13	Cipro D13	Levo D13
Veh D0								
Cipro D0	*							
Levo D0	*	ns						
Cipro D6	*	*	*					
Levo D6	*	*	*	*				
Veh D13	*	*	*	*	*			
Cipro D13	*	*	0.057		*	ns		
Levo D13	*	*	*	*	0.057	*	0.057	

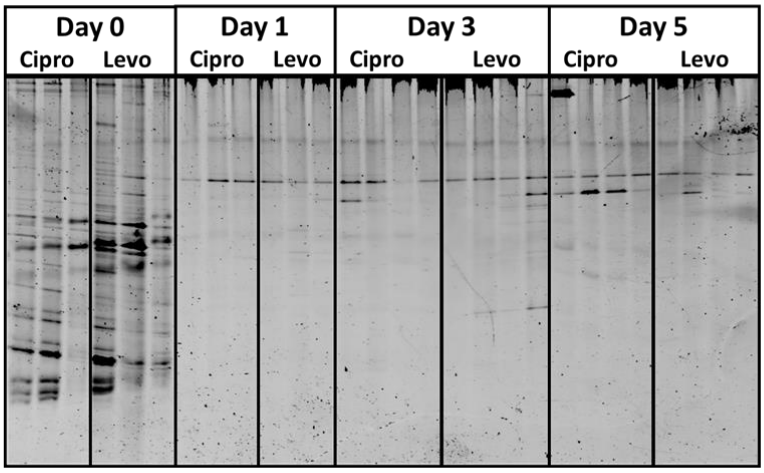
Figure 4.29: Fluoroquinolones cause significant dysbiosis by day 6 of treatment Wild type BALB/c mice were treated by oral gavage with either *dH_2O* Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=4/group) for 14 days. Faecal samples were taken daily. A) DGGE of faecal bacterial DNA taken on day 0, 6 and 13. B) Clustering of faecal DNA samples after fluoroquinolone treatment, comparing day 6 (left), and day 13 (right) with day 0 DNA. C) Significance of differences in faecal DNA after 6 or 13 days of fluoroquinolone treatment. (* P<0.05) tested using R with ADONIS Permutational Multivariate Analysis of Variance.

After examining day 6 samples, it was clear that dysbiosis occurred early in a treatment course, i.e. during the first week. As a result, another DGGE was carried out, this time with DNA samples from day 0, 1, 3 and 5. Again, due to lane limitations of the gel, a compromise had to be struck between number of samples needed for statistical significance, and number of timepoints to look at. Again, the results were quite stark and surprising with clear dysbiosis occurring after only 24 hours of fluoroquinolone treatment (figure 4.30). Visually, the amount of bacterial DNA in the gel is greatly reduced, with many bands only being present in day 0 samples. Statistically, after day 1, only levofloxacin causes a significant difference as compared to day 0 equivalents, and after 3 and 5 days both treatments are significantly different from day 0. The alteration on day 1 is likely due to only having n=3 for day 0 and 1 samples, vs n=4 for day 3 and 5, especially given the fact that there is no significant difference between the antibiotic treated cohorts (ciprofloxacin vs. levofloxacin) themselves at day 1.

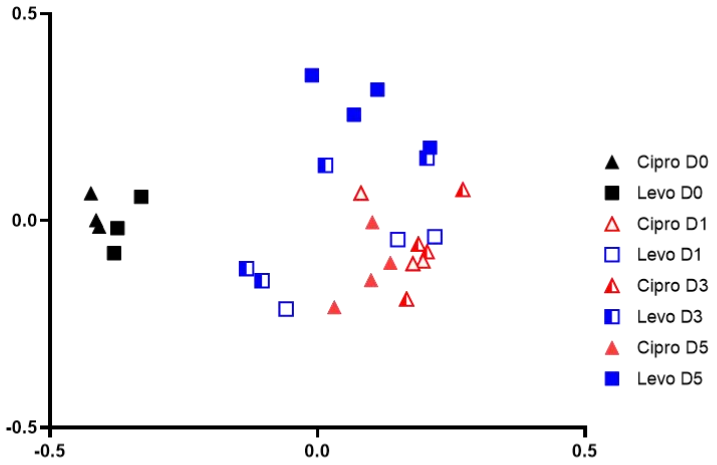
Interestingly, as before certain bands persisted after treatment, seemingly taking advantage of a lack of competition due to loss of other species'. One particular band persisted in all treatments and timepoints, and another band persisted only in Cipro-treated samples

These two experiments show that fluoroquinolones show distinct dysbiotic phenotypes occurring very soon after the start of treatment. Levofloxacin causes significant dysbiosis after one day of treatment which the clustering demonstrate is variable across the treatment time course, while ciprofloxacin takes longer to establish dysbiosis, but once established remains more constant from day 3.

A



B



C

	Cipro D0	Levo D0	Cipro D1	Levo D1	Cipro D3	Levo D3	Cipro D5	Levo D5
Cipro D0								
Levo D0	ns							
Cipro D1	ns	ns						
Levo D1	ns	ns	ns					
Cipro D3	*	*	ns	ns				
Levo D3	*	*	ns	ns	ns			
Cipro D5	*	*	ns	ns	ns	ns		
Levo D5	*	*	*	*	*	ns	*	

Figure 4.30: Fluoroquinolones cause significant dysbiosis after 24h of treatment Wild type BALB/c mice were treated by oral gavage with either *dH_2O* Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=3-4/group) for 14 days. Faecal samples were taken daily. A) DGGE of faecal bacterial DNA taken on day 0, 1, 3 and 5. B) Clustering of faecal DNA samples after fluoroquinolone treatment, comparing day1, 3 or 5 with day 0 DNA. C) Significance of differences in faecal DNA after 1, 3 or 5 days of fluoroquinolone treatment. (* P<0.05) tested using R with ADONIS Permutational Multivariate Analysis of Variance.

4.7 Levofloxacin treatment increases colonic crypt length and goblet cell count

Given the observed intestinal dysbiosis, strongly linked to intestinal morphology(Kennedy et al., 2018), we next examined alterations in intestinal histology examining crypt lengths and goblet cell number.

Interestingly, examining crypt length demonstrated that although ciprofloxacin treatment produced no alteration in crypt length following 7 days of treatment, Levofloxacin significantly increased crypt length as compared to vehicle treated controls (Fig. 4.31). Also, though not significant, there was a trend towards reduced goblet cell number in the crypts of animals treated with Cipro for 7 days. This trend did not occur in animals treated with Levo.

Collectively, these data indicate that although immune alterations were minimal in the intestinal tract, dysbiosis and intestinal morphology were greatly impacted.

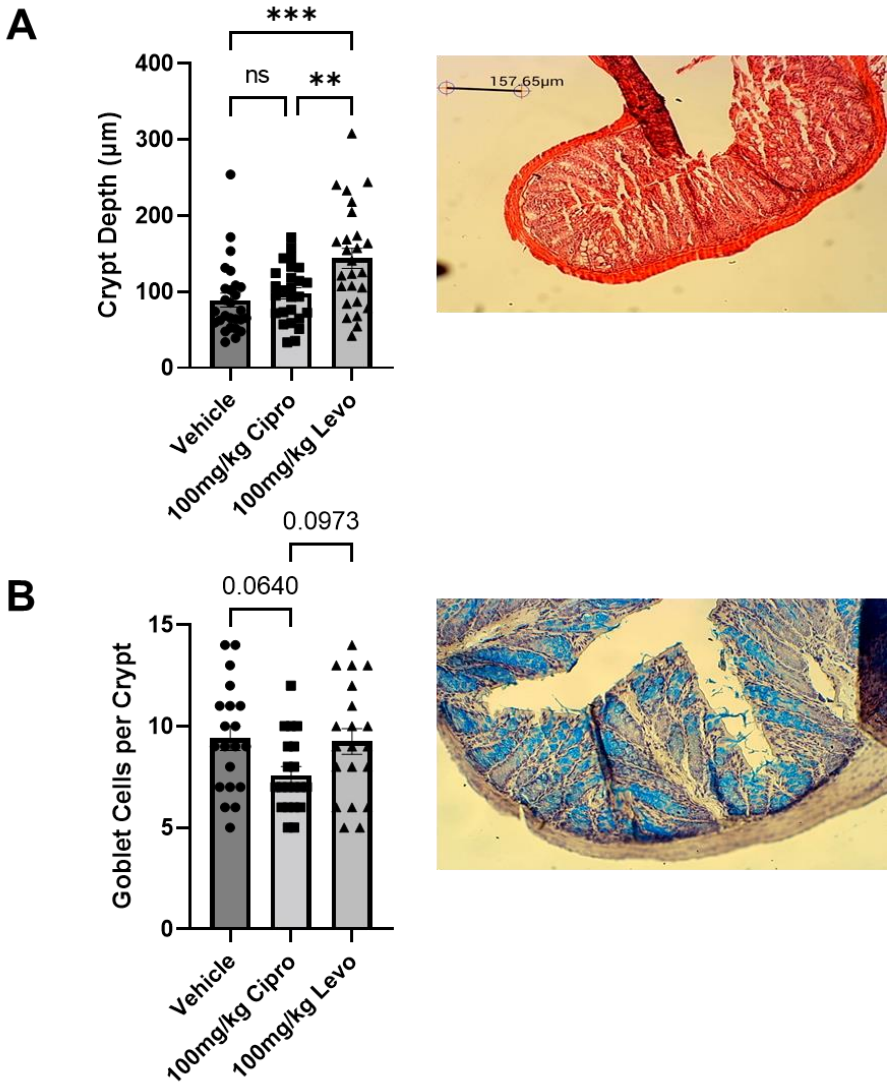


Figure 4.31: Fluoroquinolones affect gut morphology after 7 days of treatment *Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. Large intestines were harvested every 7 days. A: Large intestine crypt depths of mice treated with vehicle, Cipro, or Levo for 7 days, with a representative image. B: Goblet cell counts per crypt of mice treated with vehicle, Cipro, or Levo for 7 days, with a representative image.*

4.8 Fluoroquinolone treatment produces distinct weight loss phenotypes *in vivo*

Antibiotic treatment is associated with short-term weight loss(Miao et al., 2020b) as well as long-term weight gain (Schulfer et al., 2019) and animals were therefore weighed daily in addition to the beneficial readout that weight gives to overall health monitoring. BALB/c mice were externally sourced and aged-matched and there were no differences between starting weights of animals in any treatment group prior to antibiotic administration (Fig.4.32A); indicating percentage changes would give a reliable indication of weight alterations over the antibiotic treatment timecourse.

When treated with ciprofloxacin animals lost weight significantly from day one of treatment as compared to vehicle treated controls (Fig.4.32). This significance remained consistent until treatment ceased at day 14, with animals quickly returning to non-significant weights at days 15 and 16, but again remained significantly reduced weights until day 21 (Fig.4.32 and Table 4.1). Levofloxacin treated animals also had significant weight loss as compared to vehicle treated controls, this time commencing at day 2 of treatment (Fig.4.32). Even after 7 days of recovery, no levofloxacin treated animals had regained weight to the extent that they were no longer significantly different from vehicle-treated animals (Fig.4.32 and Table 4.1). On comparing antibiotic treated groups, although the ciprofloxacin weight loss appeared more constant while levofloxacin had 2 phases of more severe weight loss, beyond the difference at day 1 post treatment, no significant difference was seen between ciprofloxacin and levofloxacin induced weight loss (Fig.4.32 and Table 4.1). Collectively, this indicates that fluoroquinolones induce sustained weight loss in uninfected naive animals, although the mechanisms driving this pathology are unclear, albeit unlikely to be driven by myeloid immune populations in the gut and more likely related to the differing of intestinal dysbiosis.

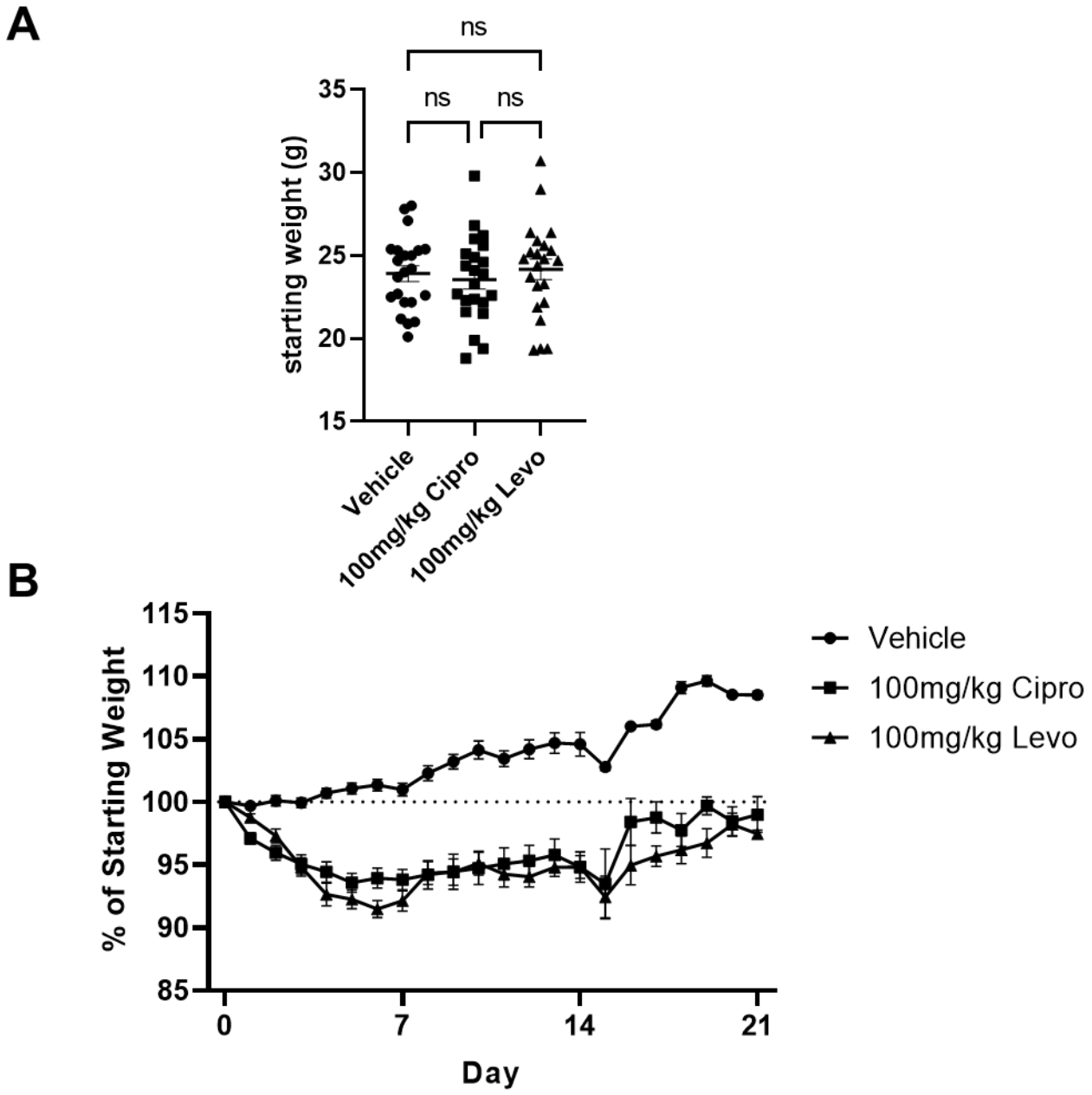


Figure 4.32: Fluoroquinolones cause significant weight loss vs control *Wild type BALB/c mice were treated by oral gavage with either dH_2O Vehicle, 100mg/kg Ciprofloxacin or 100mg/kg Levofloxacin twice daily (N=22/group) for 14 days, with a 7 day treatment-free 'washout period'. A: Starting weights of all animals before treatment commenced. Significance between groups was tested using Tukey's multiple comparison following one-way ANOVA. B: Percentage daily weight change (vs starting weight) of treated and control mice over 14 days of treatment.*

Table 4.1: ***The significance of differences between mean weights of mice given mock dose, 100mg/kg Ciprofloxacin, and 100mg/kg Levofloxacin****. *, P<0.05; **, P<0.01; ***, P<0.005; ****, P<0.0001; N.S., not significant using REML Mixed-effects analysis followed by Tukey's multiple comparisons test.*

Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Control vs Cipro 100mg/kg	ns	****	****	****	****	****	****	****	****	****	****	****	****	****	****	ns	ns	*	*	**	*	*
Control vs Levo 100mg/kg	ns	ns	***	****	****	****	****	****	****	****	****	****	****	****	****	*	*	**	**	**	*	****
Cipro 100mg/kg vs Levo 100mg/kg	ns	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

4.9 Discussion

In this chapter, wild type uninfected C57BL/6 and BALB/c mice were treated with a human- like dose regime of Ciprofloxacin and Levofloxacin. They were given a 100mg/kg dose via oral gavage twice a day for 14 days, followed by a 7 day 'washout' period. A wide variety of measurements and samples were taken, in order to broadly assess any effects the fluoroquinolones had on the animals.

Building upon *in-vitro* findings with *in-vivo* experiments give a wider context, with more organism-wide variables, one of the most significant being the presence of the microbiome. Because of these inter-strain differences mentioned above, especially with regards to macrophages, it was also important to examine the *in-vivo* effects of fluoroquinolones in multiple strains of mice.

4.9.1 Fluoroquinolone treatment increases M1 lung macrophage polarisation *in vivo*

As mentioned previously, any immunomodulatory effects fluoroquinolones have on lung cells are of particular importance. Fluoroquinolones are prescribed to treat a variety of severe respiratory tract infections, and as such any negative effects on the lung immune system could have serious consequences. As mentioned in the previous chapter macrophage studies have been strongly associated with alterations induced via fluoroquinolones. Our *in vitro* studies suggested that these effects were significant on developing macrophages and not established macrophage BMDM populations. Interestingly, our *in vivo* data only identified differences in the lung where we were able to distinguish long-lived resident macrophages, either crudely in BALB/c mice via BAL or in C57BL/6 mice via CD11b expression. In both cases, fluoroquinolones drove increased NOS2 to significance in C57BL/6 and reduced CD206 in BALB/c expression only in the short lived populations in corroboration with our *in vitro* studies. This finding was only seen in day 14 in BLAB/c mice and was lost

upon the wash out period. This implies the effect on polarisation takes time to develop, and is reversible upon cessation of treatment.

This seem specific to fluoroquinolones as M2 polarisation is seen in pleural macrophages of the lung in following isoniazid, rifampicin, pyrazinamide and ethambutol combination treatment in TB patients(Wang et al., 2017). Moreover, gut dysbiosis via broad antibiotic use promotes lung M2 Macrophage Polarization and results in allergic airway inflammation (Kim et al., 2014). However, other studies looking for M2 polarisation inhibitors identified the broad acting, fluoroquinolone alternative prescribed, doxycycline as an inhibiter of M2-type macrophage polarisation (He and Marneros, 2014). Therefore, fluoroquinolones could also be a novel M1 polariser of macrophages, albeit specifically to the lung. In agreement with our *in vitro* studies, a recent publication has shown that broad antibiotic depletion alters macrophage metabolism in the colon, with increased expression of genes involved in glycolysis and oxidative phosphorylation, as well as mitochondrial function, with CD206 being also reduced somewhat paradoxically (Scott et al., 2022). Interestingly, mitochondrial potential was significantly elevated in the subset of CD4+Tim4− macrophages suggesting alterations may be due to metabolic changes in CD4+Tim4− replenished macrophages, as opposed to issue-resident (CD4+Tim4+) (Scott et al., 2022) in corroboration with our *in vitro* and *in vivo* studies albeit in a different immune compartment potentially due to the differing antibiotic usage.

4.9.2 Fluoroquinolone treatment and myeloid cell phenotype

Every 7 days, various tissues were harvested in order to assess any wider immunomodulatory effects of fluoroquinolone treatment *in-vivo* beyond macrophage polarisation. The spleen allows for any changes in circulating immune cells to be determined and gives an overall systemic read out. The immune tissues closest to this dysbiosis are the large intestine and mesenteric lymph node, also known the GALT (gut-associated lymphoid tissue). It therefore stands to reason that the first immune populations to be affected by this fluoroquinolone-mediated dysbiosis will be those of the GALT. Finally, the

lung tissue as the key site of bacterial infection was also analysed. All isolated cells were stained with a broad myeloid antibody panel and cytokine antibody panel.

Overall increased haematopoiesis has been linked to ciprofloxacin (Dalhoff, 2005). However we saw no alteration in any myeloid, or lymphocyte, population at the percentage level in any of the tissues examined during the 14 day dosing regimen of either ciprofloxacin or levofloxacin. Neutrophils actively internalise fluoroquinolones(Hotta et al., 2002) enhancing bactericidal mechanism, but no reports exist on grand changes of this cell type beyond individual reports of neutropenia, and this was for moxifloxacin (Chen and Van Buren, 2017). Similarly, monocytes are also able to absorb fluoroquinolones(Bounds et al., 2000), but no reports of alterations could be found. NK cells have been shown to increase in the ovaries of envofloxacin treated dogs (Albrizio et al., 2015), while ciprofloxacin and levofloxacin are associated with 5 cases of human eosinophilia(Sharifzadeh et al., 2021). Moreover, the effect of fluoroquinolones on dendritic cells is limited. Overall, our data therefore supports the literature in that minimal changes in myeloid populations in terms of percentage are driven by ciprofloxacin or levofloxacin. However, our macrophage polarisation data does indicate a more in depth cell subset analysis may uncover specific actions.

Despite this variability, significant differences were seen in the mesenteric lymph node following the 7 day wash out period, potentially likely in part due to this data being from only one independent experiment. Levofloxacin treated MLN cells were found to show significantly increased neutrophils, eosinophils and NK cells. Cipro-treated MLNs showed increased macrophages, as well as reduced NK and T cells vs control. It should be noted however, that T cells were negatively gated, and as such a confirmatory panel should be performed before any conclusions made. However, this was only observed in the mLN and recent reports have demonstrated that re-exposure of antibiotic-treated mice to conventional microbiota induces a long-term, macrophage-dependent increase in inflammatory T helper 1 (TH1) responses in the colon and sustained

dysbiosis (Scott et al., 2018). This was thought to be driven by short-chain fatty acid production via the microbiome and altered the metabolic behaviour of macrophages(Scott et al., 2018).So in conclusion further repeats of the data should be produced to further investigate the reliability of this data, with perhaps rescue via the addition of butyrate to further elucidate mechanisms.

Beyond immune cellular populations and as previously mentioned in chapter 3, fluoroquinolones are linked to several immunomodulatory roles. Interestingly ciprofloxacin but not levofloxacin at 100mg/kg have been shown to significantly reduce serum TNFα, but not IL-1b or IL-6, following LPS treatment (Ogino et al., 2009b). Although we did not monitor serum cytokine levels are overall CD45 readouts in several tissues indicated there was no alteration in overall immune TNFα or IFNγ secretion, although the broad use of CD45 population may have missed subtle cell specific cytokine changes. An extensive systematic review demonstrated cytokine alterations of fluoroquinolones were only observed *in vivo* when delivered with another stimulant (Dalhoff, 2005). Immunomodulatory effects, including increased haematopoiesis, were attributed to fluoroquinolones with a cyclopropyl-moiety at the position N1 of the quinolone core structure, including ciprofloxacin, as well asmoxifloxacin, grepafloxacin, sparfloxacin(Dalhoff, 2005). The immunomodulatory effects due to intracellular cyclic AMP and phosphodiesterases, on transcription factors such as NF-kappa B, activator protein 1 and a triggering effect on the eukaryotic equivalent of bacterial SOS response(Dalhoff, 2005). Moreover, fluoroquinolones have recently been shown to drive the production of IL-2(Assar et al., 2021).

Despite these increases in IL-2 and haematopoiesis we did not see any major effects in T or B-cell populations in any tissue examined, mLN, spleen, LILP and lung during the 14 day treatment, but did see alterations at in the mLN during the wash out period as mentioned above. The previous studies on IL-2 did rely on cell lines(Riesbeck and Forsgren, 1994) or *in vitro* human T-cells alone(Riesbeck et al., 1989) and therefore do not truly recapitulate the natural *in vivo* environment. However, our adaptive immune analysis was based on

negative flow gating and a more concentrated specific panel would truly uncover any alterations. Despite occasional significant differences, no overall trends were seen to indicate fluoroquinolone treatment affected myeloid cell subtype populations.

4.9.3 Fluoroquinolones produce distinct dysbiotic phenotypes *in vivo*

Faecal samples were taken from every animal daily during the 21 day experiment. This allows any dysbiosis caused by fluoroquinolone treatment to be tracked and quantified. Loss or alteration of the gut microbiome leads to subsequent loss of faecal bacterial DNA, with the advantage of being able to monitor this while the animal is alive.

Firstly, samples were taken from day 0, 6 and 13 to represent one and two weeks of treatment. Both Cipro and Levo treated samples were significantly different from their day 0 equivalent at both day 6 and 13. Interestingly, considerable bacterial regrowth was seen in Cipro at day 13, despite treatment still being ongoing. This again fits with the fact that Levo is a newer and thus more effective antibiotic (Garrison, 2003). It was clear after this experiment that dysbiosis occurred during the first week of treatment. As such, samples were analysed from day 0, 1, 3 and 5.

Surprisingly, it was found that major dysbiosis occurred after only 24 hours of fluoroquinolone treatment. There was a considerable visual loss of bacteria after 1 day of fluoroquinolone treatment, though this was only statistically significant in Levo-treated samples. Both treatments caused a significant difference by day 3.

The distinct nature of Cipro and Levo's weight loss is also seen in their dysbiosis. By day 5, the remaining bands of Cipro and Levo-treated faecal samples were significantly different from each other, not just from untreated samples.

Gu et al. (2020) treated C57BL/6 mice with a short 4-day course of Levofloxacin and measured dysbiosis compared with other antibiotics (Cefoperazone/Sulbactam, Meropenem and Aztreonam) using faecal sampling. After the 4 day Levofloxacin course, the faecal microbiome composition was

significantly altered compared to before treatment. At day 60 of the experiment, composition had returned to near baseline, but still differed somewhat. In comparison, the microbiome of mice treated with Cefoperazone/Sulbactam and Meropenem had returned fully to baseline after 60 days. In terms of phylum composition, Levofloxacin treatment caused a huge increase in *Firmicutes* and reduction in *Bacteroidetes* at day 4. Microbial diversity was also measured using the Chao1 and Shannon indices. In the case of the Chao1 index, diversity was significantly decreased after Levo treatment versus D1 at all timepoints including d60, though it did significantly increase between d8 and d60. The Shannon index gave similar results, albeit with no significant difference between d1 and d60, implying a greater recovery of diversity after 60 days. Interestingly, Gu et al also measured levels of inflammatory cytokines in the serum on day 60. Levofloxacin was the only antibiotic that resulted in no change in cytokine levels vs non-treated controls corroborating our analysis of immune TNFα and IFNγ production in several tissues.

This study provided an insight into the longer-term microbial impact of Levofloxacin with a 60 day timepoint, effectively representing a longer-term post-treatment washout period. It is limited, however, in that faecal samples were only taken after the cessation of the treatment course. This means that whilst the timeline of recovery and 'end' of Levo-induced dysbiosis is explored, the speed of onset remains unknown. Addition of more timepoints to this study, perhaps weekly, would increase the resolution of their data. It could be the case that most of the microbial recovery occurs in the first 14 days post treatment, or it could be a much more gradual process. It is possible that, in the case of Levo where full recovery was not achieved at D60, simply more time was needed. Conversely, the different mechanism of Levo compared to the beta lactams in the study, may cause irreversible damage to the microbial composition and diversity. In addition, the 4 day course of Levofloxacin used in this study is unrealistic, with longer courses the norm. Short courses as seen here are only advised for 'uncomplicated urinary tract infections' (3 days), for which Levofloxacin is only prescribed if there are no alternative treatments available (due to the risk of side effects) (FDA, 2017).. A typical course of levofloxacin would be between 7 and 14 days, and can stretch up to 60 days in the case of

Anthrax post-exposure prophylaxis (FDA, 2017). It would be interesting to know whether length of course has an impact on dysbiosis and microbial recovery. It could be hypothesised that once ‘the damage has been done’, (in our case after 24h), increased length of treatment course has little effect on recovery.

This hypothesis can be tested by studies into the effects of long-term fluoroquinolone treatment courses on the gut microbiome. Levast et al. (2021) examined the effect of long-term antibiotic treatment on the gut microbiome of patients with bone and joint infections. Several different antibiotics were examined, with Fluoroquinolones being the most common type. The average length of course was 64 days, ranging from less than 20 to over 120 days. Most courses were either 6 weeks or 12 weeks. The fluoroquinolone category in this study comprised of Ciprofloxacin, Levofloxacin and Ofloxacin, a second-generation fluoroquinolone. Unfortunately there was no data split by individual fluoroquinolone. Levast et al found that fluoroquinolones produced the greatest impact upon the microbiome at the end of treatment, with a lower Shannon diversity index and richness than other antibiotics. The effect was relatively short lived however, recovering to pre-treatment levels at follow-up two weeks after cessation of treatment. In contrast, while non-fluoroquinolones showed a lesser reduced diversity and richness at the end of treatment, this did not recover by follow-up. Although this study did not compare different lengths of fluoroquinolone treatment course, they do back up the hypothesis stated above. Even after over 60 days of fluoroquinolone treatment recovery was still seen, to an even greater extent than other studies saw after 4 day treatment (Gu et al., 2020).

It would have been useful if this article has differentiated between the fluoroquinolones, as it is clear from data shown above that they affect the gut microbiome differently, particularly at the end of treatment. Levo causes far more loss of microbiome than Cipro after prolonged treatment. Based on publicly available prescribing data, Cipro is far more commonly prescribed than Levo and so it could be assumed that the grouped data in this study follows a similar trend. It should be noted that Levast et al’s work examined dysbiosis in a system with infection present, rather than a naïve organism. Thus, pathogenic microbes are present which would not occur in a naïve system, which may

impact the commensal microbiome. This is somewhat alleviated in this case however, as the patients in this study suffered from bone and joint infections, which are largely isolated and distinct from the gut microflora. In fact, the isolated nature of bone and joint infections is one of the primary reasons that they are so hard to treat, requiring these extraordinarily long antibiotic treatment regimens (Ferry et al., 2019).

We did observe some lung alterations following levofloxacin treatment, but not ciprofloxacin. Overall, fluoroquinolones have no reports in mice of alterations in lung pathology. Lung fluoroquinolone delivery has been slightly associated with sudden Cardiac Death (Assimon et al., 2022). Moreover, 100mg/kg of ciprofloxacin impaired cardiorespiratory development when delivered to newborn mice(Bourgeois et al., 2016). However, limited lung pathology is reported, with sparse case reports of Ciprofloxacin-induced acute interstitial pneumonitis in a 68 year old patient, who actually improved upon switching to levofloxacin (Steiger et al., 2004).

4.9.4 Fluoroquinolone treatment produces distinct weight loss Phenotypes *in vivo*

Animals were weighed every day in order to observe and monitor their overall health. Any loss greater than 20% of their starting weight would have been considered a welfare issue and the animal euthanised. This did not occur during any experiments. It was immediately obvious that fluoroquinolone treatment caused weight loss in BALB/c mice, in both fluoroquinolones examined. Ciprofloxacin caused significant weight loss after only one day of treatment while Levofloxacin caused significant weight loss after 2 days of treatment. Neither treatment fully returned to levels of PBS control mice even after the 7 day washout period, though weight was gained.

The two drugs caused subtly different patterns of weight loss. Cipro caused a slower, more gradual loss that was maintained for longer. Conversely, Levo caused a steeper decline that recovered slightly after 7 days (though still significantly different from PBS Vehicle). Data on weight is relatively limited due to the ‘secondary’ nature of weight data in research and is usually used during antibiotic studies in infected animals. Interestingly, ciprofloxacin has been

shown to delay weight gain in new born uninfected mice(Bourgeois et al., 2016). Studies have been carried out, however, into the impact of strain on weight gain and metabolic effects caused by feeding mice a high fat diet. Interestingly, BALB/c mice have been shown to be largely protected from HFD-induced metabolic effects (Montgomery et al., 2013). Whilst there were no differences in mitochondrial metabolism or oxidative stress, they did not show increased body weight, increased insulin resistance, or excess lipid accumulation in the liver. All of these were seen in mice of the other strains tested, including C57BL/6. BALB/c mice did however show increased whole-body adiposity along with the other strains. Their resistance to HFD induced weight gain could be inferred as to be similar to an increased susceptibility to weight loss, through a lack of 'protection mechanisms' afforded by lipid uptake from that occurring in their diet.

Interestingly, work by Bongers et al investigated the mechanisms involved in antibiotic induced weight loss discovering several pathways responsible. One key factor is involved in antibiotics placed in the drinking water with a bitter taste causing water aversion and strong weight loss in the typical four-drug regimen of neomycin, vancomycin, metronidazole and ampicillin (Bongers et al., 2022), while this was not observed in cefoperazone or enrofloxacin/ampicillin via the drinking water (Bongers et al., 2022). This has been long established and why some researchers use artificial sweetener in the drinking water to avoid this aversion, however we utilised gavage to avoid the addition of sweetener into altered parameters as well as to ensure dosage level and time of occurrence. Intraperitoneal delivery of antibiotics also caused weight loss, while this was not observed in germ-free animals the authors suggesting changes being therefore down to microbial alterations(Bongers et al., 2022)

Our differential pattern of weight loss in ciprofloxacin and levofloxacin could therefore be linked to the difference in generation of fluoroquinolone between Cipro and Levo. Levo is a newer drug than Cipro, a 3rd generation fluoroquinolone as opposed to 2nd generation. It could therefore be expected that Levo would be a more effective antibiotic, and therefore likely to have more of an effect on the gut microbiome. The greater antimicrobial effect was

confirmed in this chapter via DGGE, with Levofloxacin causing a greater visual loss of bands than Ciprofloxacin. The link between this dysbiosis and weight loss is less simple however. Many studies have in fact shown that dysbiosis is a contributing factor to obesity. Aoun et al. (2020) carried out a review into the role of the gut microbiome on obesity, taking into account animal and human studies. It was found that dysbiosis is a contributing factor to obesity, in particular an increased ratio between *Firmicutes* and *Bacteroidetes* species (John and Mullin, 2016). Differences between specific species were not established here. Future sequencing work would enable this to be determined. Antibiotic-induced dysbiosis has been shown to have long lasting effects on weight and health. Subtheraputic antibiotic dosing has been common in agriculture as a growth promoter for many years albeit with unclear mechanisms. Cho et al. (2012) gave mice subtheraputic doses (1µg/g body weight) of Penicillin, Vancomycin, a combination of the two, or Chlortetracycline for seven weeks in drinking water. They found that while the mice did not gain more weight that control animals, they did exhibit increased body adiposity, as well as altered metabolic hormone levels. In addition, mice who were given the cephalosporin Ceftriaxone for the first two weeks of their life showed exacerbated the negative physiological effects of a high fat diet later in life (Miao et al., 2021). These effects are not irreversible however. After microbial depletion by antibiotics, both the diversity and functionality of the gut microbiome can be restored using a faecal microbiota transplant (Guirro et al., 2019). As is clear, the vast majority of research into the subject of antibiotic treatment and weight links antibiotic dysbiosis to weight gain, rather than weight loss as seen here. Some studies have linked antibiotic treatment to weight loss however. Adult mice treated with a one week course of Ceftriaxone showed significantly impaired weight gain compared to control, which was partially rescued with the addition of probiotics. Antibiotic-induced weight loss remained after the cessation of treatment for a further week (Miao et al., 2020a). This study is in by far the minority however. On the whole, antibiotic use is much more widely associated with weight gain and obesity than weight loss.

Beyond microbial driven differences and based on our *in vitro* direct effects of fluoroquinolones, in terms of the mechanisms driving fluoroquinolone induced

weight-loss, it has been well established that antibiotics can directly modulate intestinal epithelial cells. These may be metabolic- interestingly fluoroquinolones reduce rectal temperature in uninfected mice(Miyazaki et al., 2008). More recently, it was shown that one third of the antibiotic induced changes in host intestinal epithelial gene expression could be attributed to direct regulation of their expression by the antibiotics, and not by a shift to a different microbiota composition (Morgun et al., 2015). Key cells in driving alterations in feeding are the enteroendocrine cells, as mentioned previously.

Interestingly, broad acting antibiotics, a combination of amoxicillin, vancomycin, and metronidazole from postnatal day 10 to 20 broadly increased the expression of several EEC markers as well as specific transcript changes via RNA-seq for several peptide hormones including somatostatin, neurotensin, PYY and cholecystokinin(Garcia et al., 2021). Interestingly, several of these peptides have been associated with inflammation and infection based changes in weight(Worthington et al., 2018a, Worthington, 2015a), with cholecystokinin solely responsible for weight loss induced via inflammation driven by the helminth small intestinal infection *Trichinella spiralis* (Worthington et al., 2013). Alternatively, cytokines can drive weight loss in animals(Matthys and Billiau, 1997), although TNFα, IL-6 and IL1b are thought to be the key factors involved(Baicus et al., 2012), our observations of no altered TNFα in any tissues negates this as being likely, although we did not measure serum cytokine levels. The next chapter will focus further on potential mechanisms of fluoroquinolone induced weight loss.

4.9.5 Conclusions

Overall, these data indicate the fluoroquinolones ciprofloxacin and levofloxacin have limited influence on myeloid cell populations in terms of percentages, while they may alter immune populations following a wash out period, although this requires further confirmation. In corroboration of our earlier *in vitro* BMDM studies, ciprofloxacin drives M1 polarisation but specifically in the lung short lived macrophage population. This may be a direct modulation as demonstrated by our *in vitro* studies in chapter 3 or via the significant dysbiosis caused by Ciprofloxacin and Levofloxacin after just 24 hours of treatment, which was maintained throughout the treatment course. There was, however, more recolonisation in Cipro-treated animals than Levo at the 13 day timepoint.

Finally, both fluoroquinolones drive weight loss over the course of treatment with subtle alterations in phenotype, which will be examined, in the next chapter.

Chapter 5: Investigating fluoroquinolone induced weight loss *in vivo*

5.1 Introduction

Earlier observations that a human-like course of fluoroquinolones induced weight loss in uninfected BALB/c mice so far lacked a mechanism. No alterations were seen in the weight loss inducing cytokine TNFα, suggesting the weight loss may be driven by other mechanisms. We therefore focussed on the intestine as the site of delivery of the antibiotics and major area of dysbiosis. The epithelium is the barrier of the microbiome from the immune system and it has been shown that one third of the antibiotic induced changes in host intestinal epithelial gene expression could be attributed to direct regulation of their expression by the antibiotics (Morgun et al., 2015), while the remaining 2/3 are a result of dysbiosis which we clearly saw from our microbiome analysis. Given that the release of peptide hormones from enteroendocrine cells are the natural regulators of appetite (Gribble and Reimann, 2016a, Gribble and Reimann, 2019a) and that antibiotics have been shown to upregulate several peptide hormones (Garcia et al., 2021), we focussed on the potential for EEC-peptide hormone regulation of fluoroquinolone induced weight loss.

Cholecystokinin (CCK) has previously been shown to be responsible for weight loss induced via inflammation driven by the helminth small intestinal infection *Trichinella spiralis* (Worthington et al., 2013). We therefore utilised CCK-KO mice and in-house C57BL/6 wild-type control strain mice, treating with a human-like course of fluoroquinolones, 100mg/kg Ciprofloxacin or Levofloxacin twice daily for two weeks via oral gavage. They were then monitored for a further 7 day ‘washout period’. This dosing technique allowed for precise dose-control per animal, ensuring every animal received the same amount of antibiotic.

A wide variety of samples were taken during these experiments in order to maximise the data that could be obtained from them (Fig. 5.1). Peptide hormones also have immunomodulatory roles(Worthington et al., 2018a) so we again assessed our innate immune readouts to assess direct modulation or, if responsible, alterations in antibiotic induced weight loss.

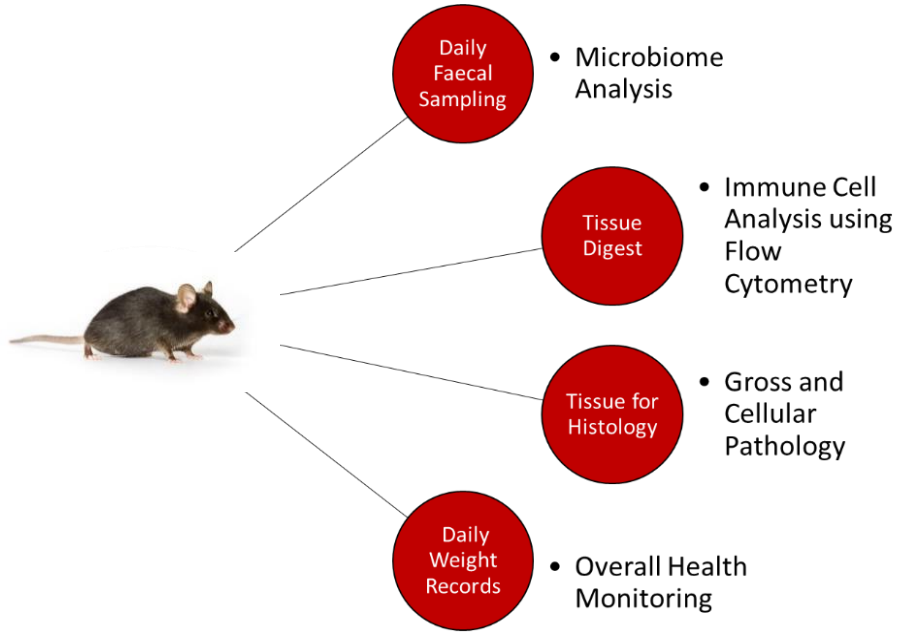


Figure 5.1. *In vivo* Experimental Design
Schematic showing the variety of samples taken during in vivo experiments. C57BL/6 and CCK-KO mice were treated with 100mg/kg Ciprofloxacin or Levofloxacin twice daily for two weeks via oral gavage. Mice were weighed daily to monitor their health and tissues were harvested at day 7, 14 and 21 following a washout no antibiotic period. Spleen, lung, large intestine, and mesenteric lymph node were processed for analysis via flow cytometry and histology, while faecal samples were taken daily in order to track any fluoroquinolone-induced dysbiosis.

The main objectives of the work in this chapter were:

I. **To investigate the effect of the fluoroquinolones ciprofloxacin and levofloxacin on weight in wild-type C57BL/6 strain and CCK-KO**
II. **To investigate the immunomodulatory effect of the fluoroquinolones ciprofloxacin and levofloxacin on innate immune cells in wild-type C57BL/6 strain and CCK-KO**
III. **To histologically investigate gut and lung pathology in wild-type C57BL/6 strain and CCK-KO treated with ciprofloxacin and levofloxacin**
IV. **To investigate the *in vivo* lung M1 polarisation of fluoroquinolones ciprofloxacin and levofloxacin ciprofloxacin and levofloxacin in CCK-KO mice.**

5.2 Fluoroquinolone Antibiotics Cause weight loss in C57BL/6 mice which is partly driven by the peptide hormone cholecystokinin

Antibiotic treatment is associated with short-term weight loss(Miao et al., 2020b) as well as long-term weight gain (Schulfer et al., 2019) and our observations in the BALB/c strain indicated fluoroquinolone induced weight loss. Animals were therefore weighed daily in addition to the beneficial readout that weight gives to overall health monitoring. C57BL/6 mice were bred in-house as a wild-type control to CCK-KO in-house strain (Lay et al., 1999a).

Starting weights were measured, particularly with the in-house sourcing of mice meaning age-matching was less precise as purchased BALB/c strain. Starting weights remained consistent between genotypes and the 1st and 2nd experiments, with the exception of CCKLacZ mice in experiment 3 being significantly heavier than their wild-type counterparts (Fig. 5.2). However, importantly when the data from different experiments are combined and treatment groups compared, there was no significant difference between any of the groups (Fig. 5.2).

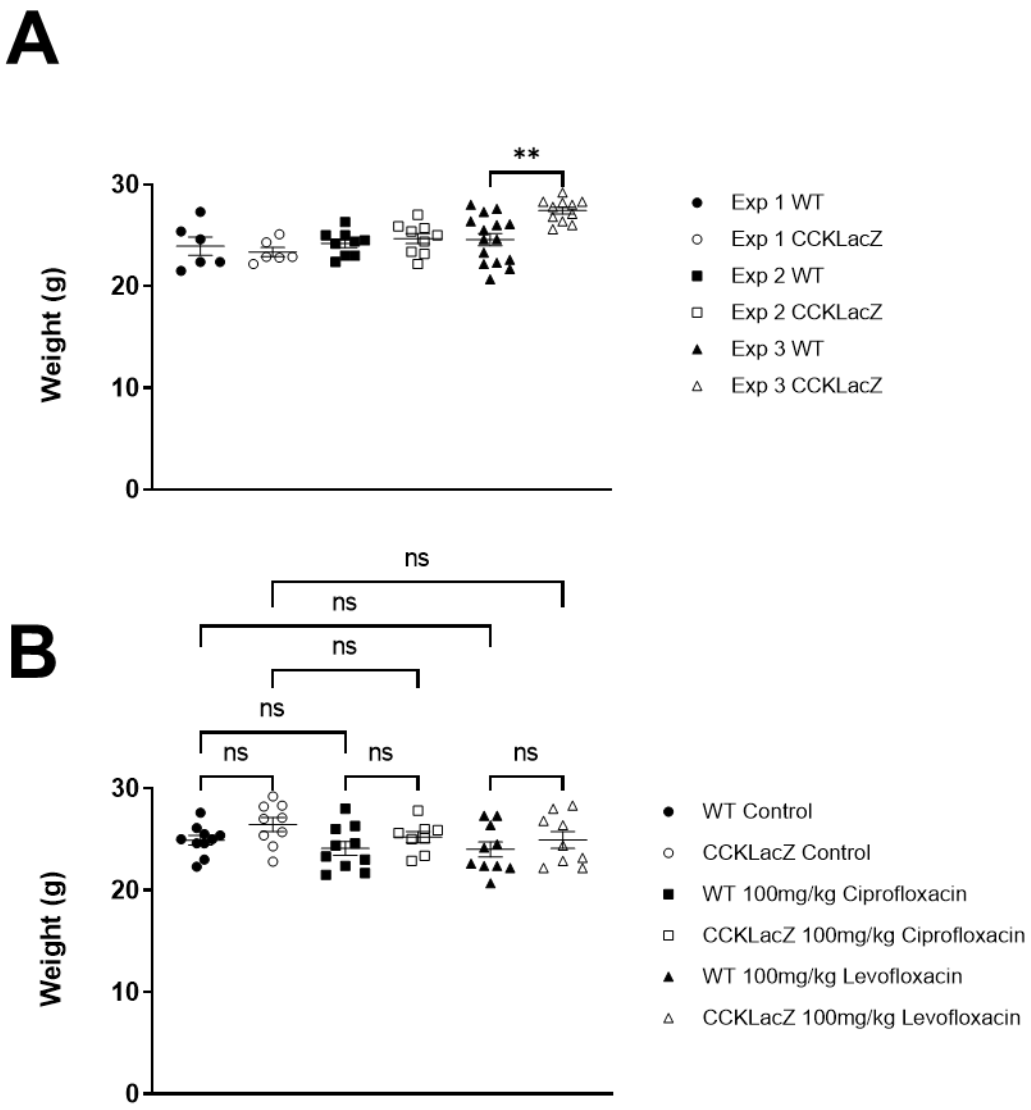


Figure 5.2 C57BL/6 and WT mice have similar weights

*C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H2O mock dose via oral gavage (N=9/group, 8/group CCKLacZ Cipro). A: Starting weights of WT and CCKLacZ animals in each experiment. B:Starting weights of all animals split by treatment. *, P<0.05; **, 0.01; ***, 0.005; ****, 0.0001; N.S. Significance between groups was tested using Sidak's multiple comparisons test following one-way ANOVA.*

In C57BL/6 mice ciprofloxacin treatment caused significant weight loss from day 2 of treatment as compared to PBS vehicle controls, the treated group maintaining significance until day 8 when they being similar to PBS vehicle controls (Fig. 5.3 and Table 5.1). When treated with levofloxacin animals lost significant weight at day 5 as compared to PBS vehicle controls, which became more significant as the treatment course progressed, and never recovered to vehicle treated weight levels throughout the 14 day time-course (Fig. 5.3 and Table 5.1).

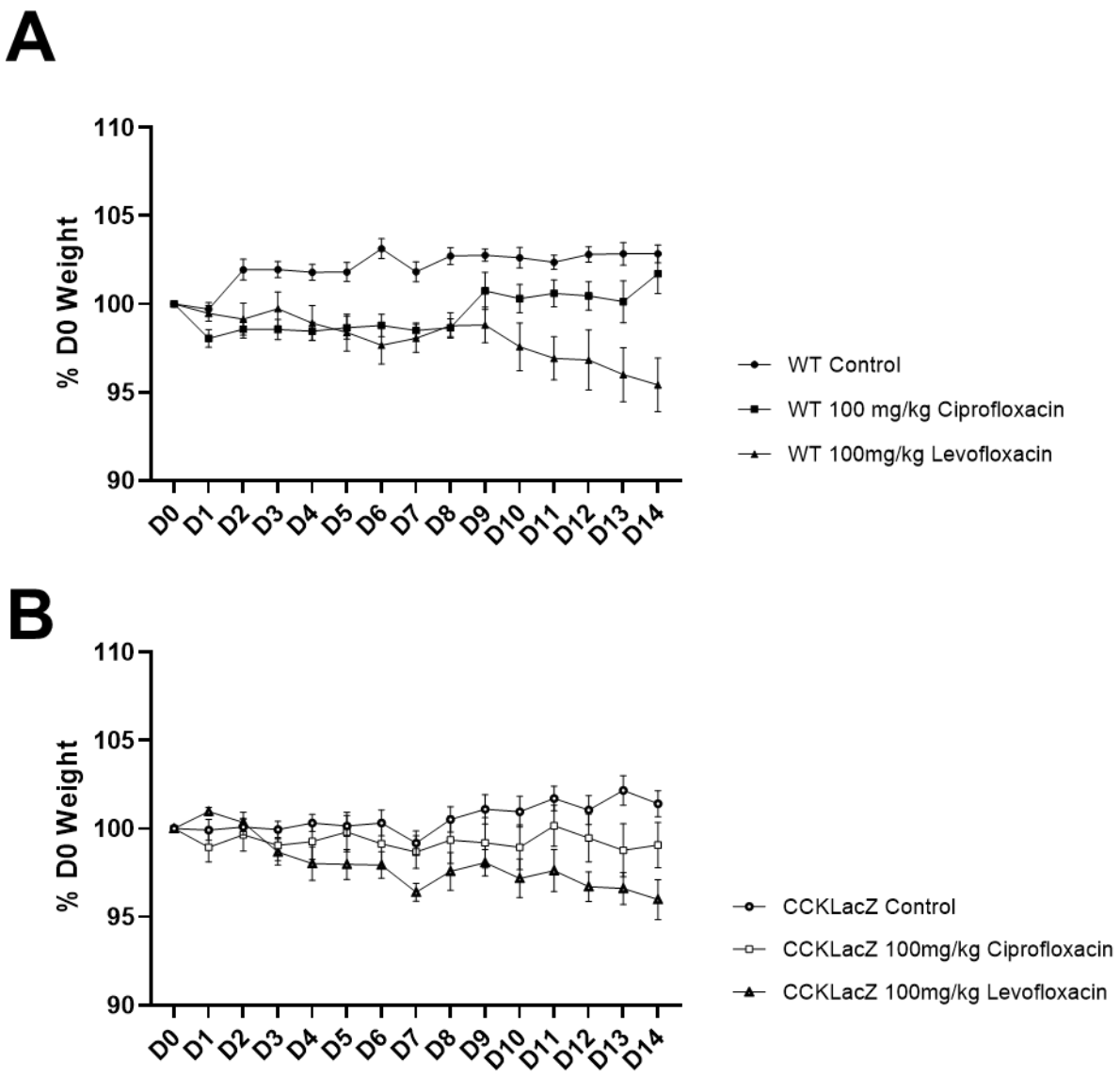


Figure 5.3 CCK is responsible for ciprofloxacin induced weight loss in C57BL/6 mice

C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H2O mock dose via oral gavage (N=9/group, 8/group CCKLacZ Cipro). A: Percentage daily weight change (vs D0 weight) of fluoroquinolone or vehicle treated C57BL/6 mice over 14 days of treatment (N=9/group). B: Percentage weight change of (vs D0 weight) of fluoroquinolone or vehicle treated CCKLacZ mice over 14 days of treatment (N=8/group). Significance shown in figure 5.4.

Table 5.1: Significance of percentage weight change in WT and CCKLacZ mice treated with fluoroquinolones vs control. *(*, P<0.05; **, P<0.01; ***, P<0.005; ****, P<0.0001; N.S., not significant using REML Mixed effects analysis followed by Sidak's multiple comparisons test.)*

Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
WT Control vs WT Ciprofloxacin 100mg/kg	ns	ns	*	*	*	*	***	*	*	ns	ns	ns	ns	ns	ns
WT Control vs WT Levofloxacin 100mg/kg	ns	ns	ns	ns	ns	*	****	**	*	*	***	***	****	****	****
CCKLacZ Control vs CCKLacZ Ciprofloxacin 100mg/kg	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CCKLacZ Control vs CCKLacZ Levofloxacin 100mg/kg	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	**	****	***

CCK was fully protective against ciprofloxacin-mediated weight loss, with CCKLacZ animals not losing significant weight vs vehicle control at any point during the 14 day time course of the experiment (Fig. 5.3 and Table 5.1). Levofloxacin caused significant weight loss by day 10 in CCK KO animals as compared to PBS vehicle controls, showing a delayed onset of weight loss as compared to day 5 in C57BL/6 comparisons (Fig. 5.3 and Table 5.1).

Collectively, these data indicate that ciprofloxacin and levofloxacin produce distinct weight loss responses in C57BL/6 mice and the peptide hormone CCK is solely responsible for ciprofloxacin induced weight loss and plays a partial role in levofloxacin.

5.3 Fluoroquinolone treatment and inhibited CCK driven weight loss does not alter immune cell populations in the C57BL/6 spleen

To further investigate whether fluoroquinolone treatment affected the overall phenotype of circulating immune cells *in vivo*, we utilised a broad flow cytometry panel in C57BL/6 mice, and to ascertain whether CCK or the weight loss induced had an impact on myeloid cells, in CCK KO mice.

Spleens were harvested at the day 7 and 14 timepoints. We first examined cellularity of the spleen across the time course and observed no significant differences between vehicle and fluoroquinolone treated animals, both with and without CCK (Fig. 5.4).

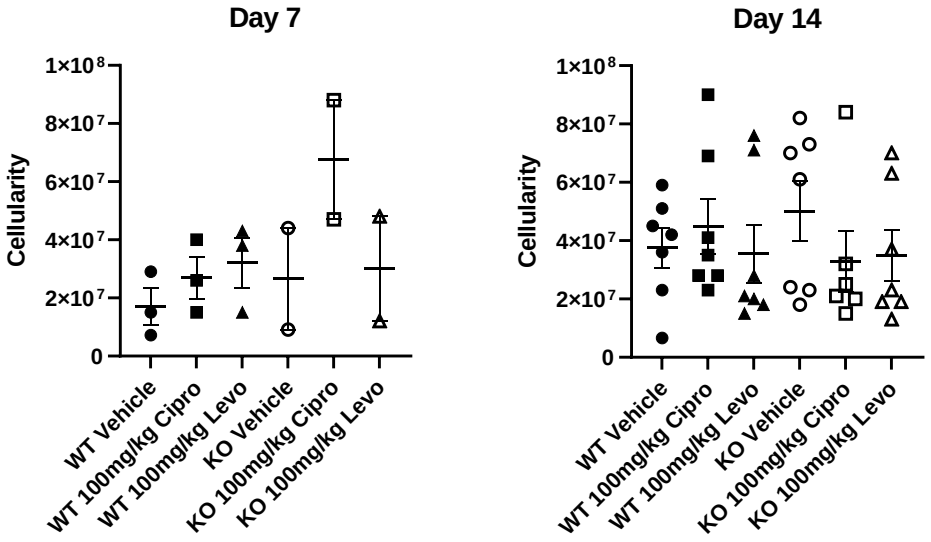


Figure 5.4: Splenic Cellularity is not influenced by ciprofloxacin, levofloxacin or the lack of CCK induced weight loss. *C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=9/group, 8/group CCKLacZ Cipro). Spleens were harvested every 7 days. Cellularity of spleen samples after 7 and 14 days of fluoroquinolone treatment. Significance tested using one-way ANOVA followed by Sidak's multiple comparisons test.*

At both day 7 and 14 the lack of CCK did not have an effect on the %CD45+ splenocytes as compared to wild-type controls (Fig. 5.5). Moreover, neither fluoroquinolone treatment altered the percentage of CD45+ splenocytes in the wild-type or CCK KO mice across the timecourse of treatment as compared to equivalent PBS vehicle treated controls (Fig. 5.5).

We next examined the granulocyte Neutrophil and Eosinophil populations in the spleen. At both day 7 and 14 the lack of CCK did not have an effect on the % of neutrophils or eosinophils as compared to wild-type controls (Fig. 5.6). Moreover, neither fluoroquinolone treatment altered the percentage of either spleen neutrophils or eosinophils in the wild-type or CCK KO mice across the

14 day timecourse of ciprofloxacin or levofloxacin as compared to equivalent PBS vehicle treated controls (Fig. 5.6).

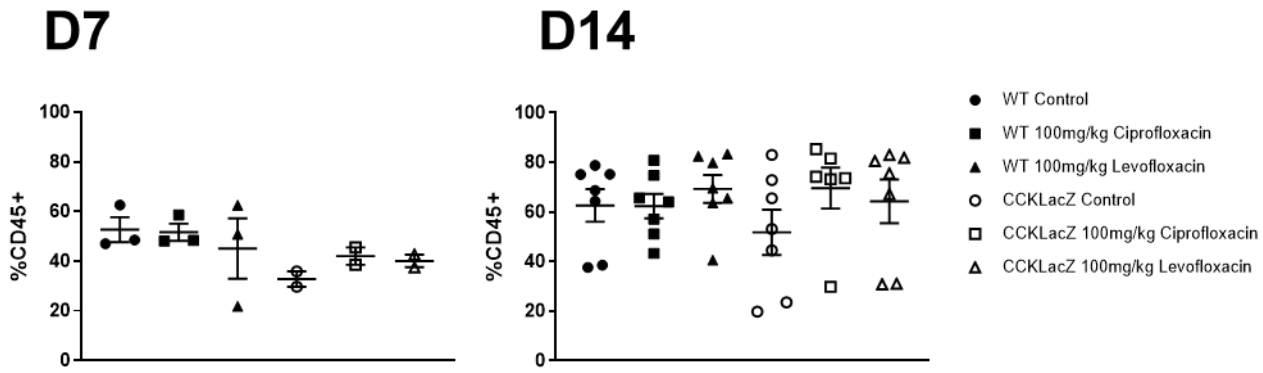


Figure 5.5: CD45+ splenocyte percentages are not influenced by ciprofloxacin, levofloxacin or the lack of CCK induced weight loss. *C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H2O mock dose via oral gavage for 14 days (N=9/group, 8/group CCKLacZ Cipro). Spleens were harvested every 7 days. Percentage of CD45+ cells in the spleen after 7 or 14 days of fluoroquinolone treatment. Significance tested using one-way ANOVA followed by Sidak's multiple comparisons test.*

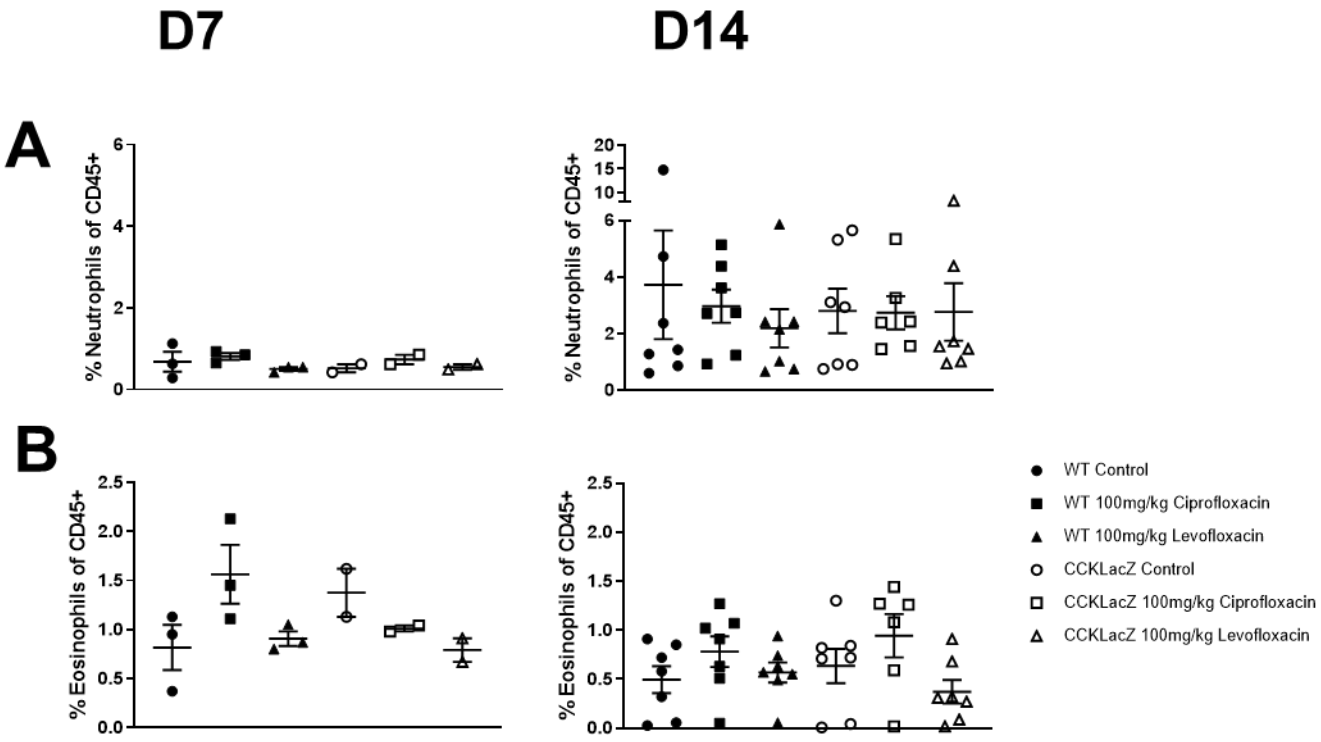


Figure 5.6: Splenic granulocytes percentages are not influenced by ciprofloxacin, levofloxacin or the lack of CCK induced weight loss. *C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=9/group, 8/group CCKLacZ Cipro). Spleens were harvested every 7 days. A) Percentage of Neutrophils in the spleen after 7 or 14 days of fluoroquinolone treatment. B) Percentage of Eosinophils in the spleen after 7 or 14 days of fluoroquinolone treatment. Significance tested using one-way ANOVA followed by Sidak's multiple comparisons test.*

Finally we examined the monocyte/macrophage and DC populations within the spleen. At both day 7 and 14 the lack of CCK did not have an effect on the % of monocyte/macrophage or DC populations as compared to wild-type controls (Fig. 5.7). Moreover, neither fluoroquinolone treatment altered the percentage of either spleen monocyte/macrophage and DC populations in the wild-type or CCK KO mice across the 14 day timecourse of ciprofloxacin or levofloxacin as compared to equivalent PBS vehicle treated controls (Fig. 5.7).

Collectively, these data indicate that both ciprofloxacin and levofloxacin treatment have minimal impact on spleen immune cell population percentages in the C57BL/6 background across the time course of our experimental analysis. Moreover, CCK itself and the inhibited weight loss driven by fluoroquinolone treatment in the absence of CCK do not influence splenic immune populations.

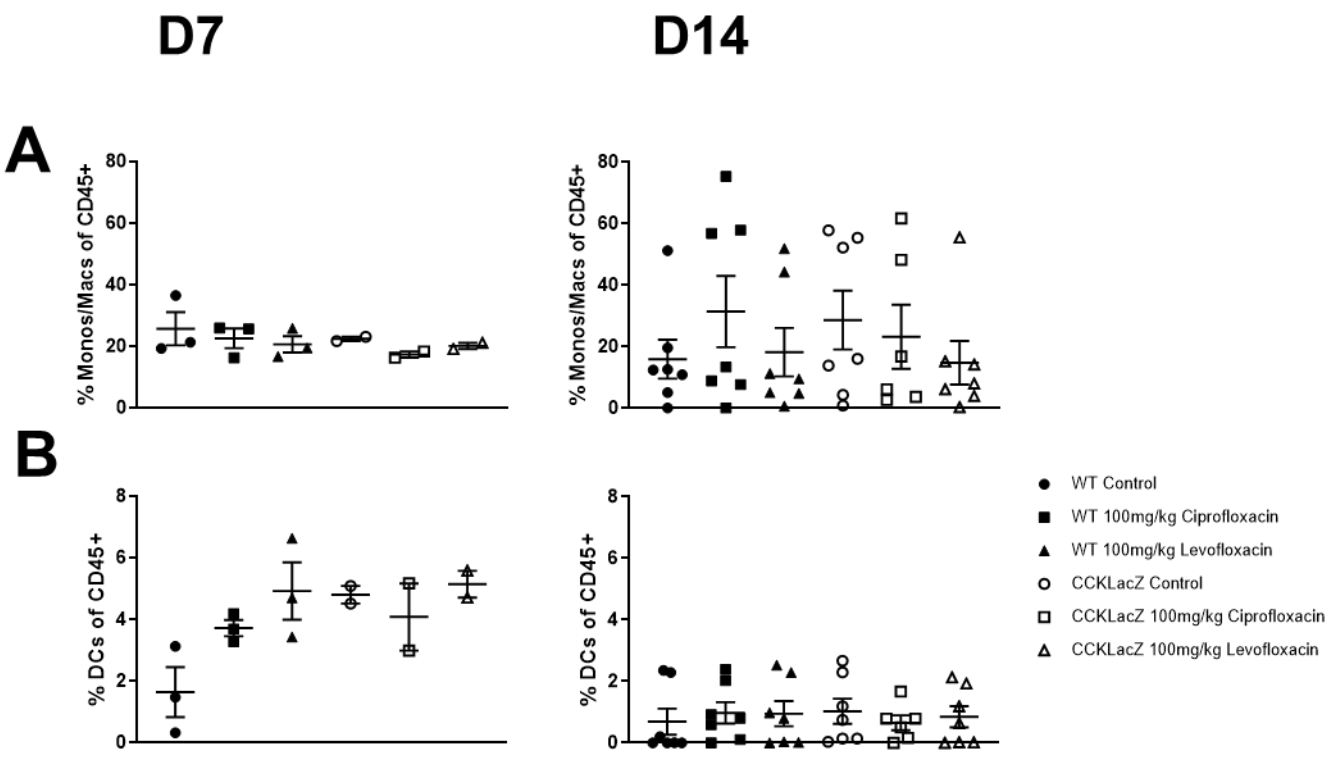


Figure 5.7: Splenic monocytes and APCs percentages are not influenced by ciprofloxacin, levofloxacin or the lack of CCK induced weight loss. *C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=9/group, 8/group CCKLacZ Cipro). Spleens were harvested every 7 days. A) Percentage of Monocytes and Macrophages in the spleen after 7 or 14 days of fluoroquinolone treatment. B) Percentage of Dendritic Cells in the spleen after 7 or 14 days of fluoroquinolone treatment. Significance tested using one-way ANOVA followed by Sidak's multiple comparisons test.*

5.4 Fluoroquinolone treatment and inhibited CCK driven weight loss does not alter immune cell populations in the C57BL/6 mLN

Next focussing on the intestine as the site of inoculation and microbial dysbiosis caused by antibiotic dosing, we first examined cellularity of the mLN across the time course. There were no significant differences between treatment groups (Fig. 5.8).

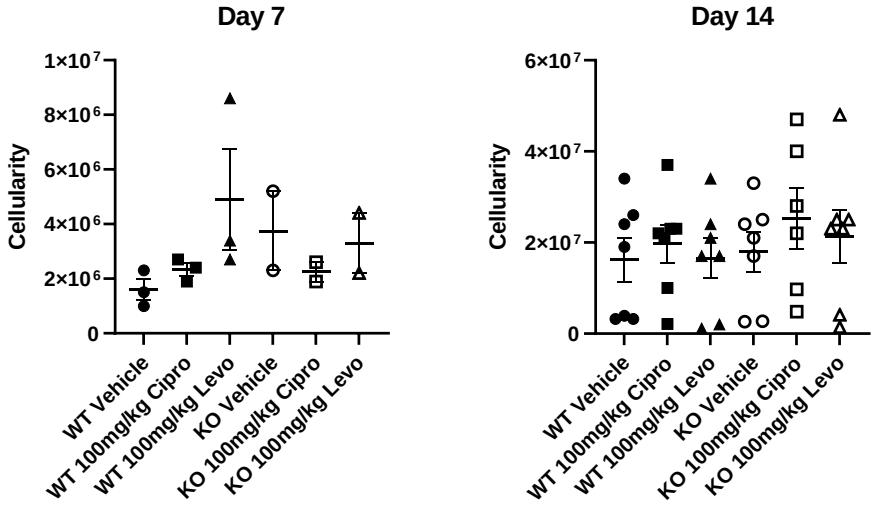


Figure 5.8 mLN Cellularity is not influenced by ciprofloxacin, levofloxacin or the lack of CCK induced weight loss *C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=9/group, 8/group CCKLacZ Cipro). Mesenteric lymph nodes were harvested every 7 days. Cellularity of MLN samples after 7 and 14 days of fluoroquinolone treatment. Significance tested using one-way ANOVA followed by Sidak's multiple comparisons test.*

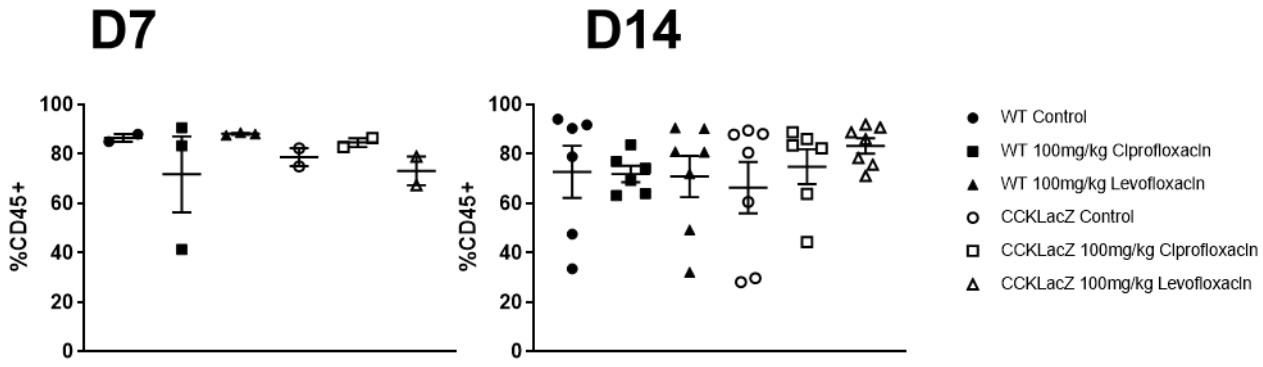


Figure 5.9: CD45+ mLN percentages are not influenced by ciprofloxacin, levofloxacin or the lack of CCK induced weight loss *C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=9/group, 8/group CCKLacZ Cipro). Mesenteric Lymph nodes were harvested every 7 days. Percentage of CD45+ cells in the MLN after 7 or 14 days of fluoroquinolone treatment. Significance tested using one-way ANOVA followed by Sidak's multiple comparisons test.*

As CCK is primarily a gut hormone, it was important to determine whether the phenotype of the gut-associated lymphoid tissue was affected by its absence during fluoroquinolone treatment. The absence of CCK did not affect the percentage of CD45+ cells in the MLN at in untreated animals as compared to wild-types (Fig. 5.9). Moreover, both ciprofloxacin and levofloxacin treatments did not significantly alter CD45+ mLN populations at day 7 nor day 14 post treatment as compared to PBS vehicle controls, true for both wild-type C57BL/6 and CCK KO mice (Fig. 5.9).

We next examined the granulocyte Neutrophil and Eosinophil populations in the mLN. At both day 7 and 14 the lack of CCK did not have an effect on the % of neutrophils or eosinophils as compared to wild-type controls (Fig. 5.10). Moreover, neither fluoroquinolone treatment altered the percentage of either mLN neutrophils or eosinophils in the wild-type or CCK KO mice across the 14 day timecourse of ciprofloxacin or levofloxacin as compared to equivalent PBS vehicle treated controls (Fig. 5.10).

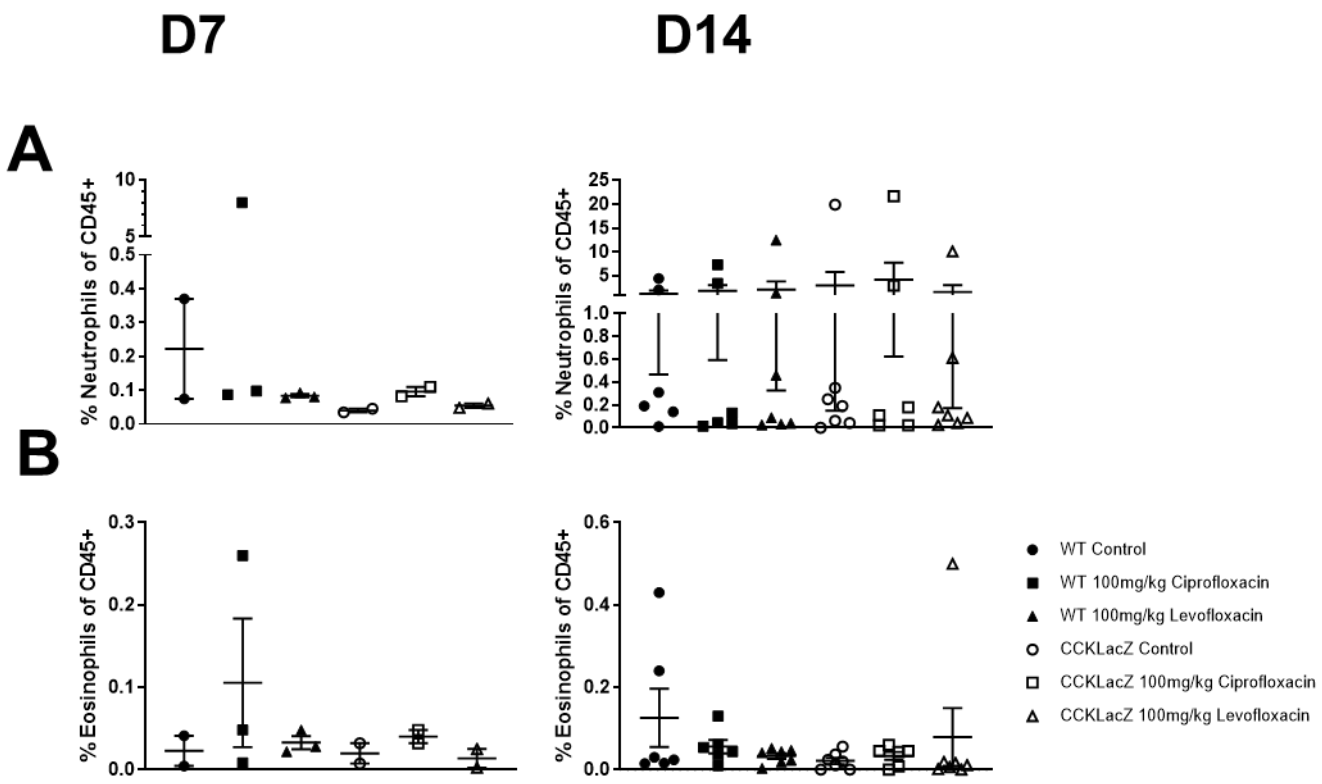


Figure 5.10: mLN granulocytes percentages are not influenced by ciprofloxacin, levofloxacin or the lack of CCK induced weight loss. *C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=9/group, 8/group CCKLacZ Cipro). Mesenteric Lymph nodes were harvested every 7 days. A) Percentage of Neutrophils in the MLN after 7 or 14 days of fluoroquinolone treatment. B) Percentage of Eosinophils in the MLN after 7 or 14 days of fluoroquinolone treatment. Significance tested using one-way ANOVA followed by Sidak's multiple comparisons test.*

Finally we examined the monocyte/macrophage and DC populations within the mLN. At both day 7 and 14 the lack of CCK did not have an effect on the % of monocyte/macrophage or DC populations as compared to wild-type controls (Fig. 5.11). Moreover, neither fluoroquinolone treatment altered the percentage of either mLN monocyte/macrophage and DC populations in the wild-type or CCK KO mice across the 14 day timecourse of ciprofloxacin or levofloxacin as compared to equivalent PBS vehicle treated controls (Fig. 5.11).

Collectively, these data indicate that both ciprofloxacin and levofloxacin treatment have minimal impact on mLN immune cell population percentages in the C57BL/6 background across the time course of our experimental analysis. Moreover, CCK itself and the inhibited weight loss driven by fluoroquinolone treatment in the absence of CCK do not influence mLN immune populations at the examined level.

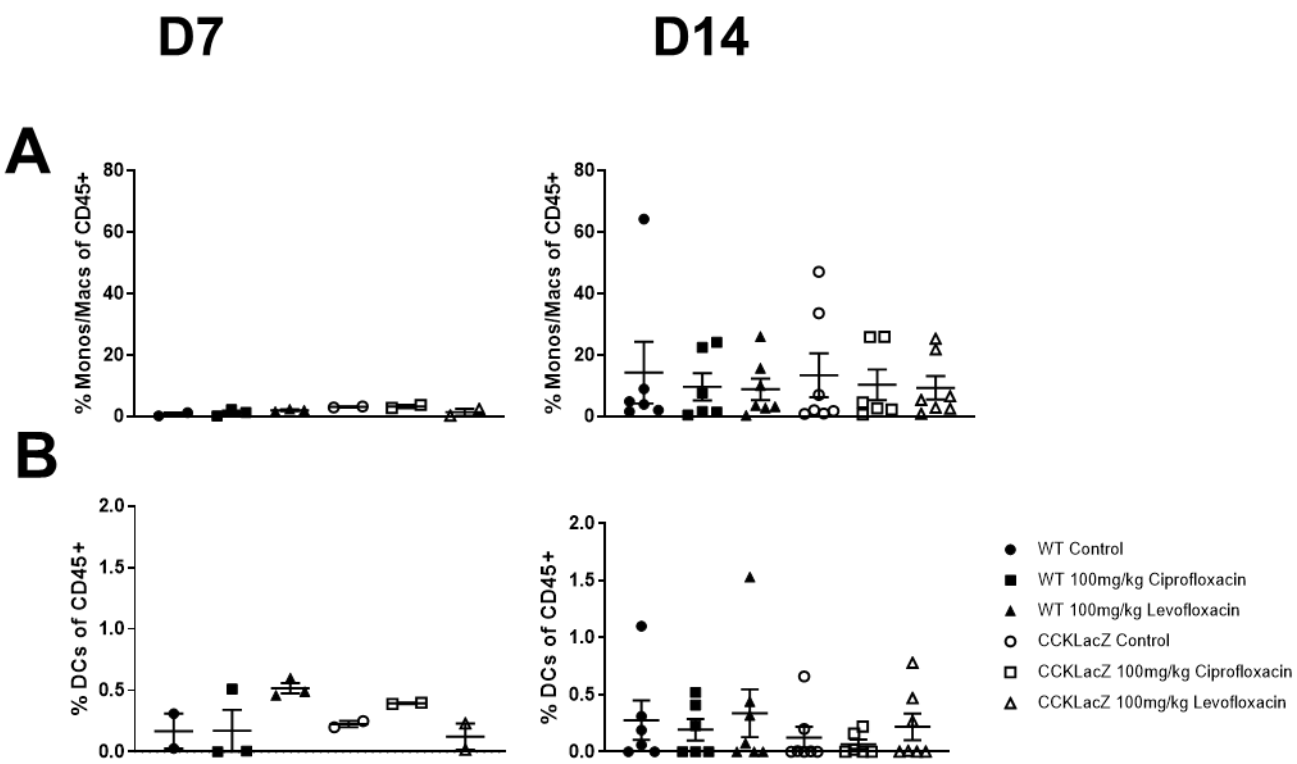


Figure 5.11: mLN monocytes and APCs percentages are not influenced by ciprofloxacin, levofloxacin or the lack of CCK induced weight loss. *C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=9/group, 8/group CCKLacZ Cipro). Mesenteric Lymph nodes were harvested every 7 days. A) Percentage of Monocytes and Macrophages in the MLN after 7 or 14 days of fluoroquinolone treatment. B) Percentage of Dendritic Cells in the MLN after 7 or 14 days of fluoroquinolone treatment. Significance tested using one-way ANOVA followed by Sidak's multiple comparisons test.*

5.5 Fluoroquinolone antibiotic treatment produces colonic goblet cell hypoplasia

To further investigate whether fluoroquinolone treatment or lack of CCK induced weight loss affected overall pathology in the gut, we enumerated Alcian Blue-Periodic Acid Schiff (AB-PAS) stained colonic sections of wild-type C57BL/6 and CCK KO mice at day 14 post fluoroquinolone treatment as an indicator of colonic barrier integrity

Ciprofloxacin and levofloxacin treated wild-type mice had a significantly reduced number of goblet cells per CCU in comparison to PBS vehicle treated controls (Fig.5.12), which corresponded with a visible reduction in AB-PAS stained goblet cells (Fig.5.12). No antibiotic-specific effects were seen, as there was no significant difference in the average number of goblet cells per CCU between Ciprofloxacin and Levofloxacin treated wild-type mice (Fig. 5.12).

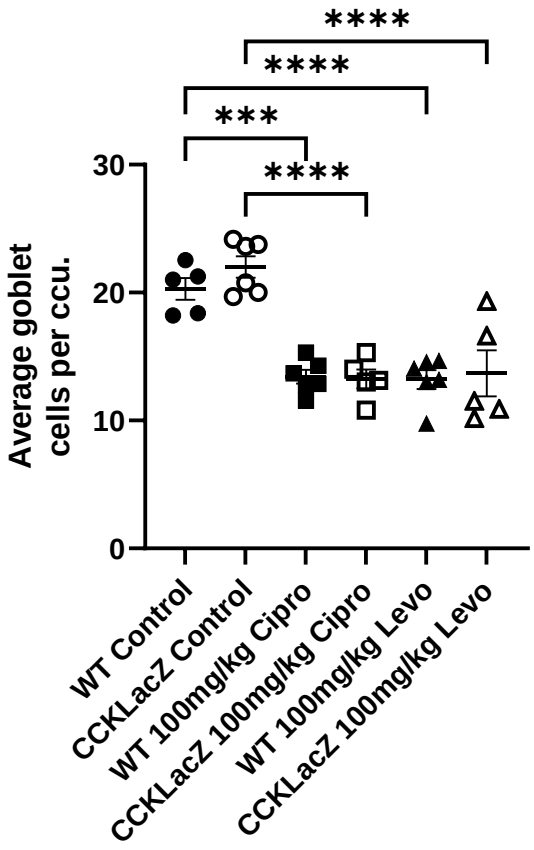


Figure 5.12 Fluoroquinolone treatment significantly reduces the average number of goblet cells per colonic crypt unit (CCU) in both wild-type and CCK KO mice*. C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=9/group, 8/group CCKLacZ Cipro). Colonic sections were excised, fixed in Carnoy's fixative, and stained with Alcian Blue-Periodic Acid Schiff (AB-PAS) stain. The average number of goblet cells per colonic crypt unit (CCU) was calculated from goblet cells counts from 20 random CCUs. Data was obtained from three independent experiments (n=5/6 mice per treatment group). ***, P<0.005 and **** P<0.0001 from a Sidak's multiple comparisons test following ANOVA...*

Moreover, the lack of CCK and hence fluoroquinolone induced weight loss did not influence the goblet cell hypoplasia, with comparable levels of goblet cells in all PBS, ciprofloxacin and levofloxacin groups (Fig.5.12 and 13).

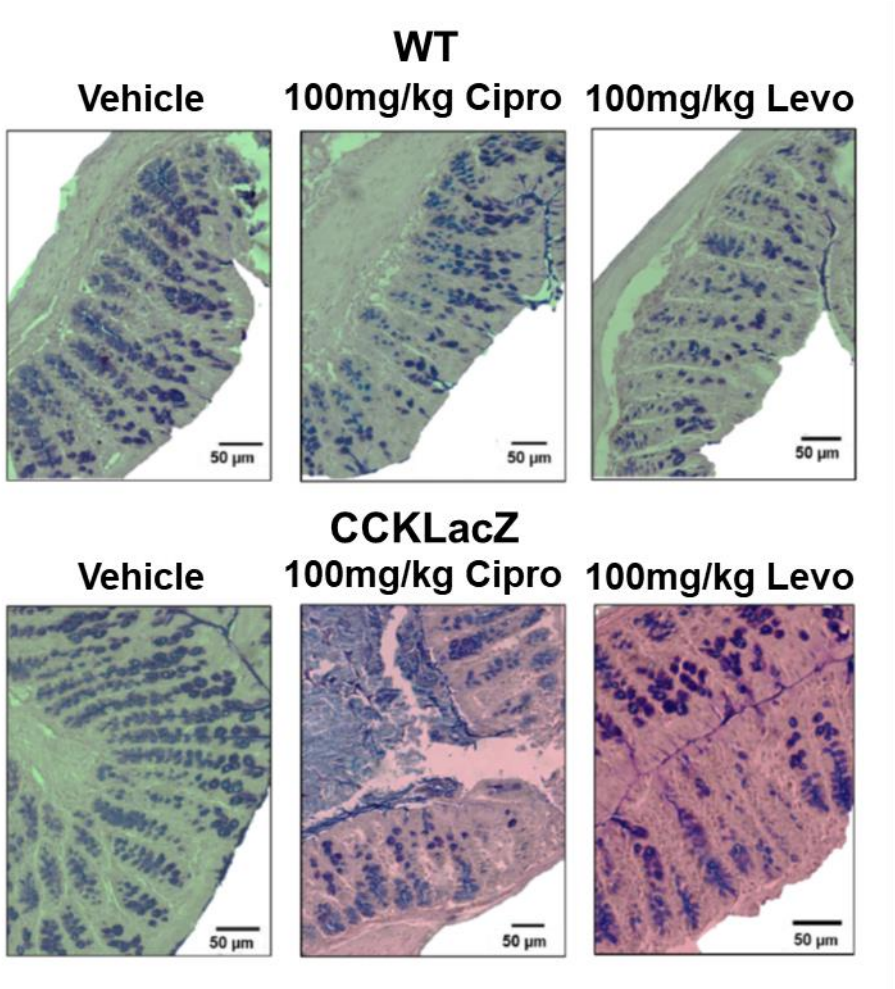


Figure 5.13 Representative images of colonic goblet cell goblet cells in both wild-type and CCK KO mice following fluoroquinolone treatments. *C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=9/group, 8/group CCKLacZ Cipro). Colonic sections were excised, fixed in Carnoy's fixative, and stained with Alcian Blue-Periodic Acid Schiff (AB-PAS) stain.*

We next analysed colonic crypt depth, as an indicator of inflammation. Examining colonic architecture, we saw no significant difference between wild-type and CCK KO crypt size in PBS treated controls, but an overall trend for reduced crypt length in both groups following ciprofloxacin and levofloxacin

treatments. This however only reached significance in the levofloxacin treated CCK KO group as compared to the vehicle PBS treated cohort (Fig.5.14).

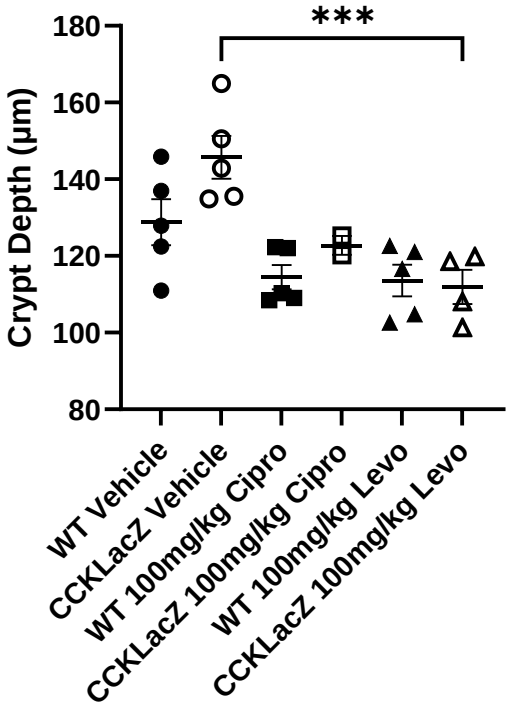


Figure 5.14 Levofloxacin treatment reduced the colonic crypt length in CCK KO mice. *C57BL/6 CCK+/+and CCK-/-mice were dosed twice daily via oral gavage with 100 µL of PBS control, Ciprofloxacin (Cipro) or Levofloxacin (Levo) at a concentration of 100 mg/kg before being euthanised at 14 days. Colonic sections were excised, fixed in Carnoy's fixative, and stained with Alcian Blue-Periodic Acid Schiff (AB-PAS) stain. Colonic section photographs were used to measure the colonic crypt length in 20 random colonic crypts and an average calculated. Data (n=5/6 mice per treatment group) was obtained from three independent experiments. ***P<0.005 from a Sidak's multiple comparisons test following a one-way ANOVA and error bars represent the standard error of independent experiment means.*

Collectively, these data indicate that although CCK itself does not influence goblet cell numbers or architecture, fluoroquinolones have a subtle effect on crypt size and a significant goblet cell hypoplasia in the colonic tissues.

5.6 Fluoroquinolone treatment and inhibited CCK driven weight loss does not alter immune cell populations in the C57BL/6 lung

We next repeated the flow cytometry panels on lung tissue from wild-type and CCK KO animals every 7 days. These are particularly important niches as any immunomodulatory effects fluoroquinolones have on lung cells are of particular importance. Fluoroquinolones are prescribed to treat a variety of severe respiratory tract infections, and as such, any negative effects on the lung immune system could have serious consequences, while positive would be in addition to direct bactericidal modulation.

We first examined cellularity of the lung across the time course and found no significant differences (Fig. 5.15).

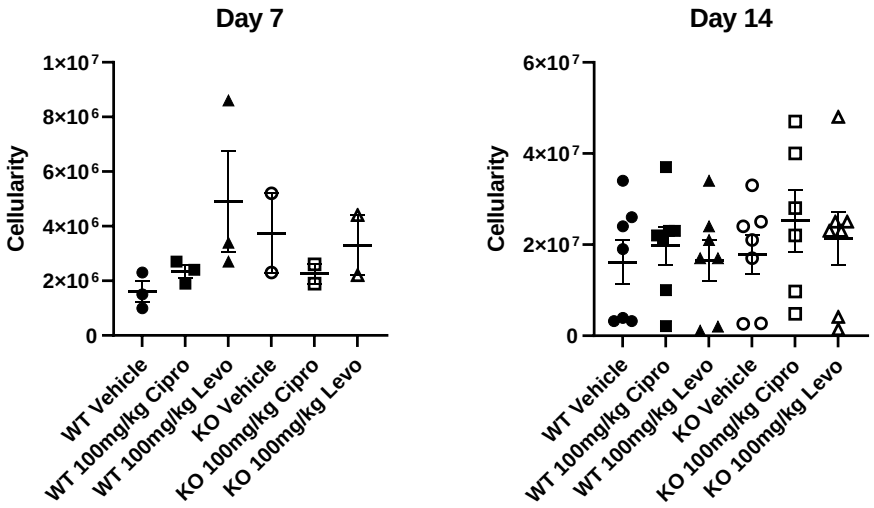


Figure 5.15 Lung Cellularity is not influenced by ciprofloxacin, levofloxacin or the lack of CCK induced weight loss *C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=9/group, 8/group CCKLacZ Cipro). Lungs were harvested every 7 days. Cellularity of lung samples after 7 and 14 days of fluoroquinolone treatment. Significance tested using one-way ANOVA followed by Sidak's multiple comparisons test.*

The absence of CCK did not affect the percentage of CD45+ cells in the lung in untreated animals as compared to wild-types (Fig. 5.16). Moreover, both ciprofloxacin and levofloxacin treatments did not significantly alter CD45+ percentage lung populations at day 7 nor day 14 post treatment as compared to PBS vehicle controls, true for both wild-type C57BL/6 and CCK KO mice (Fig. 5.16).

We next examined the granulocyte Neutrophil and Eosinophil populations in the lung. At both day 7 and 14 the lack of CCK did not have an effect on the % of neutrophils or eosinophils as compared to wild-type controls (Fig. 5.17). Moreover, neither fluoroquinolone treatment altered the percentage of either lung neutrophils or eosinophils in the wild-type or CCK KO mice across the 14 day timecourse of ciprofloxacin or levofloxacin as compared to equivalent PBS vehicle treated controls (Fig. 5.17).

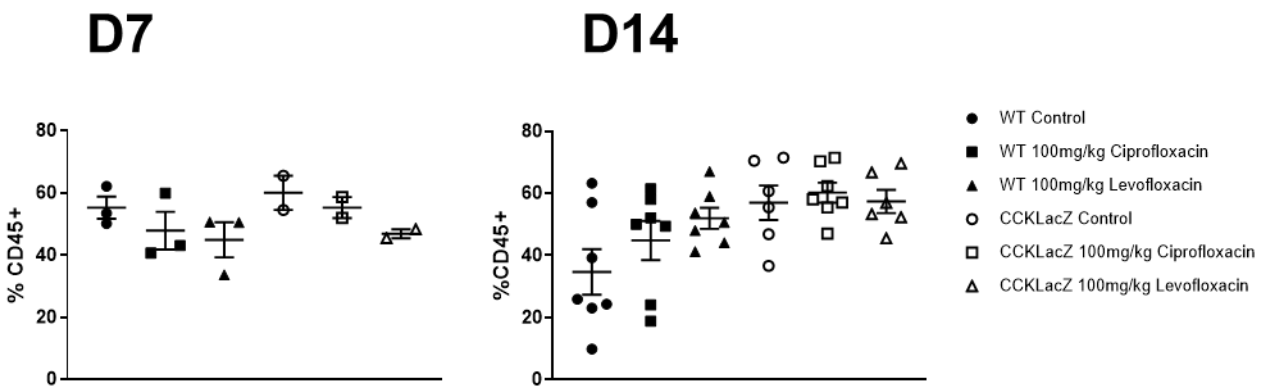


Figure 5.16: CD45+ Lung Percentage is not influenced by ciprofloxacin, levofloxacin or the lack of CCK induced weight loss *C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=9/group, 8/group CCKLacZ Cipro). Lungs were harvested every 7 days. Percentage of CD45+ cells in the Lung after 7 or 14 days of fluoroquinolone treatment. Significance tested using one-way ANOVA followed by Sidak's multiple comparisons test.*

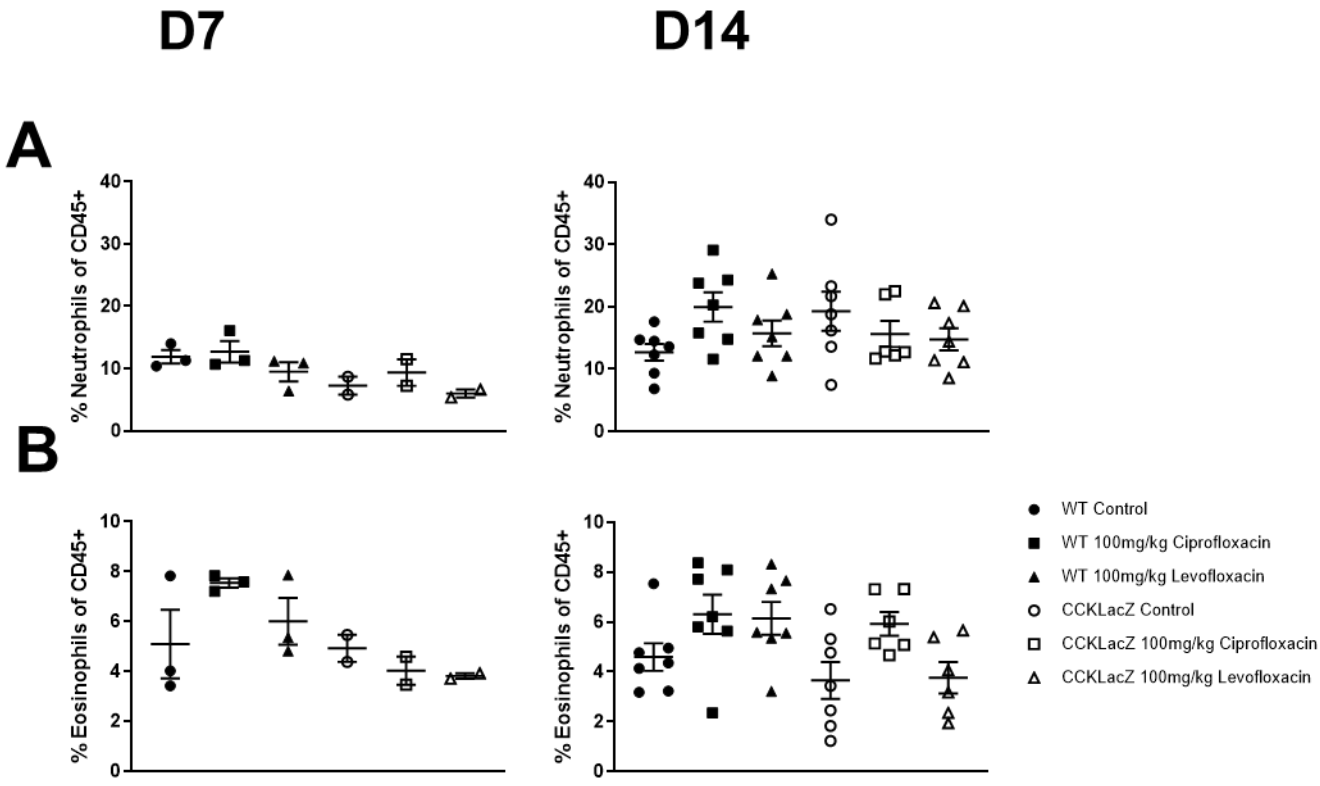


Figure 5.17 Fluoroquinolones do not affect lung granulocyte populations with and without CCK *C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=9/group, 8/group CCKLacZ Cipro). Lungs were harvested every 7 days. A) Percentage of Neutrophils in the Lung after 7 or 14 days of fluoroquinolone treatment. B) Percentage of Eosinophils in the Lung after 7 or 14 days of fluoroquinolone treatment. Significance tested using one-way ANOVA followed by Sidak's multiple comparisons test.*

Finally we examined the monocyte/macrophage and DC populations within the lung. At both day 7 and 14 the lack of CCK did not have an effect on the % of monocyte/macrophage or DC populations as compared to wild-type controls (Fig. 5.18). Moreover, neither fluoroquinolone treatment altered the percentage of either lung monocyte/macrophage and DC populations in the wild-type or CCK KO mice across the 14 day timecourse of ciprofloxacin or levofloxacin as compared to equivalent PBS vehicle treated controls (Fig. 5.18).

Collectively, these data indicate that both ciprofloxacin and levofloxacin treatment have minimal impact on lung immune cell population percentages in the C57BL/6 background across the time course of our experimental analysis. Moreover, CCK itself and the inhibited weight loss driven by fluoroquinolone treatment in the absence of CCK do not influence lung immune populations.

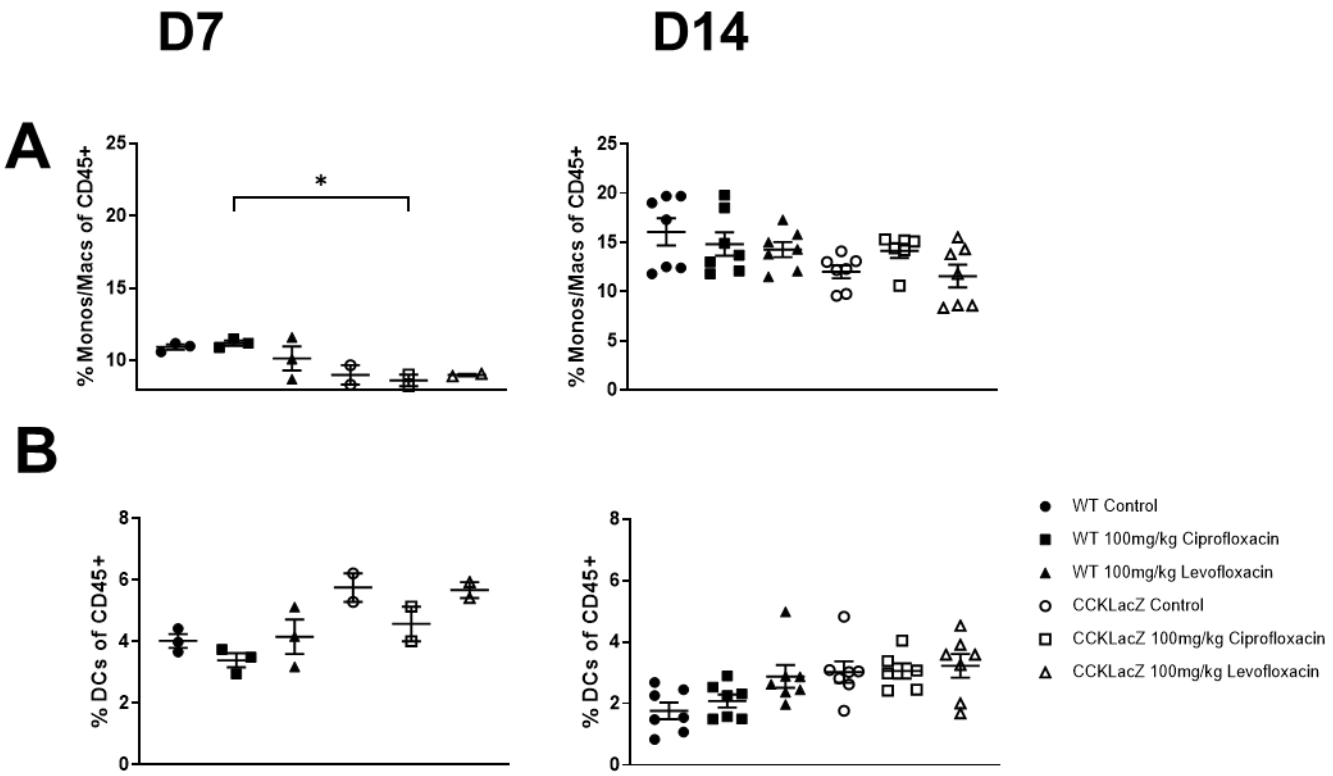


Figure 5.18 Antigen presenting cell levels are not affected by fluoroquinolones with or without the presence of CCK

C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=9/group, 8/group CCKLacZ Cipro). Lungs were harvested every 7 days. A) Percentage of Monocytes and Macrophages in the Lung after 7 or 14 days of fluoroquinolone treatment. B) Percentage of Dendritic Cells in the Lung after 7 or 14 days of fluoroquinolone treatment. Significance tested using one-way ANOVA followed by Sidak's multiple comparisons test.

5.6 The lack of CCK does not alter fluoroquinolone induced M1 lung macrophage polarisation *in vivo*

My previous data indicated that the lung is a specific site of macrophage modulation following fluoroquinolone treatment, driving a pro-inflammatory M1 phenotype specifically within the short-lived, monocyte derived macrophage population. Given this strong finding, it was next important to assess if the lack of CCK itself, or the induced weight loss it drives, altered the M1 polarisation in the lung, or other tissues, following ciprofloxacin treatment.

To give an overall readout of the peripheral state of macrophage polarisation we first examined splenic macrophages and saw no difference in NOS2 M1 populations in either of the ciprofloxacin or levofloxacin treated groups in both wild-type and CCK KO mice as compared to PBS vehicle controls across the time-course of treatment (Fig.5.19).

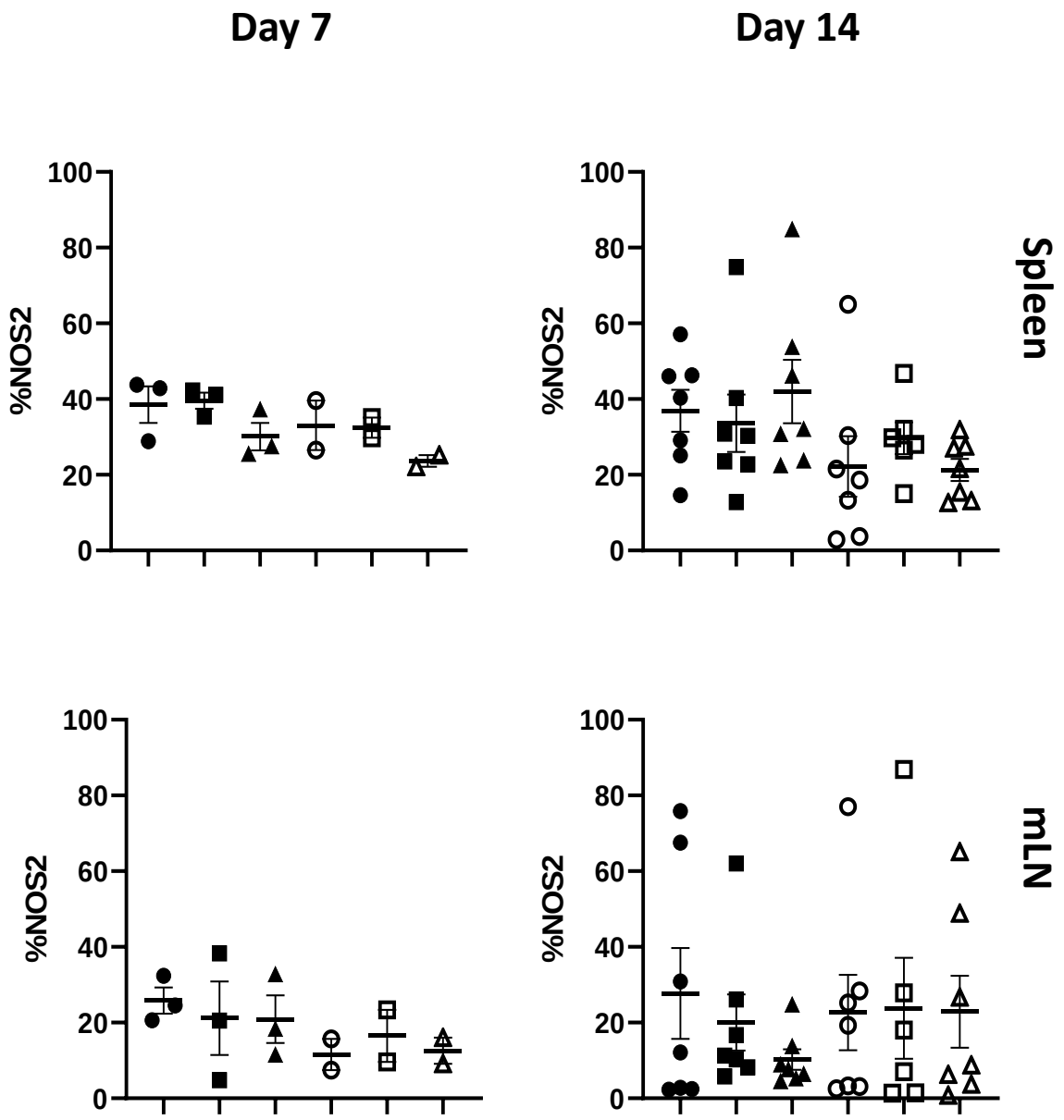


Figure 5.19: Cholecystokinin has no effect on fluoroquinolone-induced Splenic and mLN M1 macrophage polarisation *C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=7/group). Spleens and mLNs were harvested at 7 and 14 days. Percentage of macrophages in the spleen and lung expressing NOS2 *, P<0.05; **, 0.01; ***, 0.005; ****, 0.0001; Significance tested using one-way ANOVA followed by Sidak's multiple comparisons test.*

Given the strong intestinal resident microflora and location of CCK EECs, we next analysed macrophage populations in the gut draining mLN. mLN macrophage populations had no difference in NOS2 M1 populations in wild-type or CCK KO mice following either of the ciprofloxacin or levofloxacin treatments as compared to PBS vehicle controls across the time course of treatment (Fig.5.19).

We next analysed reciprocal M2 populations in both the spleen and mLN utilising CD206 surface marker and again saw no changes in populations at either day 7 or 14 in either the wild-type or CCK KO animals following either fluoroquinolone treatment, as compared to PBS vehicle controls (Fig. 5.20).

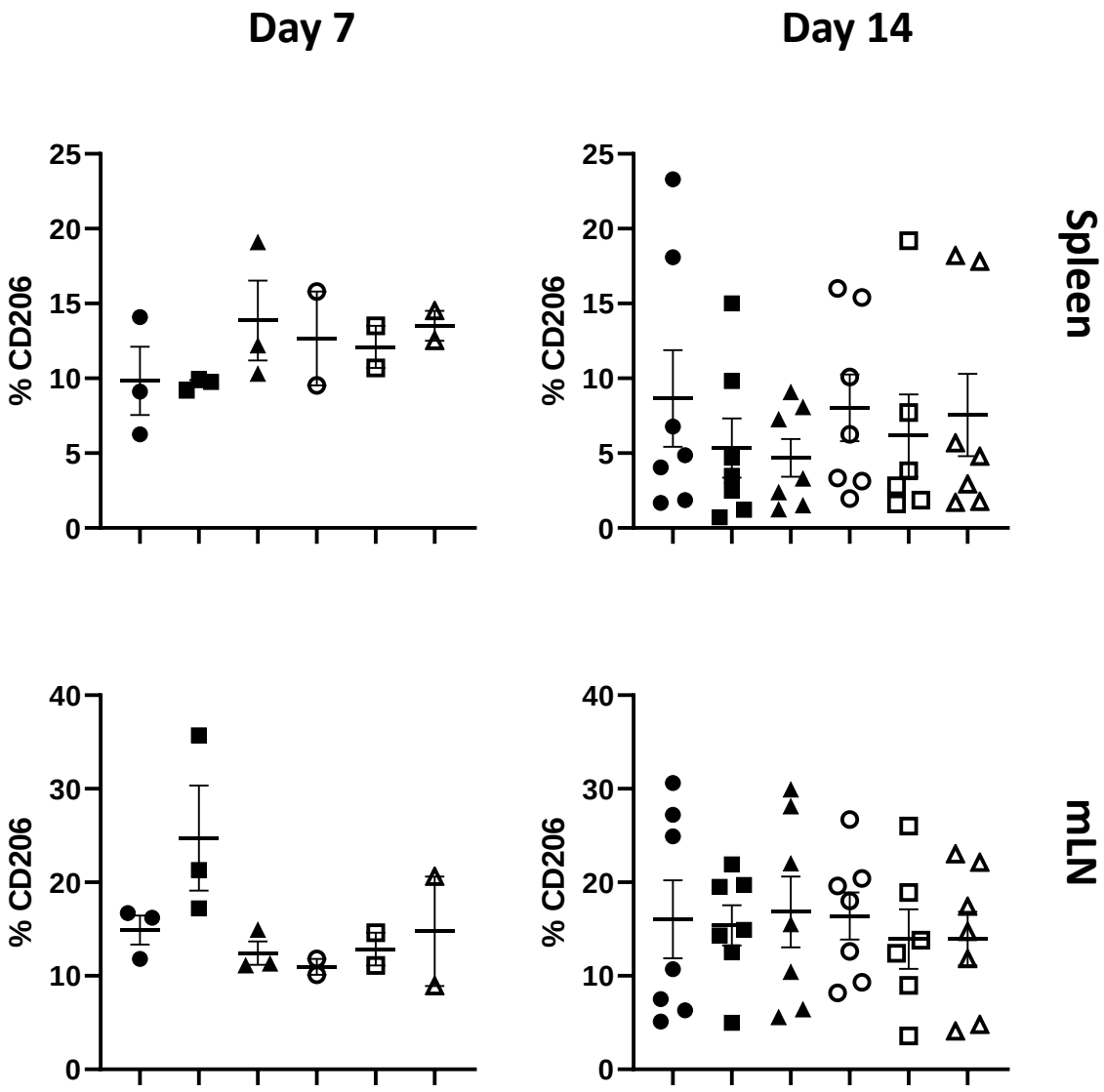


Figure 5.20: Cholecystokinin has no effect on fluoroquinolone-induced Splenic and mLN M2 macrophage polarisation *C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=7/group). Spleens and mLNs were harvested at 7 and 14 days. Percentage of macrophages in the spleen and lung expressing CD206 *, P<0.05; **, 0.01; ***, 0.005; ****, 0.0001; Significance tested using one-way ANOVA followed by Sidak's multiple comparisons test.*

Utilising the lower expression of CD11b in the CD45+/CD64+ macrophage population we were able to broadly distinguish between alveolar macrophages and the remaining interstitial macrophage, monocyte/macrophage population(Misharin et al., 2013a). Examining the expression of NOS2 in the tissue-resident alveolar macrophage population we saw no alteration in M1 populations in either the wild-type or CCK KO mice following either fluoroquinolone treatment as compared to PBS vehicle controls across the time course of treatment (Fig. 5.21).

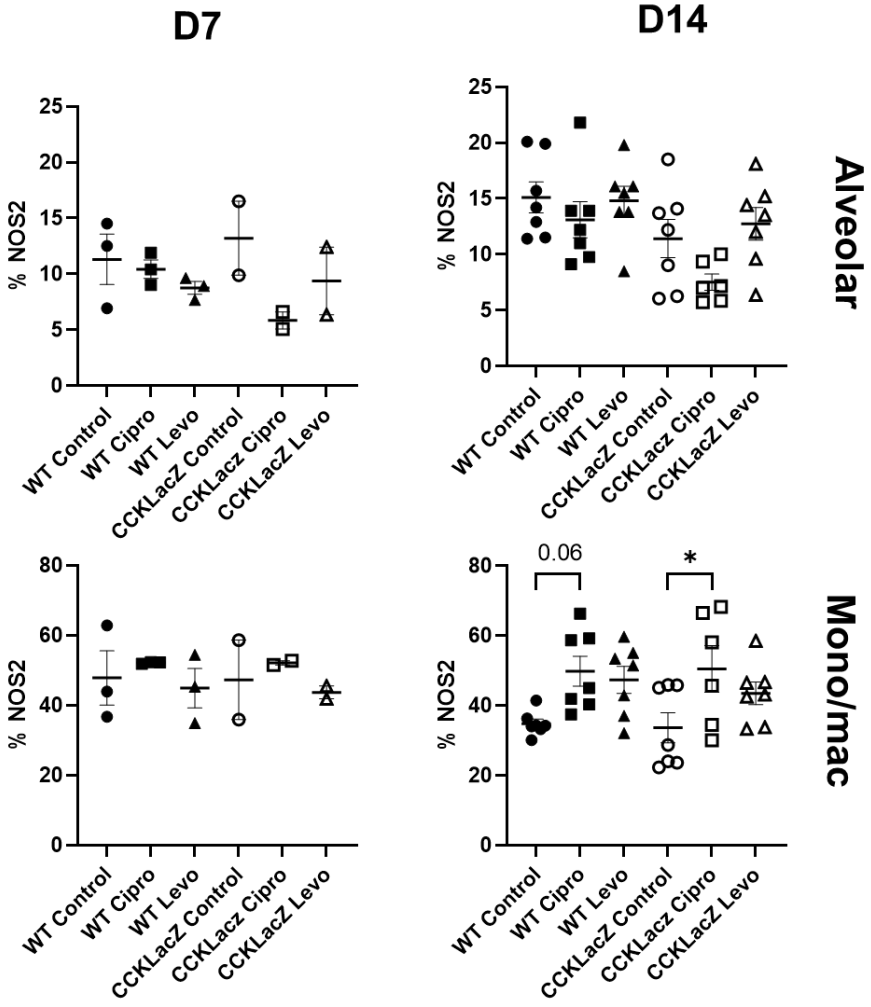


Figure 5.21 M1 interstitial lung macrophage polarisation is increased more in CCKLacZ mice than in WT after Cipro treatment *C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=7/group). Lungs were harvested at 7 and 14 days. Percentage of either alveolar macrophages or monocyte/macrophages in the lung expressing NOS2 *, P<0.05; **, 0.01; ***, 0.005; ****, 0.0001; Significance tested using one-way ANOVA followed by Sidak's multiple comparisons test.*

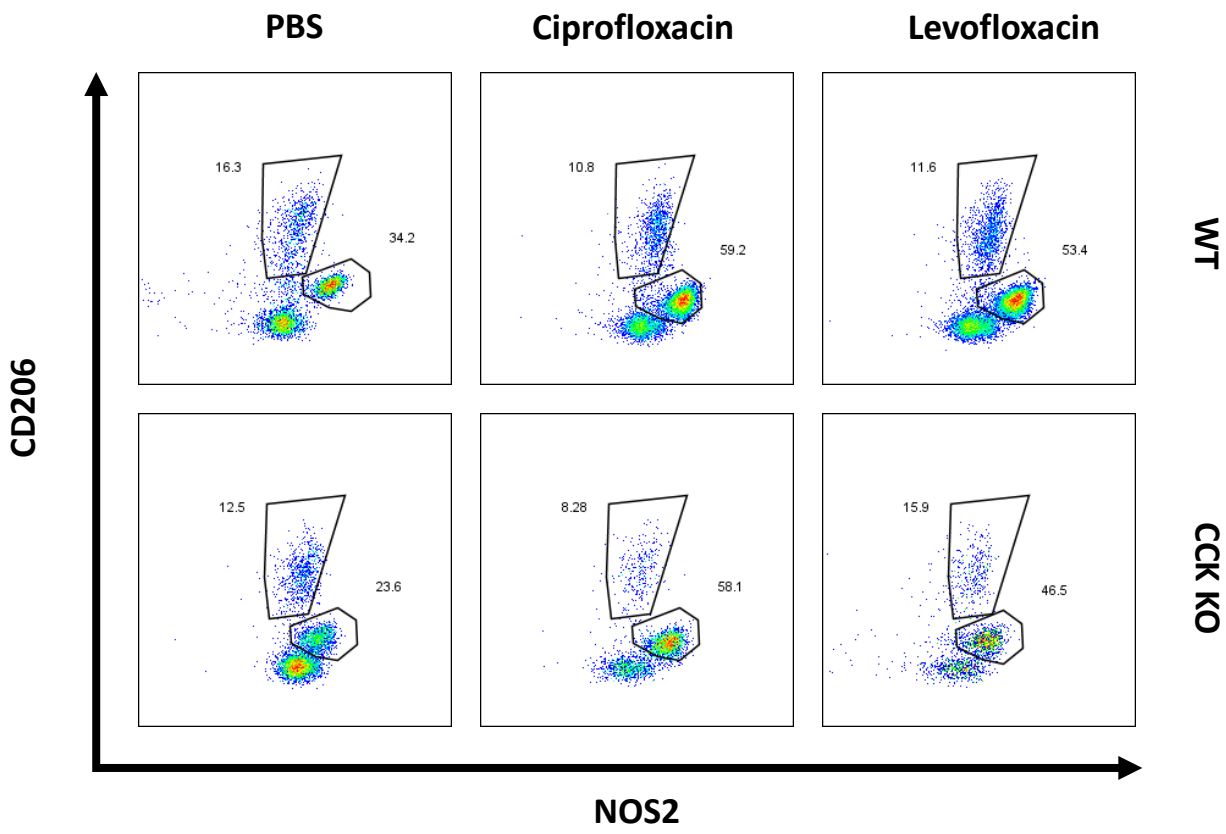


Figure 5.22: Representative flow cytometry plots showing lung M1 polarisation after fluoroquinolone treatment in WT and CCKLacZ mice *C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=7/group). Lungs were harvested at 14 days. Proportion of lung macrophages (CD11b F4/80++) positive for NOS2.*

Analysis of the short-lived and monocyte derived interstitial and monocyte/,macrophage populations(Misharin et al., 2013a) demonstrated a significant increase in M1 NOS2 expression in the ciprofloxacin treated wild-type and CCK KO animals as compared to the PBS vehicle controls at day 14 (Fig.5.21 and Fig. 5.22). Although this pattern was also seen at day 7, this did not reach significance in either group (Fig. 5.21). Interestingly in the levofloxacin treated wild-type and CCK KO groups there was no trend for increased M1 populations at day 7 and at day 14 there was no significance in the increased means seen in both the wild=type and CCK KO treated animals as compared to PBS vehicle treated cohorts (Fig.5.21 and Fig. 5.22).

We next analysed the reciprocal M2 populations and beyond a trend for a reduced average of M2 at day 14 in both wild type and CCK KO ciprofloxacin treated animals; we saw no significant changes in populations at either day 7 or 14 in either the wild-type or CCK KO animals following either fluoroquinolone treatment, as compared to PBS vehicle controls (Fig. 5.23).

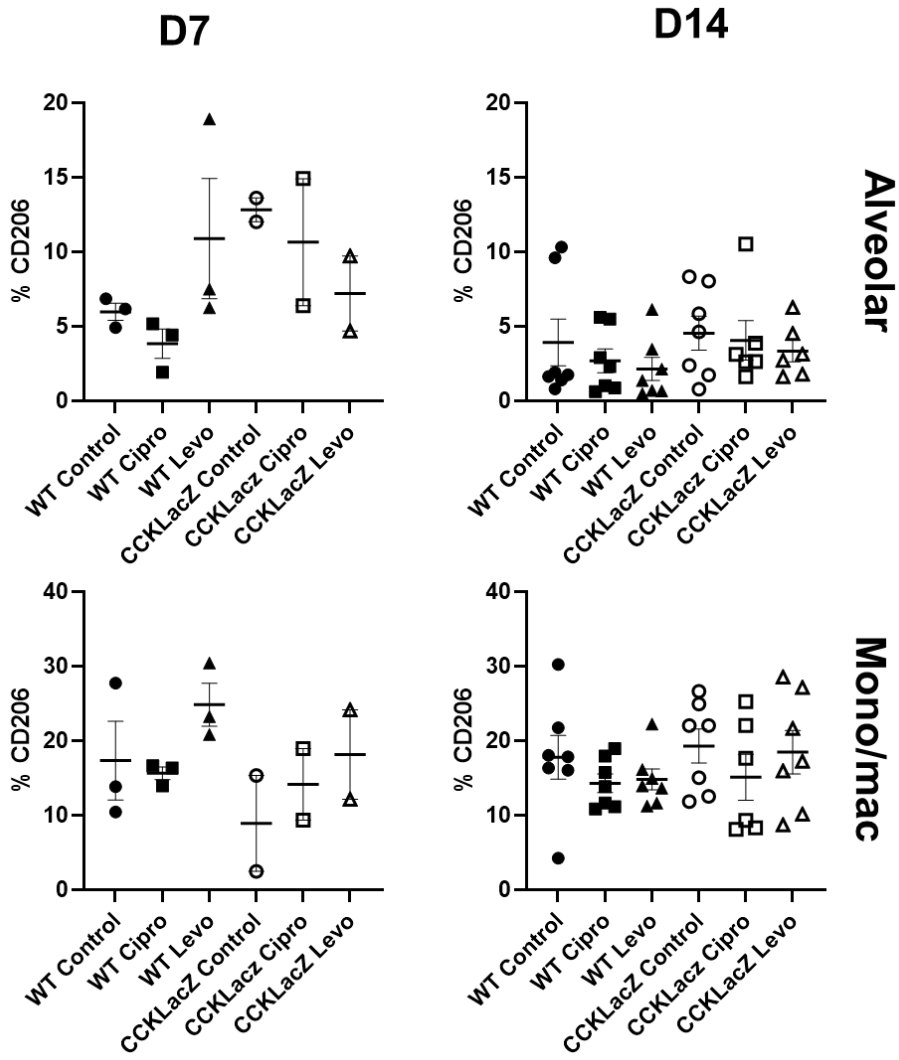


Figure 5.23* M2 *lung macrophage polarisation is not significantly affected by fluoroquinolone treatment in the presence or absence of CCK

*C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=7/group). Lungs were harvested at 7 and 14 days. Percentage of either alveolar macrophages or monocyte/macrophages in the lung expressing CD206 *, P<0.05; **, 0.01; ***, 0.005; ****, 0.0001; Significance tested using one-way ANOVA followed by Sidak's multiple comparisons test.*

Collectively, these data indicate that the lung is a specific site of macrophage modulation following ciprofloxacin treatment, driving a pro-inflammatory M1 phenotype specifically within the short-lived, monocyte derived macrophage population. This polarisation occurs at day 14, but not day 7, indicating a repeated dosage prior to polarisation occurring. Moreover, this is not influenced by CCK and is independent of the weight loss CCK drives during the dosage regime.

5.7 Fluoroquinolone antibiotic treatment does not alter lung goblet cells or alveolar spaces in wild type or CCK KO mice.

To further investigate whether fluoroquinolone treatment or lack of CCK induced weight loss affected overall pathology in the lung, we enumerated Alcian Blue-Periodic Acid Schiff (AB-PAS) stained lung sections of wild-type C57BL/6 and CCK KO mice at day 14 post fluoroquinolone treatment as an indicator of lung barrier integrity.

The lack of CCK did not influence airway goblet cell numbers as compared to wild-type mice (Fig. 5.24), and despite a trend for reduced goblet cell numbers in CCK KO levofloxacin treated animals, no significant alterations were observed following either ciprofloxacin or levofloxacin treatments in wild-type or CCK KO mice as compared to PBS vehicle treated respective cohorts (Fig. 5.24).

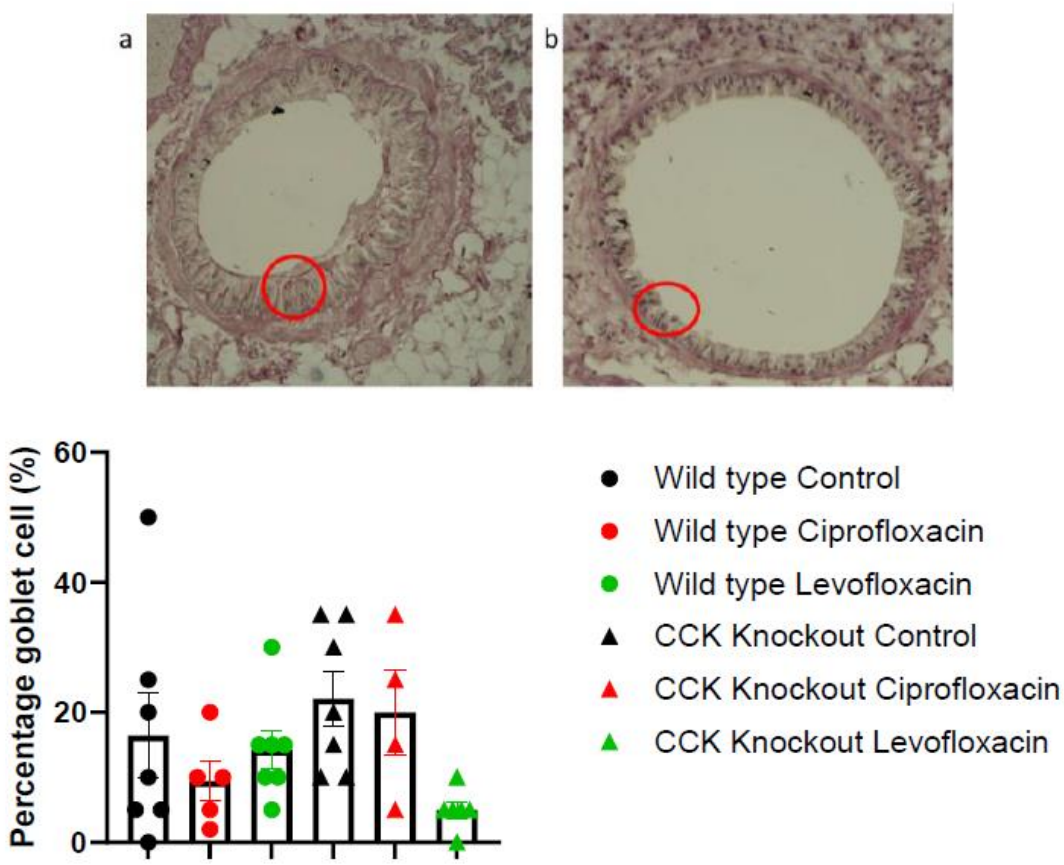


Figure 5.24 Fluoroquinolones do not significantly affect airway goblet cell numbers with or without the presence of CCK *C57BL/6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H_2O mock dose via oral gavage for 14 days (N=9/group, 8/group CCKLacZ Cipro). Lung sections were excised, fixed in Carnoy's fixative, and stained with Alcian Blue-Periodic Acid Schiff (AB-PAS) stain. The average number of goblet cells per airway was calculated. Data was obtained from three independent experiments (n=4/7 mice per treatment group). ***, P<0.005 and **** P<0.0001 from a Sidak's multiple comparisons test following ANOVA.*

Finally, to further assess lung histology we examined interstitial spaces via imageJ analysis. We observed no significant change in the levofloxacin or ciprofloxacin groups as compared to PBS vehicle treated animals and this was not altered in CCK KO mice following the 14 day timecourse of the antibiotic. (Fig.5.25 and 5.26).

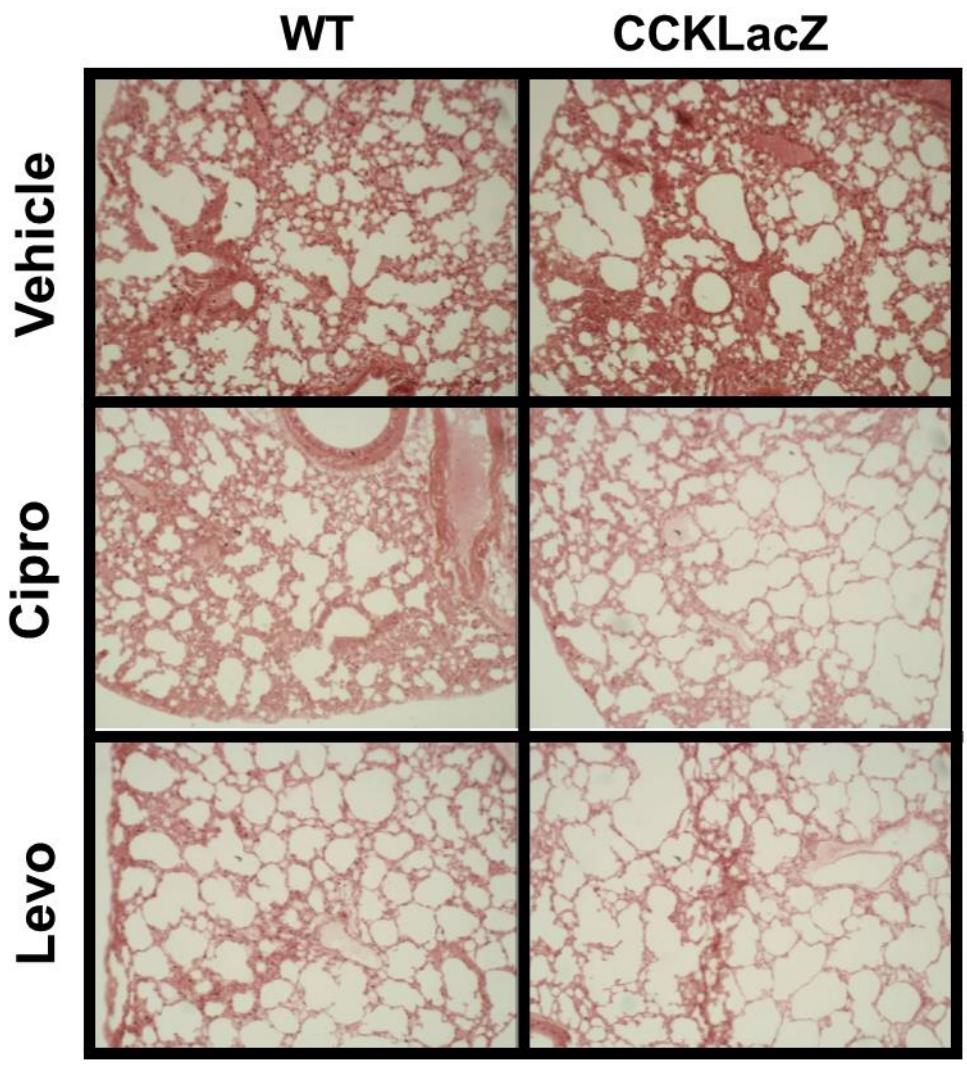


Figure 5.25 Lung surface area representative images *C57BL /6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H2O mock dose via oral gavage for 14 days (N=9/group, 8/group CCKLacZ Cipro). Lung sections were excised, fixed in Carnoy's fixative, and stained with Haematoxylin and Eosin (H&E) stain. Representative pictures of lung airways after the respective treatments, with and without the presence of CCK.*

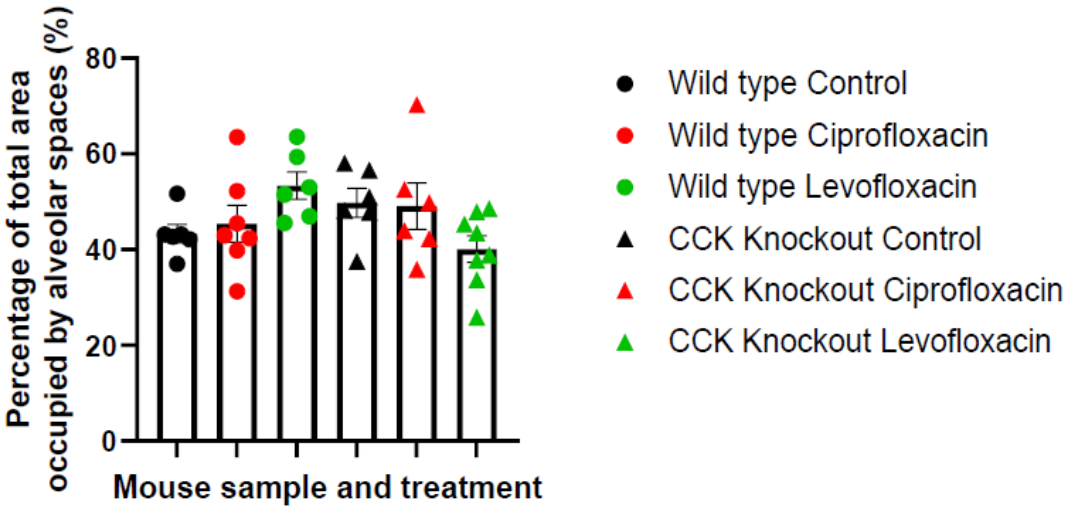


Figure 5.26: Fluoroquinolone treatment does not significantly affect lung airway surface area C57BL /6 and CCKLacZ mice were dosed twice daily with either 100mg/kg Ciprofloxacin, 100mg/kg Levofloxacin, or H2O mock dose via oral gavage for 14 days (N=9/group, 8/group CCKLacZ Cipro). Lung sections were excised, fixed in Carnoy's fixative, and stained with Haematoxylin and Eosin (H&E) stain. Representative pictures of lung airways after the respective treatments, with and without the presence of CCK.

Collectively, these data indicate that both ciprofloxacin and levofloxacin treatment have minimal impact on lung barrier function or alveolar area, and that the absence of CCK, and the accompanying weight loss, also have non-significant roles.

5.8 Discussion

The aim of this chapter was to determine whether the gut peptide hormone cholecystokinin was responsible for fluoroquinolone induced weight loss or influenced the M1 lung polarisation or other immunomodulatory effects of fluoroquinolone treatment.

5.8.1 Absence of Cholecystokinin protects against Fluoroquinolone-mediated weight loss

As the CCK KO mouse has been generated on the C57BL/6 background(Lay et al., 1999a), we first assessed if fluoroquinolones induced weight loss in this strain as seen in our previous results in BALB/c mice. In C57BL/6 antibiotic experiments, mice lost significant amounts of weight when treated with fluoroquinolone antibiotics, although this was not as apparent as the BALB/c strain. As C57BL/6 and BALB/c mice vary with regards to their immune system and microbiome, it is unsurprising that their metabolism also differs between strains. BALB/c mice have been shown to be more resistant to the metabolic effects of a high fat diet (Montgomery et al., 2013). They gain less weight and do not experience symptoms such as insulin resistance. Being less prone to obesity, it could be hypothesised that BALB/c mice are subsequently more susceptible to weight loss. Therefore it would be expected that they would lose more weight when treated with fluoroquinolones than C57BL/6 mice.

CCKLacZ mice completely lacked significant weight loss following ciprofloxacin treatment while WT mice lose significant weight between day 2 and 8. When treated with Levofloxacin, WT mice show significant weight loss from day 5 onwards. In mice lacking CCK, significant weight loss does not occur until day 10. This shows that knocking out CCK has a protective effect on weight, preventing Cipro-mediated weight loss and delaying Levo-mediated weight loss by 5 days. Previous work has demonstrated that CCK is also solely responsible for the weight loss which occurs during infection with the small intestinal helminth *Trichinella spiralis*(Worthington et al., 2013), with EEC hyperplasia occurring via CD4+ T-cells. It would be therefore interesting to examine if T-

cells are mediating weight loss here, although we saw no significant increase in T-cells or overall cytokine production in CD45 subset as seen in *T*. *spiralis* infection (Worthington et al., 2013). Infection or inflammation induced intestinal microbial dysbiosis could potentially provide a common alteration as antibiotic treatment, helminth infections known to alter the microbiome(Houlden et al., 2015).

We have processed samples for sequencing but did not perform DGGE analysis, although similar enlarged caecums were observed in both BALB/c and C57BL/6 mice, so it is likely a similar strong dysbiosis is occurring. Changes will undoubtedly be strain specific. Indeed, the differing immune responses of C57BL/6 and BALB/c mice has been shown to in turn have an effect on their microbiome. Fransen et al. (2015) showed that BALB/c mice produce a greater variety and quantity of IgA antibodies than C57BL/6 mice in both the serum and faeces. This increase in IgA correlated with increased Shannon index microbial diversity in BALB/c mice. It was hypothesised that this increased level of polyreactive IgA led to an increase in the internalisation of IgA-coated bacteria into the peyer's patches. This subsequently causes a positive feedback loop, further increasing IgA levels in BALB/c mice. Interestingly, these polyreactive IgA antibodies were already present in germ free mice, indicating their increased levels are genetic, rather than being initiated by increased microbial diversity Fransen et al. (2015).

Interestingly *T*. *spiralis* infection in C57BL/6 mice decreases the alpha diversity of the intestinal flora in the infected mouse with the genera Oscillospira from the phylum Firmicutes showing a higher abundance in the helminth-infected small and larger intestines. The genera *Bacteroides* from the phyla *Bacteroidota*, the genera Lactobacillus from the phyla *Firmicutes*, the genera Escherichia from the phyla *Proteobacteria*, and the genera *Akkermansia* from the phyla *Verrucomicrobia* displayed increased abundances in the *T*. *spiralis* positive faecal samples compared with those in the negative samples.(Liu et al., 2021). Interestingly, in BALB/c mice infection drove the abundance of the *Lachnospiraceae* NK4A136 group, *Ruminococcus 1* and *Lactococcus* to

decrease. However, the abundance of proinflammatory Parabacteroides increased over time after infection in this strain (Chen et al., 2021). Our planned sequencing of faecal samples from our experiments will allow any microbial dysbiosis parallels to be examined. Interestingly, in a previous study where levofloxacin treated animals induced weight loss, there was also significantly reduced alpha diversity and Firmicutes and *Bacteroides* (phylum Bacteroidetes) were also enriched, (Gu et al., 2020) in parallel to *T*. *spiralis* infection, yet Bacteroidetes were depleted (Gu et al., 2020). The representativeness of *Muribaculaceae*, *Rikenellaceae* decreased following levofloxacin treatment as well as *Lachnospiraceae(Gu et al., 2020)*, again similar to *T*. *spiralis* infection. Collectively, indicating that microbial dysbiosis maybe an exciting mechanism of CCK induced weight loss in both helminth and fluoroquinolone treatment.

5.8.2 Does CCK influence any effects of fluoroquinolone treatment on myeloid cell phenotype?

Different strains of laboratory mice are known to differ from one another in the nature of their immune responses. More specifically, C57BL/6 mice are known to be more aligned with a Th1-like response, whereas BALB/c align more towards a Th2 response. This is characterised largely by distinct cytokine production. C57BL/6 mice produce higher levels of IFNγ and lower levels of IL-4, with the reverse true in the case of BALB/c mice (Mills et al., 2000). It has been shown that immune differences between mouse strains are not limited to T cell responses. Watanabe et al. (2004) showed that strain differences extend to the innate immune response. Macrophages from C57BL/6 mice secreted greater levels of TNF and IL-12 in response to LPS and MALP-2 stimulation than those from BALB/c mice. In addition, BALB/c macrophages did not release NO and lysosomal enzymes in response to the stimulus. They found that there were no differences in neutrophil activity or enzyme secretion with LPS and MALP-2 stimulation. C57BL/6 and BALB/c mice also differ in their abilities to resolve inflammatory pathologies, such as in the lung. One example of this is allergic airway inflammation. After allergen inhalation, BALB/c and C57BL/6 mice both develop a Th2-driven inflammatory response. C57BL/6 mice exhibit greater eosinophilia, whereas BALB/c mice display a significant increase in

airway smooth muscle mass (Van Hove et al., 2009). This increase in airway smooth muscle mass is thought to translate to the pathology of chronic asthma, indicating that as well as BALB/c mice exhibiting a Th2 bias, they are also prone to overreactivity. More recent research into this phenomenon, however, has indicated that it does not occur due to the two strains' differing inflammatory responses. Parkinson et al. (2021) showed that, the differences in airway remodelling are likely due to compositional differences in the airway extracellular matrix between species. These differences were found to be present both during steady state and during an allergic response.

Despite these established differences, we saw no alteration of immune cell populations in either BALB/c or C57BL/6 mice at the level monitored. Though generally less so than in the BALB/c experiments, there was variation between experiments. This may have contributed to the lack of significance. At day 7, data is from one experiment and as such shows no interexperimental variation. No significances are seen here, implying lack of differences are genuine, and not merely a product of variation.

Interestingly, we also saw no alterations in CCK KO mice in the examined tissues. Peptide hormones are known to have multiple immunomodulatory pathways (Worthington, 2015b, Worthington et al., 2018a). At the most basic level CCK plays a role in detecting toxins, with EECs releasing CCK following activation of the T2R38 bitter receptor limiting the absorption of toxic substances through modulation of gut efflux membrane transporters in neighbouring epithelium (Jeon et al., 2011). CCK has also been shown to promote a Th2 and regulatory T-cell (Treg) phenotype *in vitro* (Zhang et al., 2014b). B-cells are also under the control of peptide hormones with CCK driving acetylcholine (Ach) production to recruit neutrophils independently of vagal stimulation (Reardon et al., 2013). Finally, CCK has been shown to inhibit TLR9 stimulation of plasmacytoid DCs via TNF receptor associated factor 6 signalling (Jia et al., 2014a). Despite, these multiple immunomodulatory roles we did not detect any alterations in the CCK KO treated animals as compared to WTs or PBS vehicle controls. However, we did not look into the specific pathways mentioned and mainly focussed on a general myeloid populations, so may have missed more subtle effects. However, pathology at the gut and lung

was comparable between groups. Future studies should focus on more specific pathways as highlighted in these previous publications.

5.8.3 Fluoroquinolone goblet cell alterations

One observed alteration of histology was goblet cell hypoplasia driven by fluoroquinolone treatment. Goblet cells secrete gel forming mucins to produce the mucus layer, an essential physical barrier composed of a dense inner mucus layer firmly attached to the colonic epithelium, and a thinner outer mucus layer allowing growth of interspersed commensal microbiota (Ermund et al., 2013, Johansson et al., 2013). Alcian Blue-Periodic Acid Schiff staining reveals a 14 day course of the fluoroquinolones ciprofloxacin and levofloxacin significantly drove goblet cell hypoplasia, suggesting fluoroquinolone antibiotics compromise the integrity of the mucosal barrier. This is in agreement with studies demonstrating metronidazole pre-treatment reduces the mucus layer and predisposes mice to *Citrobacter rodentium* induced colitis (Wlodarska et al., 2011). Moreover, the standard broad cocktail of antibiotics caused a predisposition to cancer in Apc^{Min+} mice (Kaur et al., 2018). The depletion in colonic goblet cells could be perhaps a result of fluoroquinolone induced goblet cell apoptosis or inhibition of goblet cell production from the basal colonic crypt stem cells. Interestingly, Kaur et al., 2018 suggested a 'microbiome-goblet cell protection' model in which antibiotic induced dysbiosis rather than the direct effect of antibiotics on goblet cells resulted in goblet cell depletion following antibiotic exposure (Kaur et al., 2018). It is important to note that crypt depth was also reduced in animals treated with fluoroquinolones. This is likely a contributing factor to the reduction in goblet cell count, as a smaller crypt will naturally have less goblet cells within it.

Commensal microbiota are known to degrade mucin glycans as a second nutritional source, and therefore the microbiota can directly influence the development and function of goblet cells (Knoop et al., 2015, Schroeder, 2019). However, whether the goblet cell hypoplasia is due to antibiotic induced microbiome dysbiosis or via a direct effect on the epithelium is widely questioned. However, ciprofloxacin treatment has a relatively minor impact on *A. muciniphila* gene expression(Cabral et al., 2020), a key microbial mucin-

degrading bacterium, albeit following a 24hour water based delivery only. In order to fully elucidate the mechanism by which fluoroquinolones alter mucosal barrier integrity, future research should focus on whether our fluoroquinolone treatment alters the composition of the commensal microbiota at a sequencing level interspersed within the outer colonic mucus layer, and whether this is linked to changes in mucus thickness. Although macrolides have been shown to prevent goblet cell hypersecretion and hyperplasia (Tamaoki et al., 1996, Ci et al., 2011, Tanabe et al., 2011) our fluoroquinolone goblet cell hypoplasia findings seem to be gut specific as no changes were observed in the lung following the 14 day time course.

We saw no impact on lung pathology following either fluoroquinolone treatments. Recent studies have demonstrated the presence of CCK receptors in rat lung tissues (Cong et al., 2003) suggesting that CCK may have a role in respiratory processes CCK receptors were positively identified in bronchial epithelial cells, alveolar epithelial cells, pulmonary macrophages, and vascular endothelial cells, there presence inhibiting the effects of endotoxin shock(Cong et al., 2003). My work therefore suggests that CCK plays no protective role in preventing fluoroquinolone-induced pathology.

Returning to the intestine we did observe that levofloxacin treated mice had colonic crypt hypoplasia in CCK KO mice, while there were no significant differences in colonic crypt lengths between wild type ciprofloxacin or levofloxacin treated mice in comparison to vehicle controls. Another study found that mice treated with broad spectrum antibiotics for 2 weeks demonstrated reduced goblet cells but no alteration in colonic architecture macro or microscopically(Aguilera et al., 2015). CCK may therefore have some anti-inflammatory roles within the gut, CCK analogues regulate intestinal tight junction, inflammation, dopaminergic neurons and α-synuclein accumulation in the colon of Parkinson's disease mouse models(Su et al., 2022) and it can protect barrier integrity following LPS induced sepsis (Luyer et al., 2005, Saia et al., 2020). However, there was no significant difference between the CCK KO and wild type equivalent pairings and strong trends did occur in the wild-type treated groups, it may be that further n numbers would perhaps untangle these disparities.

5.8.4 Does CCK influence any effects of fluoroquinolone treatment on Lung myeloid cell phenotype, or histological effects?

As with BALB/c mice, any effects of CCK on lung cells during fluoroquinolone treatment were of particular importance. This was due to severe respiratory infections being one of the most common uses of fluoroquinolones.

Interestingly, there was a significant reduction in Monocytes/Macrophages in Cipro treated CCKLacZ mice vs wild type at day 7. CCK can cause monocytes to produce inflammatory cytokines and eicosanoids (Cunningham et al., 1995), yet the day 7 timepoint was only performed once, and as such n numbers are lower than those at day 14.

Macrophage polarisation of lung cells was also measured. An increase of M1 polarisation with ciprofloxacin treatment has been seen both in BMDMs and in BALB/c mice. This was again seen in the CCK KO mice indicating CCK does not play a role in this antibiotic driven polarisation. Interestingly, CCK can inhibit macrophage activation (Miyamoto et al., 2012, Li et al., 2007, De la Fuente et al., 1995) including inducible nitric oxide synthase production (Saia et al., 2014a). So if anything increased M1 polarisation via our NOS2 readouts would be expected. However, these studies were often seen using macrophage sources from differing *in vitro* methods or other tissues such as peritoneal macrophages.

Ogino et al. (2009a) studied the effects of fluoroquinolones on cytokine production *in-vivo.* Mice were injected with LPS intraperitoneally, and serum cytokine levels measured by ELISA after 3 hours. Interestingly, in these *in-vivo* experiments, fluoroquinolones were also administered intraperitoneally, one hour before LPS injection. This differs from the *in-vivo* methods shown here, as fluoroquinolones were given via oral gavage. The IP injection method here gives peritoneal macrophages more direct contact with fluoroquinolone, but also represents a less clinically relevant scenario. The main limitation of comparing Ogino et al.'s findings with those shown here is that cytokines are produced by different types of macrophage. As is widely agreed, and has been

discussed above, macrophages are an extremely heterogeneous cell type, with diverse functions in many different locations in the body (Murray and Wynn, 2011). As such, it should not be assumed that any effects on peritoneal macrophages by fluoroquinolones would also occur in other tissues niches such as the lung.

5.8.5 Conclusions

Overall these data indicate the fluoroquinolones ciprofloxacin and levofloxacin have limited influence on myeloid cell populations in terms of percentages. In corroboration of our earlier *in vitro* BMDM and BALB/c studies ciprofloxacin drives M1 polarisation but specifically in the lung short lived macrophage population. This may be a direct modulation as demonstrated by our *in vitro* studies in chapter 3 or via the significant dysbiosis caused by these antibiotics. Both fluoroquinolones also drive a distinct goblet cell hypoplasia within the gut but not lung. CCK is key in driving the ciprofloxacin induced weight loss, potentially through specific dysbiosis and as it does not influence M1 polarisation could potentially be utilised as adjunct therapy with ciprofloxacin to maintain weight.

Chapter 6: Summary Discussion

Discussion

6.1 Background

Fluoroquinolones, whilst effective at their intended purpose-treating severe respiratory tract infections, have been linked to a variety of severe side effects. These include tendon rupture, and conditions as severe as aortic aneurysms (Daneman et al., 2015). The mechanism of these effects is not currently certain; whether they are direct effects or occur via dysbiosis. Antibiotics inevitably cause dysbiosis when they are taken- where commensal and non-pathogenic bacteria are killed alongside the target species. Commensal bacteria have such a major effect on human health-particularly in the gut that their absence can lead to a variety of different illnesses (Valdes et al., 2018).

One fluoroquinolone side effect less well studied is modulation of the immune system. It has been shown that antibiotics have the ability to modulate and suppress the effects of the immune system, in different ways. One example was Lankelma et al. (2017), who showed that antibiotic-mediated dysbiosis can harm the innate immune response, particularly the phagocytic ability of macrophages. The most worrying aspect of this was in the case of future infection, where previous doses of antibiotics lead to a decreased survival rate in subsequent infections Lankelma et al. (2017). This could have severe consequences in the real world, where antibiotics could be necessary to treat a severe infection, but would also be a ‘one time’ treatment, decreasing future survivability.

Fluoroquinolones in particular have also been shown to affect innate function directly, not via dysbiosis. Yang et al. (2017) showed how Ciprofloxacin in particular alters host metabolite production. This altered metabolism has a subsequent effect on innate immune function. They also had the effect of increasing the MIC of ciprofloxacin on other bacteria, meaning other infections would be harder to treat.

The aim of this project has been to explore the immunomodulatory effects of fluoroquinolones, and the potential role of gut hormones in driving antibiotic induced weight loss on these effects.

6.2 Levofloxacin increases M1 Macrophage Polarisation *in vitro* and *in vivo*

Preliminary *in-vitro* experiments were carried out in bone marrow-derived macrophages. These provided a sterile-reductionist model in which to study any direct effects of fluoroquinolone treatment on immune function. Macrophages are of particular relevance because, as previously mentioned, they are intrinsic to the lung innate immune response. As such, any impairment of their function would have significant effects on the body's ability to fight future respiratory infections.

One key finding from these experiments was that Levofloxacin, a 3rd generation fluoroquinolone, increases M1 macrophage polarisation. It does not do this at rest however, only in the presence of existing M1 stimuli, in this case IFNγ. This effect can be compared to a 'boost', where the presence of Levo has the ability to amplify an existing response, but not create one itself. This has potential for an interesting effect in a real world scenario, where Levo could aid in the response to separate infections that are not treatable with antibiotics, such as viral infection. A boost of M1 polarisation in an IFNγ high environment could create a somewhat positive feedback loop. It is possible that this could lead to cytokine overproduction, and possibly cytokine storm, though this would require further examination. It should be noted that an increase in M1 polarisation is not solely positive in a bacterial infection. In *M. tuberculosis* infection, bacteria actually promote a Th1 environment. This is because they are able to enter M1 macrophages when they are phagocytosed, and survive for prolonged periods within them (Byrne et al., 2015, Chacon-Salinas et al., 2005). Therefore, care must be taken in this situation, if there is a possibility of *M. tuberculosis* infection.

This IFNγ- dependence is interesting in terms of clinical context. It shows the potential for fluoroquinolones to be of benefit in situations where a response has already been initiated, facilitating additional M1 polarisation to bolster pathogen killing ability. It is worth noting that (although certainly not exclusively), fluoroquinolones are often used as post-exposure prophylaxis; where a person has been exposed to a pathogen, but no infection has been detected. In this scenario, there is unlikely to be an IFN-based response

already initiated, so this ‘boost’ provided by fluoroquinolone treatment would not occur, unless they of course had been infected in the exposure, therefore minimising immunomodulatory effects in unexposed prophylactic recipients.

Bone marrow-derived macrophages were treated with Fluoroquinolones both during their development from monocytes (day 0), and after they had matured (day 7). Polarisation was only affected when antibiotics were present from day 0 of the experiment. In a clinical context, if fluoroquinolone prophylaxis negatively affected macrophages’ ability to protect against pathogens that would be a problem, but less of a problem than if it also affected macrophages that have not yet developed. This is particularly pertinent in the case of post-exposure prophylaxis, when the treatment occurs before pathogens necessarily have time to cause symptoms of infection. It is feasible that the onset of an inhalation Anthrax infection, for example, could follow the timeline of the experiments in chapter 3. Symptoms of Anthrax are known to appear as early as 1 day and as late as 2 months after infection (CDC, 2017). Therefore, if someone were to start a course of prophylaxis on the day of exposure, symptoms may occur 7 days later, with the Fluoroquinolone already having had an effect on developing macrophages.

Fluoroquinolone treatment was shown to also drive M1 polarisation in the lung *in vivo*, but not to affect the maturation of monocytes into macrophages, with no significant difference in the levels of infiltrating monocytes in any tissues examined. Interestingly, *in vivo* at both day 7 and 21, there was no difference in polarisation. This implies the effect both takes time to develop, and also is reversible. In a clinical context, this would indicate that in order to provide sustained immune modulation in the lung, treatment must also be sustained. Whilst increased M1 lung macrophage polarisation will be beneficial in fighting a respiratory infection, an increased proinflammatory phenotype would not be beneficial after an infection has cleared. Therefore the fact that polarisation had returned to normal levels a week after cessation of treatment is ideal. It would be interesting to examine the permanence of this polarisation in the context of prolonged treatment. In a high risk environment it is quite possible that fluoroquinolone prophylaxis would occur over a longer period than a standard two week course. Future work should be carried out to determine if extended

fluoroquinolone doses have a more permanent effect on lung macrophages and influence M2 repair mediated aspect post-infection.

The cells unaffected by Fluoroquinolone treatment at the start of the course (their ‘day 7’ equivalent) would likely not be as vital at the height of a person’s symptoms, rather the cells rapidly developing in response to the emerging infection. That’s not to say that macrophages would not last the course of treatment however. Whilst circulating monocytes are short-lived, undergoing apoptosis after as little as 4 hours (Fahy et al., 1999) macrophages, particularly those residing in tissues, have a much longer lifespan (Parihar et al., 2010). Amazingly, macrophages have been found to survive for months and even years. A seminal study by van Furth and Cohn (1968) found that macrophages were a ‘final’ stage of cell development, and remained radiolabelled for as long as 8 weeks. Because of the self-sufficient nature of tissue resident macrophages as mentioned in Chapter 1, they can survive for long periods in their own tissue niche free from ‘interference’ from circulating cells (Hashimoto et al., 2013). Alveolar macrophages in particular, have been shown to have a lifespan that is comparable to that of the organism as a whole. Murphy et al. (2008) used irradiated chimeric mice to study the half-life of their alveolar macrophages, and found cells persisting after 33 weeks, and were not proliferating locally.

Our *in vivo* data demonstrated no alteration of resident alveolar macrophage polarisation and only on the interstitial short-lived population. It was notable that the increased M1 polarisation in lung macrophages corroborated with the effects seen in bone marrow macrophages. They responded the same despite being distinct types of macrophage, derived from different erythromyeloid progenitors (Epelman et al., 2014). Whilst macrophages in several tissues were examined here with lung macrophages the only showing increased M1 polarisation, future work should broaden this scope in order to ensure that the effects seen are in fact localized to the lung as it appears. It may be antibiotic specific as work on a broad acting antibody cocktail demonstrated increased M1 polarisation again in the short-lived macrophage population but this time

within the gut (Scott et al., 2022). This was also accompanied by significantly elevated mitochondrial potential in this macrophage population (Scott et al., 2022), mirroring our *in vitro* findings of altered mitochondrial metabolism.

Previous studies have shown that fluoroquinolone side effects have been reduced in patients prescribed a mitochondrial antioxidant (Lowes et al., 2009). In addition, several antibiotics have been potentially linked to the induction of psychiatric disorders via mitochondrial dysfunction (Stefano et al., 2017). As a result, BMDMs were stained with MitoTrackers in order to assess mitochondrial function *in-vitro.* Unexpectedly, after fluoroquinolone treatment, BMDMs were found to display mitochondrial hyperpolarisation- a condition where the membrane potential of the mitochondria is greater than its usual maximum. This is part of the apoptotic pathway, but is a disorder when occurring in excess. It has been previously seen in the T cells of SLE patients (Gergely et al., 2002). This fits the hypothesis, that fluoroquinolones disrupt the metabolism of macrophages, subsequently altering their polarisation and phagocytic ability.

Whilst these findings show evidence of mitochondrial dysfunction and hyperpolarisation, technical limitations meant that the conclusions made were also limited. A common method by which to determine and quantify mitochondrial function is a Seahorse analyser (Agilent). This uses probes within cell culture media to analyse the oxidative phosphorylation and glycolytic activity of cells. Thus, any reduction in function caused by fluoroquinolone treatment, in either mechanism could be quantified. A similar method could have been used, using flow cytometry to replicate the function of a Seahorse analyser, called SCENITH (Argüello et al., 2020). The required materials for this technique were sought, but administrative delays meant this could not be used in time.

After finding that Levo increased M1 polarisation in BMDMs, a key question was- does this have any functional effect? The most common way studies have assessed drugs' impact on macrophage function was by measuring their phagocytic ability. As such, phagocytosis assays were performed on cells

treated with fluoroquinolones. In contrast with findings of other studies (Azuma et al., 2001, Tawfik et al., 1990), we found that at rest and with IL-4 stimulation, Levofloxacin significantly increases the rate of phagocytosis compared to control. As with the polarisation findings, Levo (the newer drug) had a stronger effect than Cipro. Phagocytosis is an essential component of macrophage function, and generally regarded as their defining characteristic. As mentioned previously, their name even derives from their ability to ‘eat’ pathogens. The primary purpose of phagocytosis is widely accepted to be the internalisation and destruction of pathogenic and otherwise harmful objects. Both M1 proinflammatory and M2 anti-inflammatory macrophages use phagocytosis to carry out their respective functions.

As mentioned previously, one key use of fluoroquinolones is as post-exposure prophylaxis in a potential Bioterror incident. It is therefore reassuring that when this pre-treatment is replicated, there is a positive effect on pathogen clearance. Whilst our results do not match that per se, they are reassuring. They show that whilst phagocytosis is boosted in an M0 and M2 environment, it is not impaired under any conditions. This means that pre-existing macrophage polarisation would not have a negative effect on any advantages given by pre-treatment in a prophylactic scenario.

However, one potential negative effect would be a reduced intestinal goblet cell number. In C57BL/6 and CCKLacZ mice, fluoroquinolone treatment significantly reduced colonic goblet cell count. This would subsequently lead to reduced intestinal mucus, which depending on how permanent it is, could increase susceptibility to infection (Schroeder, 2019).

Importantly, this effect was not present in the lung, where goblet cell numbers were unaffected by fluoroquinolone treatment. This is of particular relevance due to the use case of fluoroquinolones- respiratory infections. If mucus in the lung was depleted by fluoroquinolone treatment, then that would make the lung more vulnerable to infection, partially negating the positive antibacterial effects of the treatment. It is unsurprising that goblet cell numbers were only affected in the colon and not the lung, due to the colon being the site where fluoroquinolones are in direct contact with the epithelium. It could be argued

that the boost in lung macrophage M1 polarisation is a justifiable trade off with the lower colon goblet cell count, due to the previously mentioned relevance of the lungs to fluoroquinolone treatment.

6.2 Ciprofloxacin treatment produces CCK dependent significant weight loss *in vivo*

After observing the macrophage immunomodulatory effects of fluoroquinolones *in-vitro* and *in-vivo*, it was important to then investigate any organism-wide effects. Both BALB/c and C57BL/6 mice underwent weight loss, albeit distinct, with both ciprofloxacin and levofloxacin. While the weight loss caused by fluoroquinolone treatment was significant, the mechanism for this was unknown. One hypothesis for this mechanism was that gut hormones- specifically cholecystokinin were involved. Cholecystokinin is released by enteroendocrine cells in the small intestine, and amongst other effects, acts as an appetite suppressant during inflammation (Worthington et al., 2018a, Worthington et al., 2013).

To test this hypothesis, CCKLacZ (KO) mice were treated with a two-week course of fluoroquinolones, as previously described. Interestingly, knocking out CCK was found to have a protective effect, preventing any significant weight loss in Cipro treated animals, and delaying significant weight loss in Levo-treated animals from day 5 (WT) to day 10. Whilst CCK has numerous effects across the body, its most notable is to signal satiety- i.e. a feeling of being ‘full’ (Fink et al., 1998). CCKLacZ mice do not secrete this hormone, and subsequently do not experience this CCK-derived satiety (though it may still occur via other peptide hormone sources). In these experiments, this provided a protective effect from the weight loss caused by fluoroquinolone treatment, showing that the weight loss is CCK-mediated. This leads to the hypothesis that fluoroquinolone treatment causes an upregulation of CCK secretion, subsequently reducing feelings of hunger and reducing feeding, causing weight loss. This appears to be fluoroquinolone-specific, as a lack of CCK had no protective effect on mice treated with Doxycycline Hyclate, albeit preliminary study (Appendix figure). However, there is still a disconnect between the antibiotic-mediated weight loss shown here and the majority of the literature,

which focuses on how antibiotic use and dysbiosis can lead to metabolic dysregulation and obesity (Cho et al., 2012), of note this includes specific reference to doxycycline (Angelakis et al., 2014).

This result uncovers the possibility of targeting CCK in order to mitigate negative effects of Fluoroquinolone treatment. A key potential scenario for this would be prophylaxis, where treatment may be prolonged, with the possibility that an infection never actually occurs. In this situation, the benefits of fluoroquinolone treatment are less likely to outweigh the potential side effects. Importantly, the M1 lung polarisation was still seen in CCK KO mice indicating that targeting CCK as an adjunct therapy would prevent weight loss while still allowing the beneficial M1 lung polarisation and phagocytosis.

The most likely way for this to occur is to treat patients with a CCK receptor antagonist alongside a course of Fluoroquinolones. This would counteract any effects that are mediated by a potential upregulation in CCK signalling caused by fluoroquinolones. The effects of this are unlikely to be as protective as knocking out CCK, but do allow for a potential treatment method. An advantage of treating with an antagonist rather than using genetic methods is that, regardless of feasibility, it is reversible. This means that any further off target effects of losing CCK (e.g. excessive feeding) would not last beyond the treatment. In addition, a challenge model should also be used, in order to determine both if fluoroquinolone mediated weight loss still occurs, and if a CCK antagonist would be still beneficial, or could even have unforeseen negative effects. Though an upregulation of CCK signalling is our hypothesis, this has not been able to be tested as of yet. To show this, CCK can be stained for in the small intestine using immunohistochemistry. An increase in staining would show CCK hyperplasia while CCK secretion could also be performed using qPCR or organoid stimulation followed by ELISA.

Notably, the effects on lung macrophage polarisation *in vivo* were reversed after cessation of treatment, whereas the weight loss was maintained. This implies, unsurprisingly, that the weight loss was not directly linked to the lung macrophage polarisation, but rather was another symptom of a more widespread systematic effect. This ties in with Fluoroquinolone-associated

disability (FQAD), where off target effects and harmful symptoms last for a far more prolonged period than the course of treatment itself. It would be interesting to track weight loss over a longer period after cessation of treatment. In documented cases of FQAD, symptoms and off-target effects of fluoroquinolones can last for months and even years (Marchant, 2018). Therefore, it would be pertinent to examine off-target effects in a rodent model for a similarly extended period.

The mechanism altering CCK is so far unknown, although, as discussed in chapter 5, similar dysbiosis occurs in *Trichinella spiralis* infection (Worthington et al., 2018a, Worthington et al., 2013), which also has CCK driven weight loss, potentially linking fluoroquinolone specific dysbiosis to CCK alterations. When BALB/c mice were treated with Cipro and Levo, it was expected that that two weeks of fluoroquinolone treatment would cause some level of dysbiosis. It was not expected though, how suddenly this would occur. After only 24h of treatment, both Cipro and Levo caused significant dysbiosis when compared to the same animal pre-treatment. Dysbiosis was greater in mice treated with Levo, with little regrowth. In Cipro-treated animals, after the initial 'hit' of dysbiosis, several species of bacteria persisted, and opportunistically became more prevalent than before the start of treatment.

The next step here would be to determine the species of bacteria that are able to persist after the Cipro treatment. The bands were excised from the gel at the time, and so could be sequenced. The lack of other bacteria after Cipro treatment gives a selective advantage to those remaining. If the remaining bacteria were harmful or potentially harmful, this could be a mechanism behind some of the symptoms of FQAD. It is well known that dysbiosis and loss of commensal bacteria has a negative effect on a wide range of health aspects. This could be occurring here, but in addition, the presence of an opportunistic pathogen may also contribute.

6.4 Conclusions

The aims of this project were to investigate off target effects of fluoroquinolone treatment, and the immunological basis for this. These off target effects are both common and potentially severe, even being classified as a syndrome-fluoroquinolone-acquired disability (FQAD). This is of particular importance due to fluoroquinolones' widespread use as post exposure prophylaxis in bioterror incidents or biological warfare.

In BMDMs, Levofloxacin was found to significantly increase M1 macrophage polarisation in the presence of existing IFNγ stimulation. IFNγ causes M1 polarisation in itself, and therefore the additional effect of Levofloxacin treatment can be thought of as a 'boost' to an existing M1 environment. This means that in addition to its intended direct antibiotic effect, Levofloxacin can also fight infections by increasing macrophages proinflammatory function in an existing type-1 immune environment. Interestingly, this would not only increase the immune system's ability to fight bacterial infections, but also potentially viral infections too.

Interestingly, *in vitro* and *in vivo* these effects on polarisation were only seen when fluoroquinolones were present with developing macrophages'. When added to the media of mature macrophages or examined *in vivo* resident lung macrophages there was no effect seen. This further exemplifies the advantages of post-exposure prophylaxis; having fluoroquinolones already in the system at the point of infection.

The underlying mechanism for these effects was unknown, though it was hypothesised that it was potentially mediated by altered mitochondrial function and we did observe mitochondrial hyperpolarisation accompanied by increased phagocytosis another beneficial side effect.

A negative side effect was that mice treated with Ciprofloxacin had a CCK dependent weight loss. This reveals the potential for the use of cholecystokinin receptor antagonists alongside a course of fluoroquinolones to provide relief against the symptoms of FQAD. This is of particular importance in cases of

post-exposure prophylaxis, where the host environment of the patient more closely resembles the naïve environments tested in this thesis.

Chapter 7: Appendices

7.1 Supplementary Figures

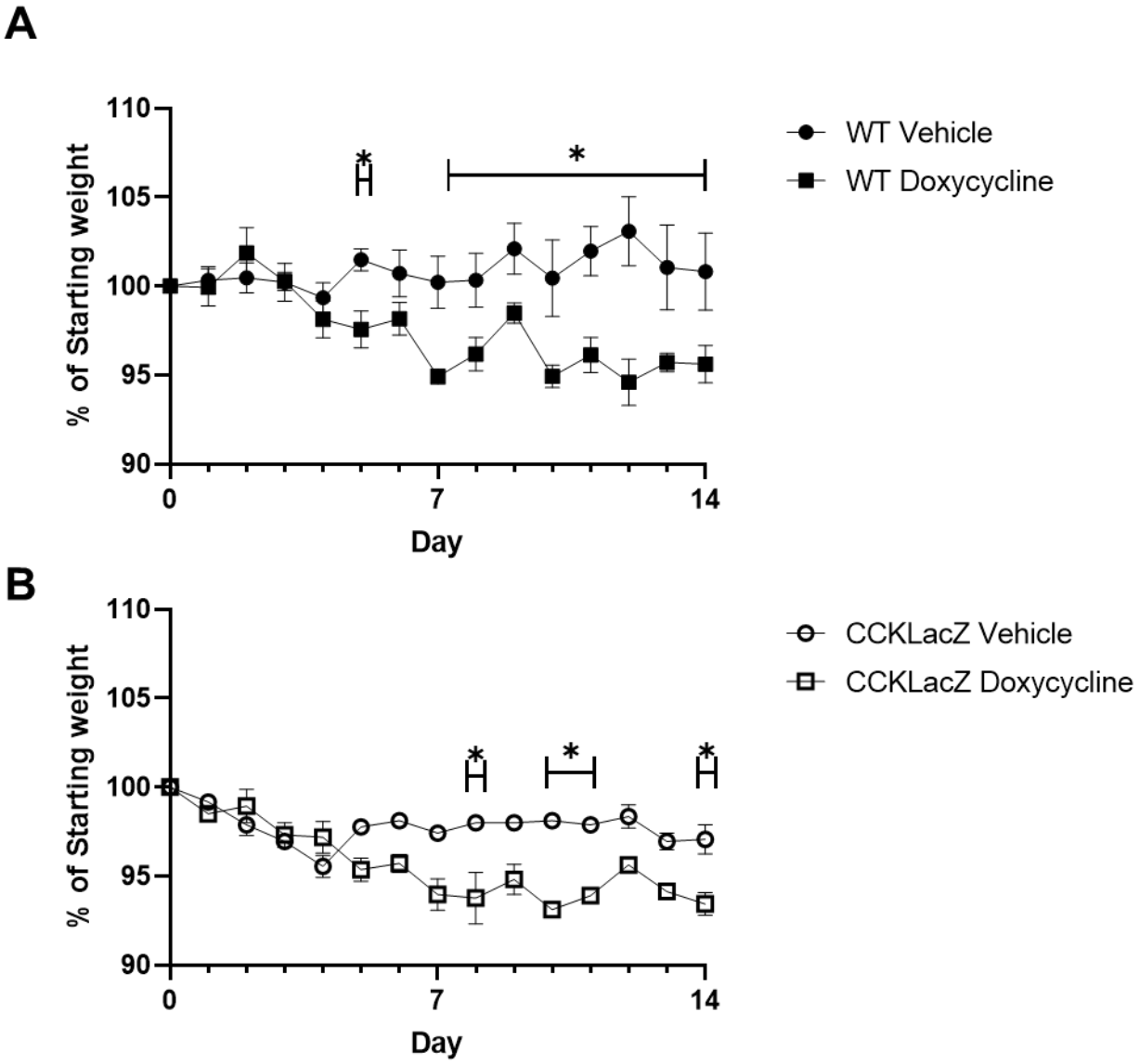


Figure 7.1: Weight loss in C57BL/6 and CCKLacZ mice after Doxycycline treatment *C57BL/6 and CCKLacZ mice were dosed daily with either 1mg Doxycycline or PBS mock dose via oral gavage (N=3/group). A: Percentage daily weight change (vs D0 weight) of fluoroquinolone or vehicle treated C57BL/6 mice over 14 days of treatment (N=9/group). B: Percentage weight change of (vs D0 weight) of fluoroquinolone or vehicle treated CCKLacZ mice over 14 days of treatment (N=3/group). *, P<0.05; **, 0.01; ***, 0.005; ****, 0.0001; N.S. Significance between groups was tested using Sidak's multiple comparisons test following two-way ANOVA.*

7.2 Media

Full BMDM Medium:

DMEM w/ Glutamax (Gibco), 10% FCS (Sigma), 20ng/ml Macrophage Colony Stimulating Factor (MCSF) (Peprotech).

Lung Digest Medium:

PBS (Sigma), 0.1mg/ml Liberase TM (Roche), 50µg/ml DNAse I (Roche)

Gut Digest Medium:

RPMI (Sigma), 10% FCS (Gibco), 0.5mg/ml Dispase(Gibco), 0.5mg/ml Collegenase (Gibco).

Full Stimulation Medium:

RPMI (Sigma), 10%FCS (Gibco), 1% Pen/Strep (Sigma), 1% L-glutamine (Sigma),, 1% Non-Essential Amino acids (Sigma),, 1% Hepes (Sigma),, 50µM β-mercaptoethanol (Sigma),.

Carnoy's Solution:

60% Absolute Ethanol, 30% Chloroform, 10% Glacial Acetic Acid (All Fisher)

7.3 Antibody Stain Panels

BMDM Activation

Antibody	Fluorophore	Concentration	Manufacturer
CD45	e506	1.5µg/ml	eBioscience
CD11b	APC	1.5µg/ml	eBioscience
F4/80	PE/Dazzle 594	1.5µg/ml	Biolegend
MHCII	AF700	1.5µg/ml	Biolegend
CD40	FITC	1.5µg/ml	Biolegend
CD80	Brilliant Violet 605	5µl/million cells	Biolegend
CD86	PE/Cy7	1.5µg/ml	Biolegend
7AAD Viability Dye	PercP/Cy5.5	5%	eBioscience

BMDM Polarisation

Antibody	Fluorophore	Concentration	Manufacturer
CD45	FITC	1.5µg/ml	eBioscience
CD64	PercP/Cy5.5	1.5µg/ml	Biolegend
CD11b	APC	1.5µg/ml	eBioscience
F4/80	PE/Dazzle 594	1.5µg/ml	Biolegend
CD206	Brilliant Violet 711	1.5µg/ml	Biolegend
iNOS	APC e780	3µg/ml	eBioscience
Zombie Aqua	KO525	1/500	Biolegend

BMDM MitoTracker

Antibody	Fluorophore	Concentration	Manufacturer
CD45	e506	1.5µg/ml	eBioscience
CD64	PercP/Cy5.5	1.5µg/ml	Biolegend
CD11b	APC	1.5µg/ml	eBioscience
F4/80	PE/Dazzle 594	1.5µg/ml	Biolegend
MitoTracker Green FM	FITC	100nM	Invitrogen
MitoTracker Orange CMTMRos	PE	250nM	Invitrogen

Mitotracker info: MitoTracker Orange CMTMRos mw: 427.37, working conc: 250nM; Mitotracker Green FM mw: 671.88, working conc: 100Nm; Stock concentration:1mM (10000X Green, 4000X Orange).

***In vivo* Surface Stain**

Antibody	Fluorophore	Concentration	Manufacturer
CD45	e506	1.5µg/ml	eBioscience
CD64	PercP	1.5µg/ml	Biolegend
CD11b	APC	1.5µg/ml	eBioscience
CD11c	PE/Cy7	1.5µg/ml	Biolegend
Ly6g	FITC	1.5µg/ml	eBioscience
Ly6c	E450	1.5µg/ml	eBioscience
CD103	PE	1.5µg/ml	Biolegend
MHCII	AF700	1.5µg/ml	BioLegend

***In vivo* Intracellular Stain**

Antibody	Fluorophore	Concentration	Manufacturer
CD45	e506	1.5µg/ml	eBioscience
CD11b	APC	1.5µg/ml	eBioscience
F4/80	PE/Dazzle 594	1.5µg/ml	Biolegend
CD64	PercP	1.5µg/ml	Biolegend
CD11c	PE/Cy7	1.5µg/ml	Biolegend
TNFα	E450	3µg/ml	eBioscience
IFNγ	FITC	3µg/ml	Biolegend

7.3 Bacterial 16S rRNA PCR primers

16S rRNA gene	341F_GC	5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG -3'
	518R	5'-ATTACC GCG GCT GCTGG-3'

Abbreviations

AM: Alveolar Macrophage

ATP: Adenosine Triphosphate

BAL: Bronchoalveolar lavage

BMDM: Bone Marrow-derived Macrophage

CBA: Cytometric Bead Array

CCK: Cholecystokinin

CCU: Colonic Crypt Unit

CDC: Center for Disease Control

Cipro: Ciprofloxacin

DC: Dendritic Cell

DGGE: Denaturing Gradient Gel Electrophoresis

Doxy: Doxycycline

EEC: Enteroendocrine Cell

ELISA: Enzyme-Linked Immunosorbent Assay

FDA: Food and Drug Administration

FMO: Fluorescence Minus One

FQAD: Fluoroquinolone-Associated Disability

GALT: Gut-Associated Lymphoid tissue

GF: Germ Free

GI: Gastrointestinal

GLP-1: Glucagon-like peptide 1

GM-CSF: Granulocyte Macrophage colony stimulating factor

HFD: High Fat Diet

IFNγ: Interferon Gamma

IM: Interstitial Macrophage

iNOS: Inducible Nitric Oxide Synthase

KO: Knockout

LDH: Lactate Dehydrogenase

Levo: Levofloxacin

LI: Large Intestine

LP: Lamina Propria

LPS: Lipopolysaccharide

M-CSF: Macrophage colony stimulating factor

MHC: Major Histocompatibility Complex

MIC: Minimum Inhibitory Concentration

mLN: Mesenteric Lymph Node

NF-κB: Nuclear factor kappa B

NK: Natural Killer

NO: Nitric Oxide

NOS2: Nitric Oxide Synthase 2

PAMP: Pattern-associated molecular pattern

PAS: Period Acid-Schiff's

PBS: Phosphate Buffered Saline

PCD: Primary Ciliary Dyskinesia

PEP: Post-exposure prophylaxis

PRR: Pathogen recognition receptor

SCFA: Short Chain Fatty Acid

SLE: Systemic Lupus Erythematosus

TGFβ: Transforming Growth Factor Beta

TLR: Toll-like receptor

TNFα: Tumour Necrosis Factor Alpha

Treg: Regulatory T cell

WHO: World Health Organisation

WT: Wild type

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