The influence of the gut microbiota on influenza vaccine-induced immunity

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Abstract

Currently, annual vaccination is widely considered the most effective method for preventing and controlling influenza virus infection. However, many individuals mount suboptimal immune responses to vaccination and the factors leading to poor immune responses are yet to be elucidated. Interestingly, it has been proposed that microorganisms that inhabit the intestinal tract, the gut microbiota, can profoundly influence many facets of the host immune system, including the strength of the immune response to influenza vaccination.

In line with these observations, we observed that short-term administration of antibiotics drastically reduced influenza vaccine-specific antibody production. In particular, antibiotic treatment diminished the frequency and activation status of multiple myeloid cell subsets in the draining lymph nodes at steady-state and following vaccination, with associated impairments in B and T_{FH} cell responses.

Composition and function of gut microbiota communities can be rapidly altered through dietary changes. Therefore, the impact of potential prebiotic and probiotic nutritional interventions on the immune response to influenza vaccination and subsequent infection was assessed. No improvement in antibody responses to influenza vaccination was observed following the nutritional interventions studies. However, oral administration of a propolis formulation led to some improvement in viral control following infection.

Collectively, this investigation indicates that alterations in microbial-associated signals leads to severe impairments in cellular responses crucial to humoral immunity and subsequent vaccine-induced antibody production. Furthermore, by altering the gut microbiota through dietary interventions, there is potential to improve immune responses to vaccination.

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Disclosure Statement

Dr. Hazel Poyntz performed and supplied the data for the long-term broad-spectrum antibiotic experiment.

With this disclosure, I declare that the content of this thesis is my own work.

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Abbreviations

ABX	Antibiotics
ADCC	Antibody-dependent cell-mediated cytotoxicity
Alum	Aluminum salts
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
BCA	Bicinchoninic acid
BCR	B cell receptor
BM	Bone marrow
BRU	Biomedical Research Unit
C	Constant
cDNA	Complementary DNA
CFU	Colony forming units
CLR	C-type lectin receptor
CS	Class-switched or class-switching
CSR	Class-switch recombination
CTL	Cytotoxic T lymphocyte
DAMP	Danger-associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
dLN	Draining lymph node
DMEM	Dulbecco's Modified Eagle Medium
DZ	Dark zone
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
FDC	Follicular dendritic cell
GC	Germinal centre
GF	Germ-free

GI	Gastrointestinal
GPCR	G-protein coupled receptor
H	Heavy
H.P.V.	Hours post-vaccination
HA	Haemagglutinin
HAU	Haemagglutination unit
HDAC	Histone deacetylase
HEV	High endothelial venule
HIV	Human immunodeficiency syndrome
IFN	Interferon
Ig	Immunoglobulin
IG	Intragastric or intragastrically
ILC	Innate lymphoid cell
IMDM	Iscoe's Modified Dulbecco's Medium
IN	Intranasal or intranasally
L	Light
LAIV	Live attenuated influenza vaccine
LN	Lymph node
LNresDC	Lymph node-resident DC
LPS	Lipopolysaccharide
LZ	Light zone
MAC	Microbiota-accessible carbohydrates
MHC	Major histocompatibility complex
MigDC	Migratory DC
MIMR	Malaghan Institute of Medical Research
mRNA	Messenger ribonucleic acid
MW	Molecular-weight
M ϕ	Macrophage or macrophages
NA	Neuraminidase

NK	Natural killer
NLR	Nucleotide-binding oligomerisation domain-like receptors
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PC	Plasma cell
PD-1	Programmed death receptor 1
PRR	Pattern recognition receptor
RLR	RIG-I-like receptors
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
SC	Subcutaneous or subcutaneously
SCS	Subcapsular sinus
SCFA	Short-chain fatty acid
SCID	Severe combined immunodeficiency
SFB	Segmented filamentous bacteria
SHM	Somatic hypermutation
sIgA	Secretory IgA
SPF	Specific-pathogen free
TCR	T cell receptor
T _{FH}	T follicular helper
T _{FR}	T follicular regulatory
T _H	T helper
T _H 1	T helper 1
T _H 2	T helper 1
T _H 17	T helper 17
TIV	Trivalent influenza vaccine
TLR	Toll-like receptor

T _{REG}	Regulatory T
V	Variable
VNAM	Vancomycin, neomycin, ampicillin and metronidazole

1 General introduction

1.1 The humoral immune response

1.1.1 Overview of the innate and adaptive immune systems

The immune system is a dynamic and diverse collection of cells and molecules, enabling protection against a wide variety of foreign insults. It consists of two arms, the innate and the adaptive immune systems, which sense the environment through receptors on, or inside, cells.

The innate immune response is rapid and a sufficient response can detect and eliminate pathogens in minutes to hours¹. The innate immune system primarily consists of an array of myeloid cells and innate lymphoid cells (ILCs), which provide initial discrimination between pathogens and innocuous stimuli. Innate immune cells possess pattern-recognition receptors (PRRs), which encompass a limited number of germ-line encoded receptors including toll-like receptors (TLRs), nucleotide-binding oligomerisation domain-like receptors (NLRs) RIG-I-like receptors (RLRs) and C-type lectin receptors (CLRs)². These are able to recognise pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively). PAMPs are molecular motifs that are conserved between a wide variety of pathogens³, while DAMPs are endogenous molecules released from host cells following stress and injury⁴. Binding of PRRs by PAMPs or DAMPs alerts innate immune cells to infectious threats and associated tissue damage, giving rise to effector responses. These rapid, antigen-independent responses include the engulfing and killing of pathogens, production of toxic molecules to directly kill or damage pathogens and secretion of molecules to recruit other innate immune cells to the site of infection¹.

The adaptive immune system mainly consists of lymphocytes that can be broadly divided into two groups: T and B cells. Both possess unique antigen receptors that are generated through rearrangement of their germline receptor

sequences, giving rise to antigen receptors with random specificity, enabling recognition of an immense diversity of pathogen-derived products⁵. Adaptive immune cells exert highly specific responses against invading pathogens. T cells orchestrate protective immune responses and directly kill infected cells, while B cells produce antibodies to neutralize and eliminate pathogens from the body. Unlike innate immune cells, after resolution of the infection, cells of the adaptive immune system can contribute to antigen-specific immunological memory. A small proportion of T and B cells can acquire a 'memory' phenotype where upon subsequent exposure to a pathogen, they mount a more rapid and powerful response than the primary response^{6,7}.

Conventional T cells can be categorised by effector function and expression of either CD4 or CD8 molecules. Each T cell has a unique T cell receptor (TCR) that recognises and binds to antigen presented on major histocompatibility (MHC) molecules by antigen presenting cells (APCs). CD8⁺ T cells recognise antigen that is presented on MHC class I and differentiate into cytotoxic T lymphocytes (CTLs), specialising in direct killing of infected cells through secretion of toxic molecules such as granzyme and perforin⁸. In contrast, CD4⁺ T cells recognise antigen presented on MHC class II and differentiate into a number of distinct T helper (T_H) cell subtypes, defined by their distinct cytokine profiles and effector functions. The major subsets are T_H1, T_H2, T_H17, regulatory T (T_{REG}) and T follicular helper (T_{FH}) cells. T_H1 cells are key effector cells in intracellular infection and produce IFN- γ and IL-2, activating macrophages and inducing effector function of CTLs⁹. T_H2 cells produce IL-4, IL-5 and IL-13 and orchestrate protection against multicellular pathogens, such as helminths, by recruiting and activating mast cells, basophils, eosinophils and directing the production of appropriate antibodies¹⁰. IL-17 producing T_H17 cells play a key role in control of bacterial and fungal infections by recruiting and activating neutrophils¹¹. T_{REG} cells produce IL-10 and TGF- β and are important in resolving inflammation and

preventing inappropriate immune responses¹². T_{FH} cells produce IL-6 and IL-21 and provide key signals to B cells for effective antibody responses¹³.

Innate immune cells play a critical role in the initiation and regulation of adaptive immune responses. T cells are unable to effectively respond to free antigen. Instead, antigen must be presented in the context of MHC with multiple secondary co-stimulatory signals from the APC to prime the T cell. The most effective APCs are dendritic cells (DCs), but macrophages (M ϕ) and B cells may also present antigen. DCs have an immature phenotype in the periphery. Upon recognition of pathogens via PRRs, DCs take up antigen and mature to an activated phenotype characterised by upregulation of costimulatory molecules, such as CD80 and 86¹⁴, as well as CCR7, which promotes cellular migration to secondary lymphoid tissues¹⁵. Here, DCs present antigen via MHC molecules to the TCR and crucial CD80/86 costimulation to CD28 on T cells¹⁴. DCs also provide additional signals, such as cytokines and chemokines, which direct the differentiation of T cells to a subset appropriate to the pathogenic environment¹⁶. This stringent process of T cell activation ensures that T cells are activated only if the antigen they present is pathogen-derived. The requirement for the MHC-TCR complex, CD80/86-CD28 co-stimulation and cytokine production provides crucial checkpoints that prevent inflammatory immune responses being mounted against innocuous environmental or self-antigens as well as driving specific immune responses that are appropriate for control of the infectious challenge.

1.1.2 Immunoglobulins

Pathogens invade the body via disruptions to epithelial barriers, by crossing host mucosal surfaces that line the respiratory, urogenital and gastrointestinal tracts, or, less commonly, by direct entry into the blood. Once infection has occurred, pathogens can also inhabit extracellular spaces of the body. In these scenarios,

infections can be controlled by antibodies secreted by B cells into extracellular spaces, termed the humoral immune response. Antibodies are antigen-specific proteins produced following immunisation, either through infection or vaccination, that bind cognate antigen, eliciting a variety of effector functions to aid in long-lasting protection against infection.

1.1.2.1 Immunoglobulin structure

Immunoglobulins (Igs) are exclusively produced by B cells and can be found in two forms, either as soluble antibodies which are present in blood and extracellular fluids and secreted across mucosal barriers, or as membrane-bound B cell receptors (BCRs). Igs consist of two heavy (H) chains linked by disulfide bonds and each heavy chain is also linked via a disulfide bond to a light (L) chain. These can be further subdivided into variable (V) regions and constant (C) regions (also known as Fc regions)¹⁷ (Figure 1.1). The amino acid sequence of the V regions are highly variable between different Ig molecules and this confers the antigen specificity of the molecule. The genome of each individual possesses many different gene segments that encode the V region of Igs. During B cell development in the bone marrow (BM), gene segments are randomly spliced together generating an extensive potential pool of B cells, each with unique variable regions that encode an Ig with an individual antigen specificity, a process known as somatic recombination⁵. Following antigen exposure, the Ig will be further diversified through somatic hypermutation (SHM), a process of random mutation in the V region antigen binding pocket¹⁸. This generates further variation in Ig specificity and enables B cells to recognise a wide range of invading pathogens that the individual may encounter throughout its lifetime. The C regions show far less variability between Ig molecules and determine the antibody isotype and, accordingly, its effector function¹⁹. The isotype, or class, of the Ig may change over the course of the immune response through a process called class-switch recombination (CSR). The Ig heavy chain locus consists of

distinct gene clusters, encoding the five Ig isotypes: IgM, IgD, IgG, IgE and IgA. During CSR, segments of this locus are deleted, bringing the promoter in line with one of the other isotype clusters, leading to sole expression of the appropriately switched Ig²⁰. Ig gene rearrangement, SHM and CSR allow B cells to alter both the specificity of the antibody and its effector function to effectively eliminate pathogens.

Figure 1.1: Structure of an immunoglobulin molecule. Immunoglobulins consist of two heavy (H) chains paired with two light (L) chains, which can be further subdivided into variable (V) regions and constant (C) or Fc regions. Chains are linked together via disulfide bonds.

1.1.2.2 Antibody effector functions

Different antibody isotypes are made in distinct sites and have varying effector functions and the isotype that dominates a response will differ according to the immunising agent and where it enters the body. Mature naïve B cells co-express IgD and IgM as surface Igs²¹. Upon encounter of their cognate antigen, activated B cells lose expression of IgD. Some will retain IgM expression, while others undergo CSR to express IgG, IgE or IgA²⁰. IgM is the first antibody produced in

the humoral response and circulates in the blood as a pentamer. IgM is a strong activator of the complement cascade, which facilitates lysis and phagocytosis of pathogens and is important in controlling infections in the bloodstream²². IgG is the predominant antibody in the blood and can be divided into four subtypes, IgG1, IgG2, IgG3 and IgG4, which are differentially produced in response to varying antigens²³. A major role of IgG is neutralisation: it can bind pathogen surfaces, preventing invasion of host cells, but is also able to activate complement²⁴. IgG antibodies can also bind virally infected cells and mediate antibody-dependent cell-mediated cytotoxicity (ADCC), where the Fc portion binds to the Fc receptor, FcγRIII, on natural killer (NK) cells, triggering release of cytotoxic granules to kill infected cells¹. IgA constitutes 70% of Ig produced by mammals and is mostly secreted at mucosal surfaces²⁵, where its primary function is to neutralise pathogens, preventing access to the mucosa^{26,27}. IgE is present at low peripheral concentrations and can be found primarily bound to granulocytes (mast cells and basophils) via FcεRI in the tissues. When antigen binds and cross-links IgE on the surface of granulocytes, they degranulate and release an array of pre-formed toxic mediators²⁸, a process that plays a key role in eliminating parasitic worm infections, as well as in the pathogenesis of allergies and asthma²⁸.

1.1.3 Generation of antibody-secreting cells

1.1.3.1 Antigen transport to the draining lymph node

The generation of high affinity, class-switched antibodies during infection or vaccination requires a concerted effort between multiple cell types across the innate and adaptive immune systems. Peripheral lymphoid organs, such as the lymph nodes (LNs), serve as a convergence point for lymphatic drainage from the tissues and are the location of many crucial immune processes, including the generation of antibody-secreting cells. LNs are spread widely throughout the

body and are highly compartmentalized in order to facilitate effective interactions between lymphocytes and antigen.

Lymphatic drainage transports foreign antigen deposited in the tissue by infection or vaccination to the LN. Antigen must reach the follicle to be recognised by B cells, and the paracortex for recognition by T cells. Antigen reaches the subcapsular sinus (SCS) of the tissue draining LN (dLN) via afferent lymphatic vessels, transported here either by migrating APCs that have taken up antigen in the tissue, or as soluble antigen that has freely drained from the tissue²⁹ (Figure 1.2). From here, low molecular-weight (MW) soluble antigens may diffuse into the follicle through pores in the SCS³⁰ or via conduits³¹. Alternately, Mφ are able to transport antigens from the SCS into the underlying follicle³². Antigen may also drain to the medulla where LN-resident DCs (LNresDC) are able to transport antigen to the follicle³³. On the other hand, antigen destined for the T cell paracortex is transported here by migratory DC (migDC) populations³⁴ or low MW antigens may also travel via the conduit network in the T cell area³⁵, where LNresDCs can sample antigen³⁶.

Figure 1.2: Organisation of the lymph node. The lymph node can be divided into three major regions: the medulla, the paracortex (T cell zone) and the follicles (B cell zones). Naïve T and B cells circulate the body, entering the LN via HEVs and B cells migrate to the follicles while T cells assemble in the paracortex. Afferent lymph drains from the tissues, carrying immune cells and soluble antigen, into the SCS. From here, antigen can enter the follicle through pores in the SCS. Antigen also enters the conduit network where it can enter the follicles or paracortex. Figure from Batista et al. 2009³⁷

1.1.3.2 Lymphocyte activation in the lymph node

Under homeostatic conditions, mature naïve T and B cells constitutively circulate between secondary lymphoid organs³⁸. Upon reaching the LN, B and T_H cells exit the blood via high endothelial venules³⁹, with B cells migrating to follicles and T_H cells entering the surrounding paracortex in pursuit of antigen⁴⁰. Following antigen exposure, DC populations present antigen and provide costimulation to effectively activate naïve T cells, which proliferate and migrate to the interface between the follicle and paracortex (T-B border)⁴¹. In parallel, B cells collect soluble antigen or antigen from APCs on surface Ig, which they

internalize and migrate to the T-B border⁴¹. This brings the two rare populations of naïve cognate B and T_H cells into proximity, allowing stable cell-cell interactions to be established⁴². Here, B cells present processed antigen via MHC II to T_H cells, which reciprocally provide crucial signals for B cell survival and differentiation, such as CD40L⁴³. B cells compete for limited T_H cell help and only those B cells possessing Igs with highest antigen affinity will survive and progress.

Selected B cells have three potential fates. A subset of activated B cells will become early memory B cells⁴⁴. Others will migrate to the outer follicle and undergo an extrafollicular response, where they differentiate into short-lived plasma cells (PCs). These secrete low-affinity antibody early following immunisation for rapid protection against infection, but only survive for around three days⁴⁵. The third option is the re-entry of B cells to the follicle where they proliferate to form the germinal centre (GC), a dynamic structure that facilitates the generation of high-affinity, class-switched long-lived PCs and memory B cells⁷.

Importantly the CD4⁺ T_H cell subset, T_{FH} cells, is required for B cell development in the GC¹³. T_{FH} cells are driven by the master regulator transcription factor, Bcl6, and are commonly characterised by the expression of programmed death receptor 1 (PD-1), the chemokine receptor CXCR5 and the secretion of IL-21¹³. T_{FH} cell development occurs at multiple stages in the LN. Initially, naïve T_H must be primed with antigen and costimulatory signals from DCs, prompting differentiation into pre-T_{FH} cells^{46,47}. These precursors then receive further signals from B cells for the full commitment to the T_{FH} lineage^{48,49}. T_{FH} cells provide important signals to aid B cell activation, proliferation differentiation and survival¹³. This includes T_{FH} cell CD40L binding to CD40 expressed by B cells, which is a critical signal to multiple stages of B cell activation and differentiation,

as well as cytokine secretion (e.g. IL-21), which potently promotes GC B cell CSR and differentiation into PCs^{50,51}.

1.1.3.3 The germinal centre reaction

Following immunisation the affinity of antibodies to antigen progressively increases, a selective process occurring in the GC that can be compared to Darwinian selection. Following activation, B cells rapidly divide in the follicle to form the GC, where they are identified by their expression of GL7 antigen⁵². By around day seven following immunisation, a mature GC can be observed, characterised by two functionally distinct areas: the dark zone (DZ) and light zone (LZ), which B cells migrate between during maturation (Figure 1.3). Ig diversification in B cells occurs in the DZ. Here, point mutations are introduced to V region genes by SHM, generating mutant B cell clones with altered antigen specificity⁵³ (Figure 1.3A). Indiscriminate mutations alter the affinity of the B cell Ig for the cognate antigen, so that B cells have varying affinities for the immunising antigen. These cells then move to the LZ where surface Ig affinity is tested in two stages (Figure 1.3B). Follicular dendritic cells (FDCs) of the LZ retain immunising antigen on their surface for an extended period of time⁵⁴, allowing only the B cells expressing functional antigen-specific surface Ig to bind and endocytose antigen, which induces survival signals within the cell, whilst others apoptose (Figure 1.3C). Secondly, the density at which B cells present antigen on MHC II is proportional to antigen affinity of the surface Ig. Those presenting higher density antigen successfully compete for the limited T_{FH} cell help, while lower affinity or non-functional mutants are neglected and apoptose^{55,56} (Figure 1.3C). B cells can undergo multiple successive rounds of Ig diversification and selection, developing a population of high-affinity B cells⁷ (Figure 1.3F). Additionally, CSR in B cells occurs in the LZ and is largely dependent on T_{FH} cell engagement of B cell CD40⁵⁷, along with cytokine

signaling, that dictates the antibody isotype - such as IL-4, which promotes switching to IgE and IgG1⁵⁸ (Figure 1.3E).

High affinity B cell clones will receive selection signals from T_{FH} cells, exit the GC and differentiate into memory B cells or long-lived PCs (Figure 1.3G and H), both of which may persist over a lifetime^{59,60}. Long-lived PCs migrate to the BM and constitutively secrete antibody, providing a first line of defence against subsequent infection⁶¹. Furthermore, memory B cells can be rapidly reactivated upon antigen encounter to proliferate and produce large quantities of class-switched, high-affinity antibody⁶².

Figure 1.3: The germinal centre reaction. The GC can be divided into two functionally distinct regions, the dark and light zones. **(A)** In the dark zone, activated B cells generate mutant clones through SHM. **(B)** Mutants move to the light zone and recognise the immunising antigen retained on FDCs, only B cells expressing functional, antigen-specific Igs receive survival signals, **(C)** whilst others apoptose. **(D)** Selected B cells then compete for T_{FH} cell help, only those with the greatest antigen affinity receive selection signals, whereas others apoptose. Cells then either **(F)** re-enter the dark zone for successive rounds of SHM, or differentiate into **(G)** PCs or **(H)** memory B cells. Figure adapted from Heesters et al.⁶³.

1.1.4 Vaccination and adjuvants

In addition to immunity conferred by natural infection, adaptive immunity to an infectious agent can be achieved by vaccination, whereby exposure to pathogen antigens in a non-virulent form induces immunological memory that protects from future pathogen exposure¹. The majority of current vaccines elicit protection via the induction of antibodies. A number of vaccines induce extremely effective antibody responses and have been successful in eradicating several infectious diseases from industrialised countries, notably polio and smallpox⁶⁴. Long-term protection is conferred by the persistence of high titres of pathogen-specific antibody or the maintenance of memory B cell populations⁶⁵.

Vaccines can be either live attenuated, where the pathogen is weakened to reduce its virulence, or non-live vaccines, consisting of inactivated microorganisms or purified pathogen components, usually proteins. Live attenuated vaccines contain organisms that are able to replicate and maintain many of the pathogen-associated signals that are provided by a live infection, triggering the activation of multiple PRRs to elicit a strong immune response⁶⁶. Non-live vaccines are a safer alternative, as no live organisms remain, however, most pathogen proteins are poorly immunogenic in the absence of danger signals provided by the live pathogen.

To address low immunogenicity, vaccines may contain an adjuvant, which provides additional 'danger signals' to increase immunogenicity of pathogen proteins⁶⁷. Licensed adjuvants that boost antibody responses to vaccines include microbial particles, such as the LPS-derived molecule monophosphoryl lipid A (MPL), aluminum salts (alum) or oil-in-water emulsions, such as MF59⁶⁸. The major function of vaccine adjuvants is to enhance the overall magnitude and affinity of vaccine-induced antibodies^{69,70}, which often means that lower or fewer doses of the vaccine are required to achieve sufficient protective antibody titres

termed seroconversion. Additionally, adjuvants may also be used to guide the type of adaptive immune response that ensues following vaccination⁶⁷.

While adjuvants are in clinical use and prove extremely effective, the mechanisms of action are incompletely understood. It was thought until recently that the primary mechanism of most adjuvants was to act as a depot for vaccine antigen in the tissue, thereby ensuring slow release and prolonged immune stimulation⁷¹. However, a number of studies have challenged this paradigm. For example, removal of the alum depot following vaccination has no effect on antibody production⁷² and it is now understood that adjuvants likely trigger innate immune responses more directly⁷¹. Many adjuvants induce a strong local inflammatory response. MF59 is able to recruit immune cells to the site of vaccination, facilitating antigen transport to the dLN^{73,74}, as well as enhancing uptake of antigen by APCs⁷⁵. Another key role of many adjuvants is to activate cells through PRRs, including the TLRs, NLRs, RLRs and CLRs. Once the receptor is bound, intracellular signalling leads to the activation of key transcription factors, such as NF- κ B, IRF-3 and IRF-7, which ultimately results in the induction of genes encoding chemokines and cytokines, as well as inducing DC maturation and expression of costimulatory and MHC molecules⁷⁶. MPL, for example, mediates adjuvant function via signaling through TLR-4⁷⁷. Studies suggest that some adjuvants, particularly alum, signal through NLRs to activate the inflammasome pathway⁷⁸. The inflammasome is a protein complex that forms following PRR activation and is able to act on cytokine precursors to convert them to an active form, including the proinflammatory cytokines IL-1 β and IL-18⁷⁸. Thus, adjuvants act via a multitude of immune pathways and evidence suggests that current effective adjuvants engage multiple mechanisms to enhance the humoral immune response to vaccination.

1.2 Influenza infection and vaccination

1.2.1 Influenza virus

Influenza is an acute respiratory virus from the Orthomyxoviridae family. Influenza infection is a significant health burden worldwide, with seasonal epidemics estimated to cause 3 to 5 million infectious cases, leading to 250,000 to 500,000 deaths per year⁷⁹. The virus infects the respiratory tract of humans and a number of other mammalian species and can cause both seasonal periods of illness and periodic pandemic outbreaks⁸⁰. In the 2015 SHIVERS survey, influenza virus was found to infect 26% of the New Zealand population in a given influenza season, of whom 20% were symptomatic⁸¹. Influenza infection clinically manifests as a high fever, cough, headache, malaise and respiratory tract inflammation, among other symptoms, which may lead to severe complications, including pneumonia, secondary bacterial infection and hemorrhagic bronchitis⁸². Furthermore, certain groups of the community show greater susceptibility to infection and subsequent complications, including young children, pregnant women, the elderly and people with chronic comorbidities, such as diabetes⁸³. Given the high incidence and risk of serious complications and mortality from influenza virus infection, effective prevention strategies are required.

The three major genera of influenza virus capable of causing human illness, influenza A, B and C, are distinguished by their distinct protein expression⁸⁴. Seasonal epidemics are largely caused by types A and B, with influenza C causing only mild respiratory illness⁸⁵. The viruses negative-sense ribonucleic acid (RNA) segments are packaged into ribonucleosome complexes and encased in a host-derived lipid envelope. A number of proteins are embedded in the envelope, most notably the glycoproteins neuraminidase (NA) and

haemagglutinin (HA), which play key roles in viral pathogenesis. Viral HA binds to sialic acid-containing receptors present on the surface of host cells, mediating attachment, fusion with host cell endosomes and subsequent endosomal membrane fusion releasing the viral genome into the cell^{86,87}. In contrast, NA enzymatically cleaves sialic acid⁸⁸ and the concerted activities of HA and NA are important in viral entry and release^{89,90}. Influenza virus infects the human host by entry into the nasal passage where it replicates in mucosal epithelial cells, eventually spreading to the lower respiratory tract. The virus has a tropism for the respiratory tract, conferred by the preference for particular sialic acid linkages present on epithelial cells here⁹¹.

The burden of influenza can, in part, be attributed to the exceptional ability for the virus to evade host immune defences, largely through the ability to constantly undergo rapid genetic evolution. Genetic variability between seasonal influenza strains occurs through genetic drift, a process by which the highly error-prone viral RNA replication machinery rapidly generates genetic mutations, leading to the constant production of viral variants expressing proteins that are not recognised by the host's existing immune repertoire⁹². Coupled with selective pressure from the host immune system, mutants able to escape the host's existing strain-specific immune defences are selected for⁹³. This results in a constantly evolving viral population that is able to successfully infect individuals with an existing immune response, allowing it to be passed through populations rapidly.

1.2.2 Influenza vaccination

Both natural infection and vaccination can confer resistance to influenza infection and severe disease. A major component of protection against influenza infection is antibody production, primarily to the viral surface proteins HA and NA⁹⁴. Following natural infection, locally produced IgA antibody to influenza

virus is secreted in the respiratory tract and is a correlate of protection against infection^{27,95}. Secretory IgA neutralises influenza virus at the mucosa and is able to prevent the virus from crossing the mucosal barrier and entering the body²⁷, as well as transporting virus that has invaded back out to the lumen⁹⁶. Furthermore, infection elicits robust levels of virus-specific IgG antibody in the serum⁹⁷, which is a strong predictor of protection against influenza infection^{27,98,99}. Influenza-specific CTL responses play a key role in limiting viral load and clearance of influenza infection, however do not appear to protect against initial infection¹⁰⁰.

1.2.2.1 Inactivated and live attenuated vaccines

Currently, annual vaccination is widely considered the most effective method for preventing and controlling influenza virus infection¹⁰¹. International surveillance data from the World Health Organisation is used to inform the most prevalent circulating strains of each influenza season^{102,103} and currently licensed vaccines contain viral material derived from three to four of the most predominant strains, referred to as trivalent or quadrivalent, respectively¹⁰³. Two major types of influenza vaccine are licensed for use: inactivated and live attenuated.

There are three types of inactivated vaccine, distinguished by the level of purification. Whole virus vaccines are simply composed of inactive whole virus⁹². Split virus vaccines are further purified through addition of detergent to dissociate the viral lipid envelope, exposing viral proteins which are formulated into the vaccine¹⁰⁴. For manufacture of subunit vaccines, viral components undergo further purification steps to isolate HA protein⁹². Due to the comparable immunogenicity of whole virus preparations, split and subunit vaccines and the lower reactogenicity of subunit vaccines^{105,106}, these are the most widely utilised, in particular the trivalent influenza subunit vaccine (TIV). TIV is administered

parenterally, either by the intramuscular or subcutaneous route. The major mechanism of protection by TIV is the induction of IgG antibody in the serum^{107,108}.

The other major licensed influenza vaccine in use is the live attenuated influenza vaccine (LAIV). LAIV is administered via an intranasal spray and contains an attenuated virus that is cold-adapted to only replicate efficiently in the nasopharynx, not the lung where pathology occurs¹⁰⁹. The vaccine more closely resembles natural infection, eliciting protection by inducing both mucosal IgA secretion and systemic IgG antibody responses¹¹⁰⁻¹¹².

1.2.3 Immune response to influenza vaccines are suboptimal

Despite widespread immunization against influenza, infection continues to cause severe illness and mortality worldwide. Current influenza vaccines currently are designed to induce primarily strain-specific immunity, and viral strains encountered may not match those included in vaccines¹¹³. Moreover, current influenza vaccines fail to protect against infection in many individuals even when the vaccine strains are well matched to circulating viruses. In individuals vaccinated with TIV and LAIV, vaccination protects against infection with matched strains in approximately 70% and 85% of people, respectively¹¹⁴. This reflects the high level of inter-individual variability in the strength of antibody responses to influenza vaccination, with antibody titres varying up to 100-fold between individuals and many individuals do not achieve seroconversion¹¹⁵. Furthermore, particular populations mount poorer antibody responses to influenza vaccination, particularly the elderly^{116,117} and children and, importantly, infection has the greatest impact on these populations¹¹⁸. The factors leading to suboptimal antibody responses in these low responding groups are yet to be fully elucidated.

It is understood that environmental factors are a key influence on human immune responses. A recent study compared an extensive panel of immune cells and soluble mediators between monozygous twins and found that non-heritable factors were the dominant determining factor of 77% of the immune parameters examined¹¹⁹, therefore surpassing the importance of genetic contributions. Furthermore, it has also been found that cohabitating, yet unrelated, individuals show 50% less immunological variation than those of the general population¹²⁰. These studies illustrate the need to elucidate the key environmental factors that influence the human immune system in order to determine interventions that will improve aberrant immune responses.

Although a number of factors have been identified to influence the efficacy of influenza vaccination, including age, immunocompetence, preexisting levels of antibody from previous infection and vaccination¹²¹, an emerging theory is that the microorganisms that inhabit the intestinal tract, the gut microbiota, may also be a contributing factor. Coinciding with similarities in immune profiles with unrelated housemates, cohabitating individuals show convergence in their gut microbial composition^{122,123}. Furthermore, gut microbial composition of the elderly¹²⁴ and young children¹²⁵ differs significantly from adults, which may be a factor in poor vaccine responses in these groups. It has also been consistently found that vaccine efficacy is much poorer in developing nations, something that may be attributable to differences in gut microbiota composition^{126,127}. There is now an accumulating body of direct evidence to support that gut microbial composition directly influences the immune response to vaccination. This will be expanded on in further chapters.

1.3 The gut microbiota

Hippocrates was quoted as saying, “all disease begins in the gut” and over 2000 years later, the relevance of his sentiment is becoming apparent. The influence of the gut microbiota on human health has recently become of significant research interest with mounting evidence demonstrating the influence these microbes have on host wellbeing. The skin, respiratory, oral, urogenital and gastrointestinal tracts of mammals offer rich ecological niches that are constitutively colonised by unique communities of microorganisms¹²⁸. The majority of these microorganisms are bacteria, with minor populations of fungi, archaea, protozoa and viruses, however for simplicity, in this thesis ‘gut microbiota’ will refer to only the bacterial communities. The gastrointestinal (GI) tract is home to the majority of these microbes, with the intestine of each human housing an estimated 39 trillion bacteria, likely exceeding the number of the individual’s own cells¹²⁹. The phyla Bacteroidetes and Firmicutes comprise more than 90% of the microbes of the human¹²⁸ and murine¹³⁰ gut microbiota. Other major phyla include Actinobacteria, Proteobacteria, Tenericutes and Verrucomicrobia, among others.

Gut microbes and their mammalian host share a symbiotic relationship, in which the host provides nutrition and favorable living conditions for microbes and, in turn, the microbiota aids in maintaining host health. The resident microbiota performs anaerobic fermentation of undigested dietary material and endogenous compounds, leading to production of a diverse collection of metabolites for use by the host¹³¹, as well as synthesising essential vitamins¹³². Additionally, the gut microbiota plays a key role in resistance to colonisation by enteric pathogens. It does so directly, by inhibiting pathogen growth through the secretion of anti-microbial molecules¹³³ and limiting nutrient availability¹³⁴, and indirectly promotes pathogen resistance by maintaining gut barrier function, for

example by stimulating mucus secretion¹³⁵. Vitally, the gut microbiota also provides signals for the development and proper function of the immune system¹³⁶, this will be expanded on in further chapters.

1.3.1 Microbiota community analysis

It is estimated that the genes of the human gut microbiota, the microbiome, contribute over 100 times the number of genes than the human genome¹³⁷. However, each individual may only possess 15% of the full microbial compliment observed in humans¹³⁸, illustrating the variability that exists between individuals. It is therefore essential to be able to identify and functionally assign the resident microbiota to elucidate key features that are important in health and disease in order to harness this knowledge to improve human health.

The development of next-generation sequencing technology has allowed the in-depth examination of the enormously complex commensal microbial communities, whether this is complete or targeted sequencing of DNA extracted from a sample, such as faeces. This has surpassed culture-dependent methods for bacterial identification, which limited analysis to the few organisms that can be grown under laboratory culture conditions¹³⁹. Analysis initially focused on phylogenetic classification which provided insight into bacterial community members that are crucial to influencing host immunity. Phylogenetic classification is performed by sequencing the gene encoding 16S ribosomal ribonucleic acid (rRNA), a prokaryotic gene that codes for part of the 30S subunit of ribosomes. This gene is conserved within bacterial and archaeal species, allowing selective sequencing of the microbiome¹⁴⁰ and importantly, it contains variable regions, allowing taxonomic classification of microbiota communities⁵⁷. Quantitative polymerase-chain reaction (qPCR) with 16S rRNA-specific primers can also be used as a cost-effective alternative to classify microbial communities

at the phyla level¹⁹. It is becoming apparent that the functional composition of the commensal microbiota is also likely to be of importance in influencing health, as many microbially-produced molecules are able to influence host homeostasis²⁰. This can be explored using whole metagenomic sequencing of DNA extracted from a sample, allowing microbial genes to be annotated and catalogued into functional categories. This approach can be extremely useful to determine changes in microbial function relating to states of health or disease²¹.

However, often the genetic potential predicted from metagenomes does not necessarily equate to functional output²⁰. Metatranscriptomics is also widely used in microbiota studies and quantifies transcriptional activity of microbial genes by sequencing messenger RNA (mRNA) transcripts²². Furthermore, translation of gut microbial genes can be quantified by metabolomics. This is often performed on faecal samples where a significant proportion of metabolites present will be microbially synthesised²³. Each technique has advantages and these can be integrated to form a comprehensive picture of microbial community composition and function, especially when comparing the microbiota between states of health and disease¹⁴¹.

1.3.2 The interface between the intestinal immune system and the microbiota

The intestine is home to the largest number of immune cells in the body, which is unsurprising considering the antigenic load presented by symbiotic and pathogenic microbes, food and other exogenous materials that constantly occupy the intestinal lumen. The intestinal immune system is tasked with preventing translocation of intestinal contents into the sterile tissues and blood in order to curb infection, as well as preventing unnecessary inflammatory responses to innocuous antigens. Perhaps counterintuitively, the maintenance of intestinal homeostasis is reliant on the constant interactions between intestinal

microbes and the mucosal immune system¹⁴². Germ-free (GF) mice are born and maintained in sterile isolators and a lack of all microbial stimuli leaves these mice highly susceptible to enteric infection compared to mice with a complete microbiota, known as specific-pathogen free (SPF) mice¹³⁶. This is due to underdevelopment in the intestinal immune system. For example, the gut microbiota is crucial for proper maintenance of the intestinal mucus layer by goblet cells, providing a first-line defence against the microbiota and pathogens¹⁴³. Gut microbes also promote the production of secretory IgA (sIgA), which binds to microbes in the intestine, protecting the epithelium from bacterial invasion and promoting tolerance to the microbiota¹⁴⁴. Furthermore, specific members of gut microbiota promote homeostasis via stimulating development of specific T cell subsets. Segmented filamentous bacteria are able to promote T_H17 skewing to protect against intestinal infection, while a community of Clostridia strains was shown to stimulate differentiation of T_{REGS} ¹⁴⁵. Mice with genetic defects in their ability to detect microbes, for example those lacking the bacterial sensor, TLR-4, or its downstream signalling molecule, MyD88, have worsened symptoms in a model of inflammatory bowel disease. Thus, it is clear that interactions between the microbiota and host immune system are crucial for maintenance of protection against local infection and inflammation.

1.3.3 Gut microbiota interactions and the systemic immune system

Intriguingly, microbiota effects extend beyond the local immune system of the intestine and it is clear that the gut microbiota provides diverse signals that are crucial for the development and function of host immunity at systemic sites. This is supported by observations that altered composition and function of the microbiota are prevalent in individuals with a number of immune-modulated diseases at sites distinct from the gut. For example, the development of allergic

diseases in childhood is associated with reduced diversity of the intestinal microbiota in infants¹⁴⁶. It has also been observed that microbial composition varies significantly between healthy individuals and those with type 1 diabetes¹⁴⁷ and rheumatoid arthritis¹⁴⁸. Furthermore, the composition of the infant gut microbiome has been shown to correlate with the strength of the antibody responses to rotavirus vaccination¹²⁷. It is understood that the microbiota communicates with its host via two major molecular signals, either by translocation of components of microbial cells or via the action of microbially-produced metabolites.

1.3.3.1 Microbial ligands

As discussed earlier, PRRs on host immune cells play a key role in sensing microbial components in order to induce cellular effector mechanisms. Traditionally, it has been understood that intestinal lumen contents are compartmentalised, preventing translocation of microbes and their components into the sterile compartments of the body¹⁴⁴. Mucus, epithelial cells and immune cells in the lamina propria as well as the liver maintain a barrier to minimise translocation to systemic circulation¹⁴⁹, as breakdown in the barrier function can be detrimental and is linked to the pathogenesis of inflammatory bowel diseases¹⁵⁰, liver disease¹⁵¹ and sepsis¹⁵². However, more recently it has been proposed that this barrier is not absolute and microbial components are able to translocate across the mucosa into the circulation and influence immunity distal from the gut. This is supported by the presence of considerable titres of gut microbiota-specific IgG in the serum of healthy humans¹⁵³ and mice¹⁵⁴. Translocation of microbial cell components has been observed directly in healthy mice, whereby labelled peptidoglycan from the commensal bacterium *Escherichia coli* was shown to undergo constitutive translocation across the gut mucosa and bind the NLR, Nod1, on neutrophils in the BM, which directly enhanced their bacterial killing capacity¹⁵⁵.

A number of other investigations have shown the requirement for sensing of bacterial components via PRRs in the development of robust immune responses distal from the gut. GF mice exhibit poor control of pulmonary infection with *Klebsiella pneumoniae* compared to SPF mice¹⁵⁶. Injection with the bacterial membrane component, lipopolysaccharide (LPS), prior to infection enhances neutrophil recruitment, chemokine and cytokine production in the lung, and restores control of infection to levels of SPF mice¹⁵⁶. Similarly, antibiotic treatment of SPF mice leads to defective T and B cell responses to influenza virus infection which could be restored by intrarectal administration of TLR agonists, mimicking the presence of bacteria in the colon¹⁵⁷. In the context of influenza vaccination, it has been found that signalling driven by microbiota-derived flagellin via TLR-5 on B cells and macrophages was required for robust IgG antibody responses to TIV vaccination¹⁵⁸. Accordingly, antibiotic-treated mice exhibited poor antibody responses to TIV, however this could be rescued by the simultaneous administration of flagellin¹⁵⁸. Sensing of microbiota components via TLRs has also been shown to be crucial for haematopoiesis. GF mice have a reduced pool of myeloid precursors and mature myeloid cells in the BM, resulting in significantly reduced frequencies of peripheral leukocytes and impaired control of systemic *Escherichia coli* infection¹⁵⁹. Strikingly, the BM myeloid compartment in GF mice could be restored by transferring heat-treated serum from SPF animals, an effect that was dependent on TLR signalling¹⁵⁹. Together these investigations show that sensing of systemic microbial components is required for the function of multiple immune cell types to respond effectively to infection and vaccination.

1.3.3.2 Microbial metabolites

The gut microbiota produces a complex collection of molecules, many of which have been shown to influence host immunity, capable of both enhancing and dampening immune responses depending on the molecule and their

physiological context. The microbiota possesses a number of digestive and metabolic enzymes that metabolise dietary components that are otherwise indigestible to the host, as well as endogenous compounds that are produced by the host or the microbiota itself. This process results in the production of a diverse collection of metabolites in the gut, many of which are absorbed through the gut mucosa and exert effects on host cells locally or systemically¹⁶⁰. Advances in characterisation of microbial metabolism through metagenomic and metabolomic analysis have identified microbial-produced molecules that have profound effects on the host immune system. The potential for small molecules to be absorbed into the circulation means they can act distally from the gut and therefore exert widespread influences on systemic immunity.

The most extensively studied of the microbiota metabolites are short-chain fatty acids (SCFAs). SCFAs are the major product of the anaerobic fermentation of digestion-resistant starches and soluble dietary fibre, known as microbiota-accessible carbohydrates (MACs) by certain bacteria in the colon¹⁶¹, explaining the very low levels of SCFA in the gut of GF mice¹⁶². The beneficial effects of dietary fibre and consequent SCFA production in disease prevention have been demonstrated in allergic airway inflammation¹⁶³, obesity¹⁶⁴ and anti-tumour immunity¹⁶⁵. Coincident with increases in these and other diseases linked with SCFAs is systematic changes in human diet, notably the reduced intake of dietary fibre¹⁶⁶. The three major SCFAs in the colon of humans and mice are acetate, butyrate and propionate¹⁶⁷, the majority of which are absorbed by the host or further utilised by commensal bacteria, with only 5-10% excreted in faeces¹⁶⁸. Beyond their use in the gut, SCFAs are easily detectable in the peripheral circulation¹⁶⁷. Here, they are available for use in tissues and have been shown to exhibit systemic immunomodulatory effects in the host via a number of mechanisms. SCFAs signal via the G-protein coupled receptors (GPCRs) GPR41, GPR43 and GPR109A with varying affinities and SCFA preference^{169,170}.

These receptors are widely expressed by immune cells, including DCs, monocytes, M ϕ , as well as in the BM^{169,171}. The pervasive immunomodulatory effect of SCFAs on host immunity is demonstrated in mice deficient in GPR43 (GPR43^{-/-}), for which the only known ligands are SCFAs. These mice exhibit exacerbated inflammatory responses in models of arthritis, allergic airway inflammation and colitis¹⁷². SCFAs also promote intestinal homeostasis by providing a primary energy source for colonic epithelial cells to support integrity of the intestinal barrier^{173,174}. Beyond the gut, SCFAs are taken up by B cells¹⁷⁵ and CD8⁺ memory T cells¹⁷⁶ and converted to acetyl CoA, fueling metabolism to drive both antibody production¹⁷⁵ and function of memory CD8⁺ T cells¹⁷⁶. SCFAs also influence the host epigenome by inhibiting histone deacetylases (HDACs)^{177,178}. HDACs induce transcriptional repression by the removal of acetyl groups from lysine residues within histones, reducing transcription enzyme accessibility to promoter regions in the DNA. Therefore, HDAC inhibitors increase gene transcription¹⁷⁹. Oral administration of SCFAs inhibits HDACs to regulate the mTOR-S6K pathway in T cells, which is important for their differentiation, promoting a T_H1 and T_H17 phenotype in peripheral T cells to enhance protection during systemic bacterial infection¹⁸⁰. Butyrate is a particularly potent HDAC inhibitor and promotes peripheral T_{REG} generation, both by directly enhancing acetylation of the T_{REG} master transcription factor locus, FoxP3, as well as reducing DC expression of pro-inflammatory cytokines¹⁸¹.

Numerous other microbial metabolites have been identified to be important for immune development and fine tuning of immune responses¹³¹. For example, the microbiota is able to synthesise a variety of polyamines which have been shown to be able to modulate systemic immune responses, including enhancing splenic B cell maturation¹⁸². Based upon the observation that the gut microbiota is able to regulate immune responses to viral infection by modulating type I interferon (IFN) signalling and robust type I IFN response is critical in anti-viral immune

responses¹⁸³, Steed and colleagues screened microbial metabolites for their ability to induce type I IFN signalling¹⁸⁴. They identified a metabolite from dietary flavonoids, desaminotyrosine, that potently enhanced signalling and subsequently promoted resistance to influenza infection¹⁸⁴. These compounds are some examples of the vast collection of metabolites produced by the gut microbiota, many of which have potent immunomodulatory properties.

1.3.4 Dysbiosis

It is clear that the gut microbiota exerts widespread influence on many aspects of host physiology, including to enhancing antibody responses to influenza vaccination, which is the focus of this investigation. It has been proposed that recent changes in the lifestyle and environmental of those in Western societies is significantly altering gut microbiota communities, a state termed dysbiosis, and this may be predisposing humans to disease¹⁸⁵.

Changes in the environment can rapidly change the composition and function of gut microbial communities and, in turn, alter essential interactions with the host. Antibiotics are a major bacterial perturbation introduced to humans in the last century and are routinely prescribed, with children in the US receiving an average of ten courses before they reach ten years old¹⁸⁶. Of concern, antibiotic use in infants leads to alterations in gut microbiota composition¹⁸⁷ and administration of antibiotics to healthy adults for only one week led to significant changes to gut microbiota communities¹⁸⁸. In both these studies, alterations were shown to persist beyond two years after treatment.

Diet also has a profound influence on gut microbiota structure and function. In two controlled feeding studies in humans, it has been observed that after just 24 hours of changes in the diet, significant alterations occur to composition of the gut microbiota^{189,190}. The Western diet is dominated by refined carbohydrates, fat

and sugar and typically contains dietary additives and low amounts of fibre¹⁹¹, in contrast to that of those living traditional lifestyles, such as hunter-gatherer societies¹⁹². Coinciding with dietary changes are significant differences between the microbiome of Westerners and of hunter-gatherers, including reduced diversity and an altered ratio of the major bacterial phyla, Bacteroidetes and Firmicutes¹⁹². Dietary impacts on the microbiota have been directly demonstrated in mice, for instance, reducing fibre intake severely reduces gut microbial diversity¹⁹³, as well as dietary additives, such as emulsifiers¹⁹⁴ and artificial sweeteners¹⁹⁵, significantly shifting the composition of microbial communities.

These changes in microbial composition through lifestyle changes are likely contributing to the rise in immunological diseases. In a number of disease states, shared microbiota characteristics within diseased populations have been determined. For example, loss of diversity of faecal microbiota communities have been found in individuals with Crohn's disease¹⁹⁶ and type 1 diabetes¹⁹⁷. Furthermore, antibiotic use in infants has been strongly linked to the development of asthma and allergy in later childhood^{187,198}. In addition, poor antibody responses to the rotavirus vaccine in infants was associated with increased Bacteroidetes and lower abundance of *Streptococcus bovis*¹²⁷. However, due to uniqueness of each individual's gut microbial community, it can be difficult to define what constitutes a health- or disease-associated microbiota. It is crucial that common features of dysbiosis are identified and their involvement in pathogenesis of disease elucidated, allowing for the rational design of microbiota-altering therapeutic approaches.

1.3.5 Beneficial modulation of the gut microbiota

While the plasticity of the microbiota can result in maladaptive changes, it also presents the exciting possibility of therapeutic modulation of the microbiota for

disease prevention or cure. Diet is considered the most affordable, safe and accessible potential therapy for modulating the gut microbiota. Diet rapidly alters gut microbiota, with a change in diet significantly altering the community composition in as little as one day^{190,199}. It has also been observed that alterations to the diet are able to reproducibly alter gut microbiota composition of different mouse strains mutant for genes important for host-microbial interactions¹⁹⁹, demonstrating that diet is strong defining factor of microbiota composition that may overshadow even genetic differences between individuals.

Given the role of the microbiota in shaping innate and adaptive immunity, a number of studies have investigated the therapeutic potential of modulation of the gut microbiota in improving immunity in infection and vaccination. A common approach used to modify the gut microbiota is the use of probiotic supplementation, defined as live microorganisms that when administered confer a health benefit to the host²⁰⁰. Additionally, MACs that selectively change the structure and function of gut microbial communities have been trialed for therapeutic use - these are referred to as prebiotics²⁰¹. Major investigations of the therapeutic benefits of probiotic and prebiotic foods in influenza infection and vaccination are summarised below.

1.3.5.1 Probiotics

Probiotics may elicit immunomodulatory effects via direct recognition of microbial molecules by the host immune system through PRRs or by modulating composition of the endogenous microbiota²⁰² and in this way have the potential to exert systemic influences on the host immune response.

Administration of probiotics to enhance protection in influenza infection and vaccination have shown some promise. Efficacy of probiotics in infection are limited to mice. It was demonstrated that administration of *Lactobacillus gasseri* promoted antiviral immune responses in the lung tissue following influenza

infection, whilst limiting excessive inflammatory cell infiltration and cytokine production in the lung²⁰³. *L. casei* administration was shown to improve control of viral load by enhancing production of inflammatory cytokines by lymphocytes in the respiratory tract²⁰⁴. In human trials, some benefit has been demonstrated with probiotic supplementation in conjunction with influenza vaccines. Benefit was observed following oral intake of *L. casei* and *Bifidobacterium animalis* in healthy adults, where administration of both probiotic strains resulted in increased vaccine-specific total IgG, IgG1 and IgG3 titres compared to placebo²⁰⁵. Furthermore, administration of *L. rhamnosus* GG prior to LAIV vaccination in healthy adults was shown to significantly enhance production of vaccine-specific neutralising antibody in the serum²⁰⁶. *L. fermentum* intake was shown to significantly enhanced the production of vaccine-specific IgA in the serum following TIV, however IgG was unchanged between probiotic and placebo groups²⁰⁷. A number of studies have also been specifically conducted in the elderly. Oral intake of *L. casei* in an elderly population significantly enhanced influenza B virus-specific antibody titres following TIV²⁰⁸. Another study found that administration of *L. paracasei* with a prebiotic fructo-oligosaccharide to the elderly showed no change in antibody production in response to TIV²⁰⁹. These studies demonstrate that probiotic supplementation shows promise in enhancing immunity in influenza infection and vaccination, however efficacy is hard to interpret as differences in strains and dosages vary significantly between studies and a strain-specific approach is clearly required.

1.3.5.2 Prebiotics

Prebiotics have the potential to significantly alter microbiota-host interactions to influence health of the host. This may be mediated by increasing the availability of substrates for microbial metabolism. For example, increased fibre intake provides fermentable polysaccharides as substrates for metabolism, therefore increasing SCFA levels in the colon and blood^{163,175,192}. Additionally, prebiotics

may encourage the growth of specific species and alter gut microbiota community structure. In areas where fibre intake is high, there is increased proportions of SCFA-producing bacteria in the gut¹⁹². Therefore, prebiotic intake may alter both the gut microbial metabolites and the community composition, and therefore microbial ligands, which have the potential to have systemic effects on host immunity.

The number of studies investigating prebiotics alone on the immune response to influenza vaccination in human trials are limited and results have been mixed. One study found that administration of a prebiotic mixture of β 2-1 fructans, inulin and oligofructose, to a healthy cohort of adults prior to TIV significantly enhanced the vaccine-induced IgG1 antibody response²¹⁰. Administration of an oligosaccharide mixture in an elderly cohort prior to influenza vaccination had no effect on vaccine-specific antibody in the serum²¹¹. Again, the type of prebiotic, dosages and duration of treatment vary widely between studies and these factors are likely to be crucial to their efficacy. It should also be noted that, unlike probiotics where effects of administration are undoubtedly microbiota-dependent, difficulty can arise in determining if a prebiotic is exerting immunomodulatory effects via the microbiota, as foods may be affecting immune pathways in other ways.

As many foods contain MACs, notably vegetables and fruits, they have prebiotic potential and may be 'functional foods': foods that deliver health benefits additional to providing basic nutrition. Further research into the potential benefit of prebiotic functional foods in immune responses to influenza vaccination are required.

1.4 Aims and hypotheses

Whilst vaccination protects against influenza infection in much of the population, a significant proportion of individuals fail to elicit sufficient protective antibody responses and a strong need remains to improve the efficacy of these vaccines. The literature presented so far demonstrates that the gut microbiota exerts an influence over many facets of the host immune system, not only in promoting homeostasis locally, but also having a pervasive effect on sites distal from the intestine. Recent investigations have identified pathways influenced by the gut microbiota to promote protective responses to both influenza infection^{157,212} and vaccination¹⁵⁸, however it is clear that the microbiota works in a multitude of ways. The first aim of this investigation was to establish an antibiotic model in which vaccine-specific antibody production was rendered defective and subsequently use this model to investigate what immune cell processes to influenza vaccination are impacted by manipulating the gut microbiota.

Furthermore, because the gut microbiota is able to significantly influence immune responses, this opens up avenues for microbiota-altering therapies for treating immune-modulated disorders and enhancing vaccine responsiveness. However, evidence for the efficacy of specific foods in altering the microbiota and enhancing the immune response to influenza vaccination are lacking. Therefore, my second aim was to test the efficacy of a number of microbiota-altering nutritional interventions to improve the immune response to influenza vaccination.

Specific aims:

- 1) To develop a model of oral antibiotic administration that negatively affects antibody responses to TIV vaccination.
- 2) To assess changes in immune cell frequency and function in the humoral immune response to TIV vaccination following microbiota alteration with antibiotic treatment.
- 3) To establish mouse models of parenteral and mucosal influenza vaccination that model low-responding human populations, characterised by suboptimal antibody production and protection against subsequent infection.
- 4) To assess the capacity of orally administered nutritional interventions with microbiota-altering potential to improve suboptimal immune responses in the influenza vaccination model.

2 Materials and Methods

2.1 Materials

2.1.1 Labware

Table 2.1: List of labware

Product	Supplier
22-gauge gavage needle	Total Lab Systems, Auckland, New Zealand
Axygen filtered, sterile, RNase free pipette tips – 10 μ L, 20 μ L, 200 μ L and 1000 μ L	Axygen Scientific, California, United States
Axygen microtubes – 0.6 mL, 1.7 mL and 2.0 mL	
Axygen pipette tips – 10 μ L, 20 μ L, 200 μ L and 1000 μ L	
BD 5 mL polystyrene round-bottom tubes	BD Biosciences, Auckland, New Zealand
BD Falcon 70 μ m cell strainer	
BD Falcon cell scrapers, 25 cm handle and 1.8 cm blade	
BD Falcon polystyrene conical tubes - 15 mL, 50 mL	
BD Falcon polystyrene flat-bottom plates - 96-well, 24-well	
BD Falcon polystyrene round-bottom plates - 96-well	

BD Falcon serological pipettes – 2 mL, 5 mL, 10 mL and 25 mL	
BD PrecisionGlide needles - 18 gauge 40 mm, 25 gauge 40 mm	
BD syringes - 1 mL, 2 mL	
BioRad multiplate 96-well PCR plates, unskirted, clear	BioRad, Auckland, New Zealand
Corning polystyrene v-bottom plates – 96-well	Sigma Aldrich, Auckland, New Zealand
Disposable carbon steel surgical blades	Swann-Morton, Sheffield, United Kingdom
Eppendorf safe-lock microcentrifuge tubes - 2ml	Sigma Aldrich, Auckland, New Zealand
Interlab sealplate classic films	Interlab, Wellington, New Zealand
MACS SmartStrainer 70 µM	Pharmaco, Auckland, New Zealand
MicroAmp 0.1 mL fast optical 96-well reaction plate with barcode	ThermoFisher Scientific, Auckland, New Zealand
MicroAmp optical adhesive film	
Nalgene cryogenic vials – 2 mL	Sigma Aldrich, Auckland, New Zealand
Nunc 96-well Maxisorb plates	ThermoFisher Scientific, Auckland, New Zealand
Qiagen 5 mm stainless steel beads	Bio-Strategy, Victoria, Australia
Sarstedt Microvette 500 serum gel tubes	Global Science and Technology Ltd, Auckland, New Zealand

Satorius Vivaspin 20 10,000 MWCO column	MicroNZ, Martinborough, New Zealand
Slide-A-Lyzer 10,000 MWCO dialysis cassettes	Thermofisher Scientific, Auckland, New Zealand
Sterilin 139 mm sterile petri dishes	

2.1.2 Reagents

Table 2.2: List of reagents

Product	Supplier
Ambion DEPC-treated H ₂ O	Thermofisher Scientific, Auckland, New Zealand
Ambion ethylenediaminetetraacetic acid (EDTA)	
Ampicillin sodium salt	Sigma Aldrich, Auckland, New Zealand
Applied Biosystems SYBR Green PCR Master Mix	Thermofisher Scientific, Auckland, New Zealand
Applied Biosystems TaqMan Fast Advanced Master Mix	
BD compensation beads anti-rat anti-hamster	BD Biosciences, Auckland, New Zealand

<i>Bifidobacterium animalis</i> subsp. <i>lactis</i> lyophilised powder	Fonterra, Auckland, New Zealand
Chloroform	Sigma Aldrich, Auckland, New Zealand
Dulbecco's phosphate buffered saline (PBS) powder	Thermofisher Scientific, Auckland, New Zealand
Ethanol (100%)	Pure Science, Porirua, New Zealand
Fresh Zespri Sungold kiwifruit	Zespri, Mount Maunganui, New Zealand
Fructose	Sigma Aldrich, Auckland, New Zealand
Gibco Dulbecco's Modified Eagle Medium (DMEM)	Thermofisher Scientific, Auckland, New Zealand
Gibco foetal bovine serum (FBS)	
Gibco Iscove's Modified Dulbecco's Medium (IMDM)	
Gibco sterile phosphate-buffered saline (PBS)	
Gibco trypan blue (0.4%)	

Glucose	Sigma Aldrich, Auckland, New Zealand
Hansell's strawberry essence	Countdown, Wellington, New Zealand
High Capacity RNA to cDNA kit	Thermofisher Scientific, Auckland, New Zealand
HRP-conjugated anti-mouse IgG secondary antibody	
HRP-conjugated anti-mouse IgG1 secondary antibody	
HRP-conjugated anti-mouse IgA secondary antibody	Southern Biotech, Birmingham, USA
HKx31 influenza A virus	Gifted from Lisa Hyland and Sam Hou, Otago University
Indian Ink	Whitcoulls, Wellington, New Zealand
Influvac® 2016 Inactivated TIV Vaccine	Mylan, Auckland, New Zealand
Invitrogen OneComp eBeads Compensation Beads	Thermofisher Scientific, Auckland, New Zealand
Invitrogen Trizol Reagent	

Isopropanol	Sigma Aldrich, Auckland, New Zealand
Metronidazole	
Mouse IgA Ready-SET-Go ELISA kit	Thermofisher Scientific, Auckland, New Zealand
Mylan Vancomycin	Pharmacy Department, Wellington Hospital, New Zealand
Neomycin trisulfate	Sigma Aldrich, Auckland, New Zealand
Pierce Bicinchoninic Acid Protein Assay (BCA assay) Kit	Thermofisher Scientific, Auckland, New Zealand
Propolis formulated with γ -cyclodextrin (Cyclopower) powder	Manuka Health, Te Awamutu, New Zealand
QIAamp Fast DNA Stool Mini Kit	BioStrategy, Auckland, New Zealand
DNase I	Roche, Mannheim, Germany
Liberase TL	
Sodium acetate	Sigma Aldrich, Auckland, New Zealand
Sodium butyrate	

Sodium propionate	
Splenda artificial sweetener	Countdown, Wellington, New Zealand
Sucrose	Sigma Aldrich, Auckland, New Zealand

2.1.3 Buffers and reagents

Flow buffer

FBS was added to PBS to give a final concentration of 2% FBS.

Anaesthetic working solution

A 10x stock solution of ketamine/xylazine anaesthetic solution was provided by the Biomedical Research Unit (BRU) at the Malaghan Institute of Medical Research (MIMR (Wellington, New Zealand)) at a concentration of 87 mg/mL of ketamine and 2.6 mg/mL of xylazine. This was diluted 1:10 in sterile PBS for a 1x working solution for intraperitoneal administration.

Lymph node digestion mix

IMDM was supplemented with 1 µg/mL liberase TL and 100 µg/mL DNase I.

2.1.4 Flow cytometry antibodies

Table 2.3: List of flow cytometry antibodies

Specificity	Fluorophore	Clone	Supplier	Catalogue #
B220/CD45R	PE-CF594	RA3-6B2	BioLegend	103254
	BV786	RA3-6B2	BD Biosciences	563894
CD11b	BUV737	M1/70	BD Biosciences	564443
CD11c	PECy7	HL3	BD Biosciences	558079
CD138	BUV737	281-2	BD Biosciences	564430
	PE	281-2	BD Biosciences	553714
CD3	PE	145-2C11	Thermofisher Scientific	12-0031-82
CD32/16 (FcγRII/III)	-	2.4G2	MIMR	-
CD326	BV711	G8.8	BD Biosciences	563134
CD4	BUV805	GK1.5	BD Biosciences	564922
CD86	BUV395	GL1	BD Biosciences	564199

CXCR5	Biotin	2G8	BD Biosciences	551960
F4/80	Biotin	BM8	BioLegend	123105
	FITC	NAWESLEE	Thermofisher Scientific	13-7691-82
GL7	FITC	GL7	BD Biosciences	553666
IgD	APC-H7	IA6-2	BD Biosciences	561305
IgG1	BV421	A85-1	BD Biosciences	562580
IgM	APC	II/41	BD Biosciences	550676
Ly6C	Pacific blue	HK1.4	BioLegend	128013
MHC II (I-A/I-E)	AF647	M5/114.15.2	BioLegend	107618
Streptavidin	BV605	-	BD Biosciences	563260
	BUV395	-	BD Biosciences	564176

2.1.5 Flow cytometry viability dyes

Table 2.4: List of flow cytometry viability dyes

Dye	Supplier
4',6-diamidino-2-phenylindole (DAPI)	Thermofisher Scientific, Auckland, New Zealand
Live/dead fixable blue dead cell dye	
Zombie NIR fixable viability dye	BioLegend, Auckland, New Zealand

2.1.6 Mice

2.1.6.1 Animal husbandry and ethics

Mice were bred and housed within the BRU at MIMR in specific-pathogen free (SPF) conditions. Unless specified, mice were maintained on autoclaved meat-free rat and mouse diet (Specialty Feeds, Western Australia) and autoclaved acidified water. The Victoria University Animal Ethics Committee granted ethical approval for experimental animal procedures and all experiments were carried out in accordance with their guidelines. Mice were treated under the animal ethics protocols 22735 and 2014R16M.

2.1.6.2 Mouse strains

C57BL/6 mice were originally sourced from the Jackson laboratory (Bar Harbor, ME, USA), and bred at the MIMR BRU.

2.2 Methods

2.2.1 Mouse manipulations

2.2.1.1 Broad-spectrum antibiotic cocktail administration

Broad-spectrum antibiotic cocktail (ABX) consisted of vancomycin at 0.5 mg/mL, neomycin at 1 mg/mL, ampicillin at 1 mg/mL and metronidazole at 1 mg/mL, dissolved in purified, acidified drinking water provided by the MIMR BRU. This was administered to mice *ad libitum* for the amount of time specified in each experiment. Splenda artificial sweetener (4 mg/mL) and Hansell's strawberry essence (2 µL/mL) were added to improve palatability of the solution.

2.2.1.2 Trivalent influenza vaccine (TIV) administration

Influvac® 2016 inactivated TIV vaccine was diluted 1:4 in sterile PBS and 200 µL was administered subcutaneously (SC) at the tail base between the dorsal and right lateral vein, with mice receiving 1/10 of the full human dose of 500 µL.

2.2.1.3 Inactivated HKx31 virus vaccine administration

Inactivated HKx31 vaccines were diluted in sterile PBS at specified concentrations. For SC vaccination, 200 µL of the vaccine was administered at the base of the tail at the specified dose. For intranasal (IN) vaccination, mice were anaesthetised by intraperitoneal injection of 200 µL 1x ketamine/xylazine solution in sterile PBS and 50 µL of the vaccine was administered IN at the specified dose.

2.2.1.4 Lymph node mapping

Indian ink was diluted 1:10 in sterile PBS. 20 µL was administered subcutaneously at the tail base between the dorsal and right lateral vein. After 2 hours, mice were euthanised, dissected and photographed to visualise ink drainage from each injection site.

2.2.1.5 Rodent diets

Meat-free rat and mouse standard diet, zero-fibre, zero-starch rodent diet (SF15-086), modified SF15-086 with 7.5% pectin, 7.5% inulin high fibre rodent diet (SF17-017), standard AIN93G rodent diet and modified AIN93G rodent diet with 8% Propolis formulated with cyclodextrin powder (25% and 75%, respectively) (SF17-055) were purchased from Specialty Feeds, Western Australia. All foods were autoclaved or irradiated prior to feeding for sterility.

Unless otherwise stated, mice were maintained on meat-free rat and mouse standard diet. Other than the specified dietary alterations, paired diets in each experiment were designed to be otherwise nutritionally equivalent. Mice were administered foods *ad libitum* for the duration specified in each experiment.

2.2.1.6 Sungold kiwifruit administration

Zespri Sungold kiwifruit (*Actinidia chinensis*) was processed to a puree for intragastric (IG) administration. The surface of whole Sungold kiwifruit were sterilised by wiping with 70% ethanol and skin was removed. Kiwifruit was blended in Brabantia Stainless Steel Blender (Briscoes, Wellington, New Zealand) to a homogenous puree which was then put through a MACS 70 μ M cell strainer in order to reach a suitable consistency for administration. Kiwifruit puree was stored at -20°C and defrosted at room temperature (RT) prior to administration. Control groups received fructose at 53 g/L, glucose at 58 g/L and sucrose at 12 g/L dissolved in autoclaved H₂O, matching the sugar profile of Sungold kiwifruit. Mice were administered solutions intragastrically using a stainless steel 22-gauge gavage needle. From day -14 to day -8 of experiments, 300 μ L of kiwifruit was administered, from day -7 to day -1 mice 400 μ L of kiwifruit was administered and from day 0 for the entirety of the experiment, 500 μ L of kiwifruit was administered. Lower doses of kiwifruit were

administered over the first 2 weeks of the experiment as 500 μL doses were not well tolerated by mice younger than 6 weeks of age.

2.2.1.7 Bifidobacterium animalis subsp. lactis administration

Bifidobacterium animalis subsp. *lactis* HN019 (*B. animalis*) was dissolved in autoclaved H_2O . Each mouse received 10^9 colony forming units (CFU) of bacteria in 200 μL IG using a stainless steel 22-gauge gavage needle. Control groups received the same volume of autoclaved H_2O .

2.2.1.8 Short-chain fatty acid administration

Solutions of single SCFAs were prepared by adding 200 mM of sodium acetate, sodium butyrate or sodium propionate to autoclaved acidified drinking water, at a concentration of 200mM and administered to mice *ad libitum* for 21 days prior to vaccination and throughout the remainder of the experiment.

A mixed SCFA solution was prepared by adding sodium acetate (70 mM), sodium butyrate (20 mM) and sodium propionate (30 mM), to autoclaved acidified water and was administered to mice *ad libitum* for 14 days prior to initial vaccination and throughout the remainder of the experiment.

2.2.1.9 Euthanising mice

Mice were euthanised by asphyxiation in a CO_2 chamber, followed by immediate cervical dislocation to ensure lethality. Mice also were euthanised by cervical dislocation or by cervical dislocation alone. Alternately, mice were euthanised by lethal anaesthesia by intraperitoneal injection of 1000 μL 1x ketamine/xylazine solution in sterile PBS.

2.2.2 Tissue collection and processing

2.2.2.1 Mouse serum collection

For non-lethal serum collection at the lateral tail vein, mice were warmed with a heat lamp to promote vasodilation prior to blood collection. A disposable sterile

surgical blade was used to make a small incision at the lateral tail vein and 3 to 4 drops of blood were collected into a Microvette gel serum collection tube. Pressure was applied to the incision until bleeding ceased. Alternatively, the submandibular vein was punctured and 3 to 4 drops of blood were collected into a Microvette gel serum collection tube.

For lethal serum collection, mice were euthanised by CO₂ asphyxiation and blood was collected by cardiac puncture into a Microvette gel serum collection tube.

Following collection, blood samples were centrifuged for 5 minutes at 13,000 × g and the serum fraction was stored at -20°C for further analysis.

2.2.2.2 Mouse faeces and caecal content collection

For non-lethal faecal sample collection, mice were placed in clean, separate cages until faecal samples could be collected.

For lethal caecal content collection, mice were euthanised by CO₂ asphyxiation and caecum was dissected and contents collected under sterile condition. All samples were stored in 0.6 mL microtubes at -70°C for further processing.

2.2.2.3 Bronchoalveolar lavage and nasal lavage collection

To quantify secretory antibody in the pulmonary airways and nasal passage, bronchoalveolar lavage (BAL) and nasal lavage were collected. Mice were euthanised by lethal anesthesia as previously described and a longitudinal cut was made at the neck of the mouse, revealing the trachea. To collect BAL, a cannulated syringe was inserted into the trachea towards the lung and 500 µL of sterile PBS was dispensed into the lung then the maximal amount was withdrawn into the syringe and collected, this was repeated once. To collect nasal lavage, a transverse, semi-lunar cut was made in the trachea, a cannulated syringe was inserted into the trachea towards the nose and 500 µL of sterile PBS

was dispensed rapidly through the nose and collected for analysis. The samples were centrifuged for 3 minutes at $13,500 \times g$ at 4°C and the supernatant was collected to exclude any red blood cells. Samples were stored at -20°C for further analysis.

2.2.2.4 Lung dissection

To quantify viral load following influenza infection, lungs were collected. Mice were euthanised by cervical dislocation and a longitudinal cut was made through the upper torso of the mouse through the ribs. Lungs were excised, any surrounding connective tissue was removed, the tissue was then placed in a cryogenic vial and immediately snap-frozen in liquid nitrogen, before being stored at -80°C for further analysis.

2.2.2.5 Mouse faecal lysate

To quantify secretory IgA in the faeces, faecal lysates were obtained. Briefly, fresh faecal pellets were weighed and homogenised in PBS containing 0.1% Tween 20 at 100 mg/ 100 μL . The samples were centrifuged for 10 minutes at $13,500 \times g$ at 4°C and the supernatant was collected. Samples were stored at -20°C until analysed.

2.2.2.6 Lymph node cell isolation

Following euthanasia by CO_2 asphyxiation, LNs were collected into cold PBS and immediately stored on ice. For flow cytometry analysis of lymphocytes, LNs were mashed through a $70 \mu\text{m}$ cell strainer and washed through with PBS to form a single-cell suspension. Cells were then centrifuged at $500 \times g$ for 5 minutes and the supernatants were discarded. Cells were resuspended in flow buffer for cell counting and preparation for flow cytometry.

For flow cytometry analysis of myeloid cells, LNs were digested to liberate cells from the organ. LNs were added to 1 mL of 1 $\mu\text{g/mL}$ liberase TL and 100 $\mu\text{g/mL}$

DNase I in IMDM, torn apart with 18 gauge needles and incubated at 37°C for 25 minutes. To LNs, 20 µL of EDTA was added and incubated for a further 5 minutes at 37°C. Cells were resuspended with a 1 mL pipette to liberate cells and then mashed through a 70 µm cell strainer and washed through with IMDM. Cells were centrifuged at 500 x g for 5 minutes and supernatants were discarded. Cells were resuspended in flow buffer for further cell counting and for use in flow cytometry.

2.2.3 Production of inactivated viral vaccines

2.2.3.1 Formalin inactivation

Formalin inactivation protocol was performed according to previous publication demonstrating complete viral inactivation²¹³. Briefly, live HKx31 virus stock at 20,480 HAU/mL was incubated at 37°C in formalin at a final concentration of 0.02% in PBS for 72 hours. Solutions were transferred to 10,000 MWCO Slide-A-Lyzer dialysis cassette and dialysed in a 1 L glass beaker containing 500 mL of DMEM. The process was repeated twice for 2 hours each and once overnight, on a IKA Colour Squid magnetic plate stirrer (Sigma). Dialysed vaccine was diluted to 1000 HAU/mL and stored at -70°C until required.

2.2.3.2 Ultraviolet light inactivation

Ultraviolet (UV) irradiation inactivation protocol was performed according to Zou and colleagues, demonstrating complete viral inactivation following greater than 30 minutes of UV irradiation²¹⁴. Briefly, live influenza A HKx31 virus stock at 20,480 HAU/mL was spread in a thin layer at 1.5 mL per sterile petri dish with a cell scraper. Petri dishes were placed in a Herasafe Class II Biological Safety Cabinet (Thermofisher Scientific) and UV radiation was applied for 45 minutes, ensuring no shadows blocked exposure of the viral suspension to radiation. Inactivated vaccine was resuspended at 10,000 HAU/ mL in PBS and stored at -

70°C until required. Annual testing ensures UV radiation in the safety cabinet reaches the required UV radiation safety standards for sterilisation.

2.2.4 Enzyme-Linked Immunosorbent Assay (ELISA)

2.2.4.1 TIV-specific IgG and IgG1

Levels of TIV-specific IgG and IgG1 antibody in mouse serum were determined by ELISA. Briefly, each well of a 96 well Nunc Maxisorb plates was coated with 100 μ L of Influvac® diluted to 25 μ L/mL in PBS and incubated overnight at 4°C. Non-specific protein binding was blocked with the addition of 150 μ L of 10% FBS in PBS and incubated for 2 hours at RT. Serum samples were serially diluted in 10% FBS in PBS and 100 μ L was added to each well. The plates were then incubated for 2 hours at RT. HRP-conjugated anti-mouse IgG and IgG1 secondary antibody were diluted in 10% FBS in PBS at 1:5000 or 1:1000. 100 μ L was added to each well and plates were incubated for 2 hours at RT. Finally, 100 μ L of OptEIA TMB substrate was added and after 5-10 minutes the reaction was stopped with 50 μ L of 1M sulphuric acid. The absorbance was read on Versa Max microplate reader at 450 nm. Before the initiation of each step, plates were washed five times with 0.05% Tween 20 in PBS, except following the blocking step. Relative antibody levels were expressed as optical density at 450 nm (O.D. 450) at the appropriate non-saturating serum concentration.

2.2.4.2 HKx31 virus-specific IgG, IgG1 and IgA

Levels of HKx31 virus-specific IgG and IgG1 antibody in mouse serum and IgA in mouse BAL and nasal lavage were determined by ELISA assay. Briefly, 96 well Nunc Maxisorb plates were coated with 100 μ L of HKx31 virus stock diluted to 10 HAU/ μ L in PBS and incubated overnight at 4°C. Non-specific protein binding was blocked by addition of 150 μ L of 10% FBS in PBS and plates were incubated for 2 hours at RT. Samples were serially diluted in 10% FBS in PBS and 100 μ L was added per well. Plates were incubated for 2 hours at RT.

HRP-conjugated anti-mouse IgG, IgG1 and IgA secondary antibodies, were diluted in 10% FBS in PBS at 1:5000, 1:1000 and 1:4000, respectively. Each well received 100 μ L of diluted secondary antibody and incubated for 2 hours at RT. Finally, 100 μ L of OptEIA TMB substrate was added to each well and 5-10 minutes later the reaction was stopped with the addition of 50 μ L 1M sulphuric acid to each well. The absorbance was read on Versa Max microplate reader at 450 nm. Before each step, plates were washed five times with 0.05% Tween 20 in PBS.

2.2.4.3 IgA

IgA levels in the serum and faeces were measured using mouse IgA Ready-SET-Go ELISA kit according to the manufacturer's instructions. Samples were serially diluted as needed for accurate readings.

2.2.5 Quantitative PCR

2.2.5.1 Lung RNA extraction

The right lung was placed in a sterile petri dish, roughly chopped with a disposable steel blade and placed in a cliplock tube with 1 mL of TRIzol reagent and two stainless steel beads. Samples were homogenised in a tissue lyser (Tissue Lyser II, Qiagen) for 2 minutes at 30 Hz. This was repeated with samples switched to the opposite arm of the tissue lyser to ensure equal homogenisation between samples. Samples were centrifuged for 10,000 $\times g$ for 3 minutes at 4°C and the supernatants were transferred to a sterile 2 mL microtube. Each sample received 200 μ L of chloroform, shaken by hand for 15 seconds and left to rest for 5 minutes at RT. Samples were then centrifuged at 12,000 $\times g$ for 15 minutes at 4°C. The transparent, colourless top layer was transferred from each sample to a sterile 1.7 mL microtube and 500 μ L of isopropanol was added. Samples were incubated at RT for 10 minutes then centrifuged for 12,000 $\times g$ for 10 minutes at 4°C. The supernatants were discarded and each sample was washed with 1 mL

of 70% ethanol and centrifuged at $10,000 \times g$ for 5 minutes at 4°C . The supernatants were discarded and samples were centrifuged at $10,000 \times g$ for 1 minute at 4°C . Excess ethanol was carefully removed, leaving the RNA pellet in the microtube. The pellet was left to air dry for 5-10 minutes and resuspended in $40 \mu\text{L}$ of DPEC-treated H_2O . RNA concentration and purity was measured on a ND-1000 Nanodrop spectrophotometer (Thermofisher Scientific). Samples were stored at -70°C until required.

2.2.5.2 RNA to cDNA conversion

RNA was converted to complementary DNA (cDNA) using the High Capacity RNA to cDNA Kit according to the manufacturers specifications. The reaction was run in a Veriti 96-well Thermo Cycler (Thermofisher Scientific), incubated for 60 minutes at 37°C followed by 5 minutes at 95°C to stop the reaction and then held at 4°C . cDNA was stored at 4°C for up to 3 days or at -20°C for long term storage until required.

2.2.5.3 Faecal bacteria DNA extraction

Bacterial DNA was extracted from faecal samples using QIAamp Fast DNA Stool Mini Kit according to the manufacturer's instructions. DNA concentration and purity was measured on a ND-1000 Nanodrop spectrophotometer (Thermofisher Scientific). Samples were stored at -20°C until required.

2.2.5.4 Quantitative PCR

Determination of influenza virus viral load in lung

A custom FAM-labelled TaqMan influenza A probe was purchased from Life Technologies for RNA analysis as described previously⁴⁸. This consisted of influenza A virus sense primer ($5' -\text{AAGACCAATCCTGTCACCTCTGA}-3'$), antisense primer ($5' -\text{CAAAGCGTCTACGCTGCAGTCC}-3'$) and probe (FAM- $5' -\text{TTTGTGTTACGCTCACCGT}-3' -\text{TAMRA}$). All primers were

specific for the influenza A virus matrix protein. VIC-labelled TaqMan *Gapdh* probes were used as an endogenous control (Assay ID NM_008084.2; Thermofisher Scientific). A qPCR reaction mix was first prepared by combining per reaction; 10 μ L TaqMan master mix, 1 μ L *Gapdh* housekeeping gene probe, 1 μ L influenza A virus probe and 5 μ L of DPEC-treated H₂O. To each well of an optical 96-well reaction plate with barcode, 17 μ L of prepared qPCR reaction mix and 3 μ L of the cDNA sample were added. The plate was covered with an optical adhesive film and centrifuged for approximately 30 seconds at 500 x g. All samples were run in duplicate and each plate included an H₂O sample as a negative control. qPCR was run on a QuantStudio 7 Flex PCR system (Thermofisher Scientific) using the standard instrument settings for SYBR green 96-well plate assay.

Determination of predominant bacteria in faeces

Proportions of dominant bacteria in the faeces were determined by quantitative PCR as previously described²¹⁵. A qPCR reaction mix was prepared by combining per reaction; 10 μ L SYBR green PCR master mix, 1 μ L each of forward and reverse primers (Supplementary table 1) and 6 μ L of DPEC-treated H₂O. To each well of an optical 96-well reaction plate with barcode, 18 μ L of prepared qPCR reaction mix and 2 μ L of the DNA sample (standardised to 1 ng/ μ L) were added. The plate was covered with an optical adhesive film and centrifuged for approximately 30 seconds at 500 x g. All samples were run in duplicate and each plate included an H₂O sample as a negative control. qPCR was run on a QuantStudio 7 Flex PCR system (Thermofisher Scientific) using the standard instrument settings for fast TaqMan 96-well plate assay.

2.2.6 Total protein quantification

Total protein was quantified using the Pierce bicinchoninic acid (BCA) protein assay according to the manufacturers instruction. Samples were serially diluted in PBS in order to obtain optimal sample protein concentrations for accurate measurements.

2.2.7 TIV vaccine AF488 protein labelling

Initially, TIV was concentrated to 2 mg protein/mL by placing on a Vivaspin 20 10,000 MWCO column and centrifuged at 3000 × *g*. Protein concentration was determined by BCA assay and vaccine was diluted to 2 mg/mL in PBS for labelling. Concentrated TIV vaccine was labelled with Alexa Fluor 488 dye using the Alexa Fluor 488 Protein Labelling Kit (Thermofisher Scientific, Auckland, New Zealand) according to the manufacturer's instructions. When specified in the experiment, the vaccine was subsequently washed. To do so, the labelled vaccine was placed on a Vivaspin 20 10,000 MWCO column and topped up to 20mL with sterile PBS, this was repeated once. Following labelling, protein concentrations were measured by BCA assay and diluted in sterile PBS to equal concentration as unlabeled TIV vaccine. All labelling procedures were performed in sterile conditions.

2.2.8 Flow cytometry

2.2.8.1 Controls

Controls used for flow cytometry experiments included; unstained cells, viability dye single-stained cells, fluorescence minus one (FMO) stained cells and single-stained compensation beads.

2.2.8.2 Cell surface staining

Flow cytometry antibodies were titrated for optimal concentration prior to use in experiments. Antibodies were centrifuged at $12,000 \times g$ and only the supernatant was taken to exclude the use of aggregated antibody. Single cell suspensions from LNs were obtained as previously described (in section 1.1.1) and counted using trypan blue exclusion. Between $1-5 \times 10^6$ cells were plated per well of a 96-well V-bottom plate, centrifuged at $500 \times g$ for 3 minutes and supernatants were discarded. If fixable viability dye was used, samples were resuspended in 50 μ L of viability dye diluted in PBS and incubated for 10 minutes on ice. Samples were washed by addition of 150 μ L of flow buffer and centrifuged for 3 minutes at $500 \times g$, supernatants were discarded. All subsequent washes were performed in this manner. Samples were resuspended in 50 μ L of flow buffer containing anti-CD16/CD32 antibody for 10 minutes to prevent non-specific binding of antibodies to Fc receptor-expressing cells. If biotinylated antibodies were used, cells were resuspended in 50 μ L of flow buffer containing the biotinylated antibody, incubated for 30 minutes at 37°C then washed twice. Cells were then resuspended in 50 μ L of cell surface antibodies diluted in flow buffer, including any streptavidin-conjugated fluorophores if used, and were incubated for 30 minutes at 4°C , washed twice and resuspended in 150 μ L of flow buffer for acquisition. DAPI viability dye was diluted in flow buffer and added to samples immediately prior to acquisition.

2.2.8.3 Intracellular staining

For antigens located within the cell, cells were permeabilised prior to staining to allow antibody to enter the cell. Cells were fixed and permeabilised using the FoxP3/Transcription Factor Staining Buffer Set (eBioscience) as per manufacturer's specifications. Briefly, cells were stained for cell surface antigens as described previously (In X.X.X) and washed twice. Prior to intracellular staining, one part fixation/permeabilisation concentrate was diluted with three

parts fixation/permeabilisation diluent to yield a 1x fixation/permeabilisation solution and one part permeabilisation buffer was diluted with nine parts PBS to yield 1x permeabilisation buffer. Cells were resuspended in 100 μ L of fixation/permeabilisation solution and incubated at 4°C for 30 minutes. Cells were washed twice with 150 μ L of permeabilisation buffer, centrifuged at 500 x g for 3 minutes and supernatants were discarded. Cells were then resuspended in 50 μ L of intracellular antibodies diluted in permeabilisation buffer and incubated for 1 hour at RT. Cells were washed twice and resuspended in 200 μ L of flow buffer for acquisition.

2.2.8.4 Acquisition and analysis

Samples were analysed on either an LSR-II special order product (BD) or an LSR Fortessa (BD) flow cytometer.

The LSR-II was equipped with the following lasers and detectors: Ultra violet laser (355 nm) UV740/35, UV450/50 and UV379/28. Violet laser (405 nm) V780/60, V720/40, V660/20, V605/40, V560/40, V525/50 and V450/50. Blue laser (488 nm) B705/70, B515/20 and B488/10. Green laser (532 nm) G780/60, G610/20 and G575/26. Red laser (640 nm) R780/60, R710/50 and R670/14.

The LSR Fortessa was equipped with the following lasers and detectors: Violet laser (405 nm) V780/60, V710/20, V660/20, V610/20, V560/40, V525/50 and V450/50. Blue violet laser (445 nm) BV504/12. Blue laser (488 nm) B685/35, B515/20 and B488/10. Green laser (532 nm) G780/60, G695/40, G670/30, G610/20 and G575/26. Red laser (640 nm) R780/60, R710/50 and R670/14.

All fluorophores were detected using laser and detector combinations that best matched their excitation and emission profile.

Following acquisition, FCS files were analysed using FlowJo software (Tree Star, San Carlos, CA, USA). For all flow cytometry analysis, cells were gated first by

excluding doublets using forward and side scatter. Dead cells were removed from analysis by gating on viability dye negative cells. Cells of interest were identified by forward and side scatter profile before immunophenotyping (Figure 2.1).

2.2.9 Statistics

Statistical analysis was performed using Prism software (GraphPad Prism Version 6.0 for MacIntosh, GraphPad Software Incorporated, USA). Figure legends state the statistical analysis used for each experiment.

For experiments comparing only two groups, statistics were obtained using a Mann Whitney test. In experiments with three or more groups, statistics were obtained using a one-way ANOVA with Bonferroni's post-test. Tukey's post-test was applied when comparing every mean. Dunnett's post-test was applied when comparing every mean with a control mean. Bonferroni's post-test was applied when a set of means were selected to compare.

In experiments comparing groups between time points, statistics were obtained using a two-way ANOVA with a Bonferroni's post-test. Relevant statistics are shown in figures.

Figure 2.1: Pre-gating strategy for flow cytometry analysis. Representative pre-gating strategy to select for single, viable cells of interest when analysing **(A)** lymphocytes or **(B)** myeloid cells and lymphocytes.

3 Evaluating the gut microbiota influence on antibody responses to influenza vaccination

3.1 Introduction

TIV vaccination acts by inducing the production of IgG isotype antibodies which protect against infection and a critical threshold of anti-influenza antibodies need to be mounted to confer protection, termed seroconversion^{107,108}. However, there is a high degree of variability in the titre of antibody reached across individuals within a population, with some individuals not reaching seroconversion. The mechanisms by which the vaccine elicits high levels of protective antibody in some individuals, whilst inducing weak responses in others, remains incompletely understood. Interestingly, transcriptome analysis of human PBMCs following TIV vaccination has revealed that expression of TLR-5, the PRR for bacterial flagellin³⁵, was strongly correlated with the level of virus-neutralising antibody in the serum of individuals³⁶. Further investigations indicated that antibody responses to TIV were severely impaired in TLR-5 deficient mice, as well as GF and antibiotic-treated mice, illustrating that microbial flagellin may act as an adjuvant to boost host antibody responses to the vaccine¹⁵⁸. TLR-5 signalling on M ϕ was demonstrated to be important for expression of CD86 and IL-6, and direct signalling through TLR-5 on B cells was shown to promote differentiation to PCs, both crucial for robust TIV-specific antibody production¹⁵⁸.

There is a growing body of research demonstrating that host recognition of immunostimulatory components of gut microbes or their metabolites can influence the development and function of many immune cell subsets to enhance local and systemic immune responses. Gut microbial signals influence the development of multiple innate immune cell populations systemically by altering haematopoiesis in primary lymphoid organs, via both TLR signalling³⁷ and microbiota-produced circulating SCFAs³⁸. Additionally, the microbiota is

required for maintaining the ability for innate immune cell populations to mount effective responses to infectious challenges. Type I interferon (IFN-I) production following viral encounter by mononuclear phagocytes is impaired in GF and antibiotic-treated mice, leading to a failure to prime NK cells and a decrease in viral control³⁹. Microbiota signalling has also been shown to be instrumental for inflammasome activation in DCs following influenza infection, promoting the migration of DCs to secondary lymphoid organs and expression of costimulatory molecules, that in turn promote T and B cell responses⁴⁰. Antibiotic administration also leads to decreased numbers of M ϕ in the lung following influenza infection, which express lower levels of CD86⁴¹. Microbiota-derived SCFAs directly enhance peripheral PC differentiation to boost baseline concentrations of IgG antibody in the blood⁴¹. This demonstrates the ubiquitous and systemic influence of the gut microbiota on various cell subsets of the innate and adaptive immune system. As the humoral immune response requires the concerted action of a number of immune cell populations, including B cells, T cells, DCs and M ϕ , it is likely that previously demonstrated microbiota effects are playing a role in the humoral immune response to TIV vaccination.

Antibiotic treatment reduces intestinal bacterial load that in turn lowers host systemic exposure of the host to microbial stimuli, including circulating microbial metabolites such as SCFAs⁴² and flavonoid degradation products⁴³, as well as bacterial cell components in the serum, such as peptidoglycan⁴⁴. Administration of broad spectrum antibiotics to adult mice does not completely ablate the microbiota⁴⁵ or impair early immune system development as is observed in GF mice⁷ and so serves as a physiologically relevant model of the dysbiotic phenotype in the human population¹³ to examine microbiota influence on immune responses to TIV.

3.2 Aims

Based on the ubiquitous influence of the microbiota on multiple immune cell compartments, it is highly likely the host is integrating many signals from the microbiota in addition to the requirement for flagellin signalling via TLR-5 to mount optimal antibody responses to TIV vaccination. Therefore, in this chapter the impact of alterations to microbial signalling on a number of cellular processes in the generation of the humoral immune response to TIV vaccination were examined by manipulating microbiota populations through antibiotic treatment.

Specific aims:

- 1) To develop a model of oral antibiotic administration that reduces antibody response to TIV vaccination.
- 2) To assess changes in immune cell frequency and function in the humoral immune response to TIV following microbiota alteration with antibiotic treatment.

3.3 Results

3.3.1 Intact microbiota communities are required for antibody response to influenza vaccination

We first sought to establish if altering the gut microbiota by administration of oral antibiotics would lead to reduced vaccine-specific antibody production. In a preliminary experiment conducted by Dr. Hazel Poyntz (MIMR), mice were administered a broad-spectrum antibiotic cocktail, consisting of vancomycin, neomycin, ampicillin and metronidazole (ABX), in the drinking water for 14 days prior to TIV vaccination and throughout the experiment. Vaccine-specific total IgG antibody and the IgG1 isotype were quantified at 14 and 28 days post-vaccination (Figure 3.1A). IgG1 antibodies were chosen to be measured because they are the dominant isotype produced in response to TIV vaccination in humans¹³ and mice^{46,47}.

ABX treatment led to a striking reduction in total IgG (Figure 3.1B) and IgG1 isotype (Figure 3.1C) in the serum at both 14 and 28 days after vaccination. However, it was observed that mice are prone to becoming dehydrated during this long period of ABX treatment. Based on this, the duration of ABX administration was reduced to just three days prior until seven days post-vaccination (Figure 3.1D), minimising dehydration as a confounding factor. Indeed, only ten days ABX treatment was sufficient to significantly reduce the serum concentration of vaccine-specific total IgG (Figure 3.1E) and IgG1 (Figure 3.1F) antibody at 14 and 28 days following vaccination.

To confirm that this ten-day course of oral ABX was indeed altering gut microbial communities, faeces were analysed by qPCR prior to ABX (day -3), on the day of vaccination (day 0) and following ten-days of ABX treatment (day 7).

Phyla-specific 16S rRNA gene-targeted primers for Firmicutes, Actinobacteria, Deferribacteres, Candidatus Saccharibacteria, Verrucomicrobia, Tenericutes and α , β and γ proteobacteria were used in the assay and phyla representation was expressed as a proportion of total bacterial RNA quantified against a universal 16S rRNA sequence. Actinobacteria, Deferribacteres, Candidatus Saccharibacteria, Tenericutes and α Proteobacteria phyla were undetectable in the faeces by this assay (Figure 3.1G). Following only three days of ABX, the frequencies of Firmicutes and Verrucomicrobia were diminished, while Actinobacteria and Bacteroidetes increased in frequency (Figure 3.1G). After ten days of ABX the faecal microbiota was further altered, with an expansion of Verrucomicrobia, Firmicutes and β proteobacteria (Figure 3.1G). It should be noted that this experiment was repeated, however the DNA extracted from the faeces was of poor quality, with low A260/A230 ratios by spectrophotometric analysis, and could not be used for qPCR (Supplementary figure 1A and B).

These data demonstrate that ten days ABX treatment significantly altered microbial phyla composition and this short treatment was able to recapitulate the detrimental effect on antibody production without the confounding factor of dehydration. Therefore, in this model, signals from the gut microbiota are important in antibody production to TIV vaccination.

Figure 3.1: Administration of antibiotics is detrimental to vaccine-specific antibody production and significantly alters faecal microbial communities. Experiment in (A-C) was conducted by Dr. Hazel Poyntz. (A) Experimental outline for long-term administration of oral antibiotics and TIV vaccination. C57BL/6 mice received a broad-spectrum antibiotic cocktail (ABX) in the drinking water, consisting of vancomycin (0.5 mg/mL), neomycin (1 mg/mL), ampicillin (1 mg/mL) and metronidazole (1 mg/mL), or drinking water alone (H₂O) *ad libitum* for 14 days prior to initial vaccination and throughout the entirety of the experiment. Mice were vaccinated with 200µL 25% TIV in PBS. At 14 and 28 days post-vaccination, serum was collected for measurement of TIV-specific antibody by ELISA. TIV-specific (B) total IgG and (C) IgG1 at days 14 and 28 following vaccination. (D) Experimental outline for short-term administration of oral antibiotics and TIV vaccination. C57BL/6 mice received ABX or H₂O *ad libitum*, for three days prior to initial vaccination until seven days following vaccination. Mice were vaccinated with 200µL 25% TIV in PBS. At 14 and 28 days following vaccination, serum was collected for measurement of TIV-specific antibody by ELISA. TIV-specific (E) total IgG and (F) IgG1 at days 14 and 28 post-vaccination. (E-F) is representative of 3 independent experiments. (G) Faecal-resident bacterial frequencies within a cage of mice throughout ABX treatment was determined by qPCR using 16S rRNA gene-targeted group-specific primers for Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, Verrucomicrobia, Candidatus Saccharibacteria, Deferribacteres and Tenericutes, and proportions were quantified against a universal 16S sequence. Proportions of bacteria are presented as the group mean. Data shown is n=4-5 mice per group. Statistical analysis of antibody titres was performed using Two-Way ANOVA with Sidak's post-test showing mean ± SEM. **p≤0.01, *** p≤0.001, **** p≤0.0001.

3.3.2 Subcutaneous TIV vaccination induces potent B cell responses in inguinal lymph nodes

Having established a model in which antibiotics reduce antibody responses to TIV vaccination, immune cell processes in the humoral immune response to TIV were next investigated in order to determine the cellular basis for the reduction in antibody production to vaccination following antibiotic treatment. To assess this, the impact of ABX on the GC response to vaccination first had to be assessed. However, in order to analyse this response, the tail base dLNs needed to be determined.

To assess vaccine drainage, mice were injected with 10% indian ink in PBS or PBS alone, between the dorsal and right or left lateral veins, and were sacrificed two hours later. As expected, dLNs could not be visualised with PBS injection (Figure 3.2A). Following injection on the right or left of the tail base ink could consistently be visualised at both the axillary and inguinal LNs on both sides (Figures 4.2B and C and quantified in Figure 3.2D). Ink drainage could not be visualised at any other LNs. To validate that SC vaccination in the tail was indeed draining to inguinal and axillary LNs, flow cytometry analysis of inguinal or axillary LN B cell and PC populations was performed at 14 days following influenza TIV vaccination and compared to LNs of mice administered PBS (Gating strategy figure 3.3). While the frequency of B cells was not increased in either set of LNs following vaccination, there was a significant increase in B cell number in the inguinal LNs following vaccination, reflecting the increased cellularity (Figure 3.2E). IgG1 producing cells could also be tracked using flow cytometry providing a cellular correlate to IgG1 antibody titres. This allows for antibody titres to be correlated with IgG1 producing cells detected by flow cytometry, as fluorescent antibodies specific for total IgG are not available. There was a significantly increased frequency and number of GC B cells (Figure 3.2F),

IgG1⁺ GC B cells (Figure 3.2G) and IgG1⁺ PCs (Figure 3.2H) in the inguinal LNs following TIV vaccination. However, in axillary LNs the number and frequency of these populations were comparable to PBS administered mice.

This data shows that the inguinal LNs are the primary dLN of SC TIV vaccination in the tail base and subsequent analyses of cellular responses were performed with these LNs.

Figure 3.2: SC TIV vaccination at the tail base drains to inguinal LN and induces potent B cell responses at 14 days post vaccination. (A-C) Representative images showing LN drainage two hours following 20 μ L SC injection of (A) PBS between the dorsal and right lateral vein, (B) 10% indian ink in PBS between the dorsal and right lateral veins and (C) 10% indian ink in PBS between the dorsal and left lateral veins of the tail of C57BL/6 mice. Red arrows show LN. This experiment is summarised in (D), indicating the number of mice in which drainage was observed to each LN. 14 days post-SC vaccination with 200 μ L 25% TIV in PBS or PBS alone at the tail base, inguinal or axillary LNs were analysed by flow cytometry. For PBS group analysis, cells from one inguinal and one axillary LN were pooled. Frequency and total number of (E) B cells, (F) GC B cells, (G) IgG1⁺ GC B cells and (H) IgG1⁺ PCs. Data shown is n=4-5 per group. Statistical analysis of (E-H) was performed using One-Way ANOVA with Tukey's post-test showing mean $\pm$ SEM. *p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001, ****p $\leq$ 0.0001.

A) B cells **B) Plasma cells**

Figure 3.3: Representative flow cytometry gating strategy for B cell and PC populations in the LNs. Gating strategy used to identify (A) B cell populations in the LNs including B cells, naïve B cells, GC B cells, IgM⁺ GC B cells, CS GC B cells and IgG1⁺ GC B cells and (B) PC populations in the LNs including PCs and IgG1⁺ PCs. Pre-gated on live, single cells of interest and arrows indicate parental gate.

3.3.3 Antibiotic treatment impairs B cell differentiation

To determine whether GC development is impaired in ABX-treated, mice were treated with ABX (Figure 3.4A) and the frequencies of B cells (Figure 3.3A) and PCs (Figure 3.3B) in the dLN at 14 days post-TIV were assessed by flow cytometry.

In this experiment, the cellularity of the dLNs did not increase following vaccination, however, ABX treatment resulted in a significant reduction in the overall cellularity of the dLN of vaccinated mice compared to H₂O controls (Figure 3.4B). Despite this, total B cells were not significantly reduced in frequency or number in ABX-treated mice (Figure 3.4C). While the frequency of B cells differentiating into GC B cells was not significantly reduced in ABX mice, the overall number of GC B cells were reduced (Figure 3.4D). The frequency and number of GC B cells of the IgM isotype that had not undergone CSR were comparable between ABX and control vaccinated mice (Figure 3.4E). However, the frequency of GC B cells that had downregulated IgM, thereby undergoing CSR, was reduced in ABX mice and this translated to a significant reduction in the numbers of CS GC B cells (Figure 3.4F). Of cells that had undergone CSR, there were comparable frequencies of the IgG1 isotype following ABX treatment, however the overall number of IgG1⁺ GC B cells was diminished (Figure 3.4G). Strikingly, the frequency and number of PCs in ABX-treated mice was significantly reduced, to levels below PBS vaccinated mice (Figure 3.4H). A comparable frequency of PCs were of the IgG1 isotype but total number of IgG1-secreting PCs was reduced in ABX-treated compared to H₂O control mice (Figure 3.4I).

Collectively, this analysis shows that CS GC B cells and PC frequencies were reduced following ABX, and this resulted in the number of IgG1⁺ PCs being

significantly reduced in number. This suggests that altering gut microbial signals impacts multiple stages of B cell development following vaccination with TIV, leading to fewer antibody-secreting PCs and the observed reduction in serum IgG1 antibody.

Figure 3.4: Antibiotic treatment impairs multiple stages of B cell differentiation following TIV vaccination. (A) Experimental outline for short-term administration of oral antibiotics and TIV vaccination. C57BL/6 mice received ABX or H₂O *ad libitum* for three days prior to vaccination until seven days following vaccination. Mice were vaccinated with 200 μ L 25% TIV in PBS or PBS alone SC at the tail base. At 14 days following vaccination, proportion and total number of (E) B cells, (F) GC B cells, (G) IgG1⁺ GC B cells, (H) PCs and (I) IgG1⁺ PCs in the inguinal or axillary LNs of mice were determined by flow cytometry analysis. Data shown is n=5-6 per group and is representative of three independent experiments. Statistical analysis of flow cytometry data was performed using One-Way ANOVA with Tukey's post-test showing mean $\pm$ SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.3.4 Antibiotic treatment reduces follicular helper cell population

Given the importance of T_{FH} derived signals in development and activity of GC B cells, it was hypothesised that ABX treatment may be leading to defective T_{FH} cell help to B cells. Therefore, the frequency of T_{FH} cells in the dLN of ABX-treated mice was assessed by flow cytometry at 14 days post-vaccination (Figures 3.5 and 3.6A).

As was observed in the previous experiment, vaccination did not increase cellularity of the dLN of H₂O controls compared to naïve mice, however the dLN of ABX-treated mice were significantly reduced in cellularity (Figure 3.6B). The frequency of CD4⁺ T cells of live cells in the dLN was not altered by vaccination and there was no significant difference in the frequency or number of CD4⁺ T cells observed between ABX-treated and untreated vaccinated mice (Figure 3.6C). Following vaccination, there was a significant increase in the frequency and number of T_{FH} phenotype CD4⁺ T cells (Figure 3.6D). There was a trend to lower frequencies of T_{FH} cells in ABX-treated mice compared to untreated controls, however the number of T_{FH} was significantly reduced (Figure 3.6D).

These data demonstrate that depletion of the microbiota with ABX reduces the number of T_{FH} cells in the dLN of TIV vaccinated mice, indistinguishable from baseline numbers, which may be a contributing factor to defects observed in B cell differentiation following vaccination.

Figure 3.5: Representative flow cytometry gating strategy for T_H cells in the LNs. Gating strategy used to identify CD4⁺ T cells (CD3⁺, CD4⁺) and T_H cells (CD3⁺, CD4⁺, CXCR5⁺, PD1⁺). Gated on live, single cells of interest and arrows indicate parental gate.

Figure 3.6: Antibiotic treatment leads to a reduction in T_{FH} cells in the dLN following vaccination. (A) Experimental outline for short-term administration of oral antibiotics and TIV vaccination. C57BL/6 mice received ABX or H₂O *ad libitum* for three days prior to initial vaccination until seven days following vaccination. Mice were vaccinated SC with 200 μ L 25% TIV in PBS or PBS alone at the tail base. At 14 days following vaccination, (B) total number of live cells in the dLNs, proportion and total number of (C) CD4⁺ T cells and (D) T_{FH} cells were determined by flow cytometry analysis. Data shown is $n=3-4$ per group. Statistical analysis of flow cytometry data was performed using One-Way ANOVA with Tukey's post-test showing mean $\pm$ SEM. * $p \leq 0.05$.

3.3.5 AF488 labelled TIV vaccine fluorescently labels cells non-specifically *in vivo*

The data presented thus far demonstrates that modifying the gut microbiota composition with oral ABX throughout the period of TIV vaccination leads to impairments in GC B cell development at multiple stages and a subsequent reduction in vaccine-specific antibody production. As DCs and M ϕ play central roles in both T and B cell activation and differentiation in the dLN⁶², microbiota alterations may be impacting the frequency or activation status of myeloid cell subsets, affecting their ability to transport and present antigen, resulting in defects in B and T_{FH} cell activity.

TIV vaccine antigen was labelled with AF488 fluorescent dye in order to track myeloid subsets that take up vaccine antigen and migrate to the dLN. This technique is routinely utilised at MIMR to track antigen uptake by phagocytic cells²¹⁶. Prior to administration, the protein content of AF488-labelled vaccine was equated to that administered in unlabeled TIV vaccination using the bicinchoninic acid (BCA) protein quantification assay. Initially, it had to be confirmed that phagocytic cells could be identified as antigen-positive by flow cytometry in the dLN and it was necessary to ensure labelling the vaccine did not alter the vaccine efficacy. To investigate this, mice were vaccinated with AF488-labelled TIV (TIV-AF488) or TIV alone and B220⁺ cells, which encompasses B cells and pDCs²¹⁷, LNresDCs, MigDCs, M ϕ and T cells were assessed for antigen uptake by flow cytometry analysis 24 or 42 hours later (Figures 4.7 and 4.8A). TIV-AF488⁺ cells were detected at both 24 and 42 hours following vaccination (Figure 3.8B and C). Of cells that were TIV-AF488⁺, significant proportions were B220⁺ cells, LNresDCs, MigDCs and M ϕ , which are phagocytic and are expected to take up the vaccine. However, 10-20% of TIV-AF488⁺ cells at 24 and 42 hours post-vaccination (H.P.V.) were T cells (Figure

3.8D), and not expected to acquire antigen. This data suggests that TIV-AF488 is binding to or being taken up by cells irrespective of their ability to phagocytose large particles. Furthermore, the total number of B220⁺ cells, MigDCs and M ϕ were significantly lower following TIV-AF488 compared to TIV vaccination alone (Figure 3.8E), suggesting the immunogenicity of TIV-AF488 is not equal to TIV or that it is somehow leading to loss of cellularity in the LN. Therefore, it will not serve as a reliable way to track TIV antigen uptake *in vivo*.

The AF488 protein labelling kit used specified purification over a silica column with no subsequent washes. I hypothesised that part of the signal observed in cells other than APCs could be due to non-specific binding of unbound AF488 dye, therefore the vaccine was washed prior to administration. A more extensive range of cell subsets were assessed for antigen uptake 24 H.P.V. by flow cytometry (Figure 3.7 and Figure 3.8F). Importantly, total numbers of all subsets of cells analysed were not significantly different between mice vaccinated with TIV-AF488 and TIV alone (Figure 3.8J), suggesting that washing rectified the issue of differential immunogenicity. However, only a small proportion of live cells could be identified as being TIV-AF488⁺, and was not statistically greater than levels as compared to TIV alone (Figure 3.8G and H). Furthermore, even after extensive washing, a large proportion of AF488⁺ cells were T cells (Figure 3.8I). This demonstrates that even with extensive washing the TIV-AF488 labels non-phagocytic cells and therefore will not be suitable to track TIV vaccine uptake *in vivo*.

Figure 3.7: Representative flow cytometry gating strategy for vaccine-labelled cells in the dLN. Gating strategies used in analysis of cell subsets after administration of TIV-AF488 purified without (**A**) and with (**B**) subsequent washing B220⁺ cells, T cells (CD3⁺), LNresDCs (CD3⁻, B220⁻, CD11c⁺, MHC II^{med}), migDCs (CD3⁻, B220⁻, CD11c⁺, MHC II^{hi}), and Mφ (CD3⁻, B220⁻, CD11b⁺ F4/80⁺). From each cell subset (red gates) the frequency of TIV-AF488⁺ cells were determined (green gate). Pre-gated on live, single cells of interest. Arrows indicate parental gate.

Figure 3.8: Fluorescently labelled TIV vaccine is acquired by non-APC populations. (A) Experimental outline for vaccination with AF488 labelled TIV vaccine (TIV-AF488). C57BL/6 mice were administered TIV-AF488 or unlabeled TIV and numbers and frequencies of AF488⁺ B220⁺ cells, T cells, migDCs and M ϕ were assessed in the dLN at 24 or 42 hours post-vaccination (H.P.V.) by flow cytometry analysis. **(B)** Plots showing TIV-AF488 uptake among live cells. **(C)** Frequency of TIV-AF488⁺ cells among live cells. **(D)** Proportion of cell subsets that are TIV-AF488⁺ among total TIV-AF488⁺ cells. **(E)** Total numbers of cell subsets. Graphs show mean $\pm$ SEM. Data shown is n=3-6 per group. Statistical significance of **(C)** was determined by One-way ANOVA with Tukey's post-test and for **(D)**, **(E)**, **(H)** and **(J)** was determined by Mann-Whitney test. *p $\leq$ 0.05, **p $\leq$ 0.01.

3.3.6 Antibiotic treatment impacts number and activation status of myeloid cells at steady-state

Unfortunately, the AF488-labelled TIV could not be used to track myeloid cells capability to transport TIV to the dLN due to non-specific labelling. To assess impact of ABX treatment on myeloid responses, instead changes in overall myeloid populations in the dLN in ABX-treated mice were analysed. Mice were administered ABX and myeloid populations assessed at steady-state to assess myeloid population status at time of vaccine antigen encounter (Figure 3.9 and 4.10A).

Three days of ABX treatment led to significant reduction in the cellularity of the dLN (Figure 3.10B). Of these live cells, there were no significant changes in the frequency of pDCs or LNresDCs (Figure 3.10C). MigDCs represented a higher proportion of the live cells in the LN in ABX-treated mice, whereas the frequency of MoDCs and M ϕ were both significantly reduced in these mice (Figure 3.10C). There were comparable numbers of LNresDCs and MigDCs between mice administered water and ABX, whereas ABX mice had significantly fewer MoDCs and M ϕ , and a trend to fewer pDCs (Figure 3.10D). The expression of the costimulatory molecule, CD86, was reduced in pDCs, LNresDCs and MigDCs, however without statistical significance.

Collectively, these data demonstrate that ABX treatment in the three days leading up to vaccination impacts myeloid populations in the vaccine-draining LN, reducing overall frequencies and expression of costimulatory molecules.

Figure 3.9: Representative flow cytometry gating strategy for myeloid cells in the LNs. Gating strategy used to identify pDCs (CD3⁻, B220⁺, CD11b⁻, Ly6C⁺) LNresDCs (CD3⁻, B220⁻, CD11c⁺ MHC II^{med}), migDCs (CD3⁻, B220⁻, CD11c⁺, MHC II^{hi}), moDCs (CD3⁻, B220⁻, CD11c^{med}, MHC II^{med}, Ly6C⁺, CD11b⁺) and Mφ (CD3⁻, B220⁻, CD11b⁺ F4/80⁺). Gated on live, single cells of interest and arrows indicate parental gate.

Figure 3.10: Antibiotic treatment decreases myeloid cell numbers and activation state. (A) Experimental outline for short-term administration of oral antibiotics. C57BL/6 mice were administered ABX or H₂O *ad libitum* for three days. Following ABX treatment, (B) the total number of live cells in the dLNs, (C) proportions of cell subsets within live cells, (D) total number of cell subsets and (E) CD86 MFI were determined by flow cytometry analysis for pDCs, LNresDCs, migDCs, moDCs and Mφ. Data shown is n=5-6 per group and representative of two independent experiments. Statistical analysis was performed using Mann-Whitney test showing mean ± SEM. *p≤0.05, **p≤0.01.

3.3.7 Antibiotic treatment impacts number and activation status of myeloid cells following vaccination

Next, to determine if similar impairments may be seen following vaccination, mice were administered ABX and sacrificed on day three following TIV vaccination for flow cytometry analysis of myeloid cell populations (Figure 3.9 and 3.11A).

A striking reduction in LN cellularity was observed in mice treated with ABX (Figure 3.11B) and significantly reduced frequency of pDCs, LNresDCs, MoDCs and M ϕ was observed, however the frequency of migDCs were not significantly reduced (Figure 3.11C). Reduced overall cellularity coupled with stark reductions in frequency of myeloid cells translated to severely reduced numbers of all myeloid populations analysed (Figure 3.11D). Furthermore, all myeloid populations analysed, excluding MoDCs, had diminished expression of costimulatory molecule CD86 (Figure 3.11E).

Collectively, these data suggest that altering microbiota-derived signals by oral ABX treatment is detrimental to the frequency and activation status of myeloid populations under homeostatic conditions and following vaccination.

Figure 3.11: Antibiotic treatment severely impacts myeloid cell frequencies and activation status following TIV vaccination. (A) Experimental outline for short-term administration of oral antibiotics and TIV vaccination. C57BL/6 mice were administered ABX or H₂O *ad libitum* for three days prior to vaccination until sacrificed three days post-vaccination. Three days following vaccination (B) the total number of live cells in the dLNs, (C) proportions of cell subsets within live cells, (D) total number of cell subsets and (E) CD86 MFI on pDCs, LNresDCs, migDCs, moDCs and Mφ was analysed by flow cytometry analysis. Data shown is n=5-7 per group and representative of two independent experiments. Statistical analysis was performed using Mann-Whitney test showing mean ± SEM. *p≤0.05, **p≤0.01.

3.4 Discussion

Previous investigations have shown that microbiota-derived flagellin has an adjuvant effect in TIV vaccination, providing a critical signal to M ϕ and B cells and is required to induce robust antibody responses⁷². The aim of this chapter was to further assess the impact of the intestinal microbiota on the humoral immune response to TIV vaccination in mice. Broad-spectrum ABX were administered to alter gut microbial communities in order to investigate the impact of the gut microbiota on the humoral immune response to TIV vaccination. A ten-day course of oral ABX was sufficient to alter gut microbial communities and severely impair vaccine-specific antibody production. Concurrently, ABX treatment impaired development of the dLN B cell response at multiple stages in the dLN following TIV vaccination. T_{FH} populations were also reduced in number in ABX-treated mice. TIV vaccine antigen was labelled to allow for tracking of antigen uptake by myeloid populations, However, this technique was not able to be optimised. Instead, total myeloid cell populations in the dLN were assessed. Multiple populations were found to be significantly reduced in number, as well as exhibiting a less activated phenotype, at both steady-state and post-vaccination in ABX-treated mice compared to untreated controls.

A ten-day course of broad spectrum ABX recapitulated the detrimental effect on antibody production observed with a six-week regime without the confounding factor of dehydration, and therefore this regime was employed. It was confirmed that ABX treatment significantly altered microbial phyla composition. Ideally, this analysis of bacterial phyla in the faeces would have been repeated in a further experiment but unfortunately the extracted DNA was of poor quality. Faecal samples from mice after both three and ten days of ABX treatment had

very low A260/A230 ratios by spectrophotometric analysis, indicating a high ratio of organic contaminants to DNA. This is likely due to a reduction in total quantity of faecal bacterial DNA following ABX treatment. Although it was not determined whether bacterial load was reduced by additional methods, other investigators observed a greater than two log reduction in the faecal bacterial load with a similar ABX regime⁷¹. These results suggest bacterial load was substantially reduced which, in turn, would restrict host circulating levels of microbiota-derived immunostimulatory PAMPs and bacterial metabolites and is likely a key factor in the effect of ABX treatment on capability to mount anti-TIV humoral response.

At 14 days post-vaccination, B cells in the dLN were analysed to determine whether ABX impaired any stage in the generation of the B cell response. A major finding was the reduction in the frequency of GC B cells that had undergone CSR in ABX-treated mice. Additionally, the frequency of cells differentiating into PCs was also reduced. Of note, defects were not universal across all B cell populations, as the number and frequency of total B cells and non-class-switched B cells were comparable in vaccinated mice following ABX treatment. The effect of ABX on multiple stages of B cell development culminated in significantly fewer IgG1⁺ PCs being produced, correlating with reduced serum IgG1 antibody titres.

B cell development is a collaborative effort, relying on concerted action between many different cells. Based on the findings in B cells, other cells crucial to GC B cell and plasma cell development were subsequently analysed. In the GC, T_{FH} cells provide important signals to aid B cell activation, proliferation, differentiation and survival^{73,74}. Signals include T_{FH} cell CD40L binding to CD40 expressed by B cells, which is a critical signal for multiple stages of GC B cell activation, differentiation and cytokine secretion such as IL-21 which potently

promotes GC B cell CSR and PC differentiation⁷⁵. Whilst the frequency of CD4⁺ T cells of the T_{FH} phenotype were comparable between ABX and water-administered groups, the total number of T_{FH} cells following vaccination in ABX-treated mice was significantly reduced. This may contribute to the impaired B cell responsiveness observed in ABX-treated mice, as GC B cell competition for T_{FH} cell help is already a response-limiting factor⁷⁶. However, as GC B cells in the dLN are also significantly reduced in number, the ratio of GC B cells to T_{FH} cells may not be different, and in this case competition for T_{FH} cell help would also remain constant. Further investigation is required to determine if the reduced number of T_{FH} cells is having a causative effect in defective B cell development.

Impaired development of the B cell and T_{FH} cell response was observed in ABX-treated mice, both of which require signals from myeloid populations for priming and differentiation. In order to determine if myeloid subsets that take up the vaccine antigen and migrate to the dLN were impaired following ABX treatment, TIV vaccine antigen was labelled with AF488 fluorescent dye. Unexpectedly, a proportion of T cells stained positively for AF488. Antigen uptake should be restricted to phagocytic cells and as T cells are non-phagocytic, this indicated cells were non-specifically acquiring antigen. This may be due to labelled HA protein in the vaccine binding the surface of cells via sialic acid residues, which are ubiquitously expressed on the surface of all cells⁷⁷, including T cells²¹⁸. This is supported by studies utilising a labelled HA probe to identify antigen-specific B cells following influenza infection, finding it was necessary to cleave sialic acid residues by pre-treating cells with NA prior to staining in order to prevent non-specific binding²¹⁹. Furthermore, only a small proportion of myeloid cells were antigen positive following vaccination and the inability to distinguish AF488⁺ myeloid populations was exacerbated by the high level of

cellular autofluorescence in the AF488 detector. Following vaccination with TIV-AF488, an increased number of events could be observed amongst the background autofluorescence - these are likely cells that have taken up low levels of antigen - resulting in the true frequency of AF488⁺ cells being under-represented. The AF488 detector has a bandpass filter with a range that optimally picks up cellular autofluorescence (505nm-525nm)²²⁰. Therefore, in future investigations, protein should be labelled with a fluorescent probe that does not fluoresce at a similar wavelength to autofluorescent cells. Due to the low frequency of antigen-positive events, the frequency of antigen positive myeloid cells in these mice is likely to be challenging to quantify. For these reasons, it was decided the labelled vaccine could not be reliably used to track vaccine uptake.

Tissue-resident myeloid populations are the first cells that encounter antigen following vaccination. It would be interesting to determine what effect ABX had on these cells prior to vaccination, which would likely affect their ability to take up vaccine antigen, migrate to the dLN and prime adaptive immune responses. However, due to time constraints this protocol could not be developed. Instead, steady-state LN-resident myeloid cells were analysed as these are also likely to play a key role in initial priming immunity and B cell responses in TIV vaccination. Small soluble antigens freely drain from the site of SC vaccination to be acquired by LNresDCs, independently of transport by migDCs, as demonstrated by the observation that DCs acquire antigen in the dLN by three hours after SC injection^{36,38}, while it takes around 12 hours for migDCs to arrive in the LN from the tissue³⁹. Following three days of ABX treatment and prior to vaccination, cellularity of the dLN was significantly reduced. Interestingly, ABX treatment had differential effects on myeloid cell subsets, significantly reducing the number and frequency of MoDCs and Mφ, however, not impacting their

expression of costimulatory marker CD86. Conversely, the number and frequency of other DC populations remained unaffected, yet they consistently expressed lower levels of CD86. It should be noted myeloid populations have different developmental origins: the pDCs and classical DCs (cDCs), which encompass migDCs and LNresDCs, develop from a common BM-derived DC precursor^{221,222}. In contrast, moDCs differentiate from circulating monocytes that migrate from the blood in response to microbial and inflammatory stimuli²²³, while M ϕ can be also be derived either from circulating monocytes²²⁴ or embryonic progenitors that are self-renewed in the periphery^{225,226}. In the absence of certain microbial signals with ABX treatment, it is feasible that replenishment of MoDCs or M ϕ in the LN by monocyte differentiation is reduced. In addition, DC and M ϕ subsets exhibit distinct expression patterns of PRRs²²⁷, therefore responding to different microbial stimuli and consequently would be differentially affected by ABX-induced dysbiosis, potentially leading to a reduced activation phenotype.

The effect of ABX on activity of myeloid populations was further demonstrated at three days following vaccination. Further reductions were observed in dLN cellularity, with all M ϕ and DC subsets drastically reduced in number. Expression of CD86 was reduced on all myeloid subsets with the exception of MoDCs, in contrast to what was observed at steady-state. Due to a general reduction in all myeloid cell subsets, it may be that a lack of microbiota signals is altering cellular trafficking to the peripheral LN to replenish or sustain myeloid cells here. Indeed, the microbiota controls pDC trafficking by sustaining constitutive levels of CCL2, which is necessary for pDC egress from the BM and their distribution in the blood and peripheral tissues²²⁸. Additionally, consistent with the role of gut microbiota signalling in maintenance of myeloid progenitor cells of BM and yolk-sac origin^{159,229}, it is also conceivable that defects in

myelopoiesis may lead to the general reduction in myeloid cell numbers observed both at baseline and post-vaccination. However, previous investigations show defects in myelopoiesis have been restricted to longer durations of ABX treatment (four to five weeks) and it is known that DCs in LNs are replaced every ten to 14 days²³⁰, so it remains to be determined if a defect in haematopoiesis could lead to reduced numbers of peripheral myeloid populations in just three to six days.

Collectively, these data demonstrate that defects in B cell development could be due to altered signalling of microbial components either directly on B cells or indirectly on cells important to B cell development, such as T cells or myeloid cell subsets. The microbiota may be directly acting on T cells via TLRs^{231,232}. Recently, T cell-intrinsic TLR signalling was shown to promote T_{FH} development in the gut and subsequent GC formation and IgA production²³³. Furthermore, the significant reduction in the number of myeloid cells and their activation status in the dLN at steady-state and following vaccination in ABX treatment likely impacts T cell activation, given that DCs supply cytokines and present antigen required for T cell priming and differentiation to T_{FH} cells^{46,47}. Fewer myeloid cells with reduced costimulatory capacity may have contributed to the lower number of T_{FH} observed following ABX treatment, thereby indirectly impacting B cell responses. DCs and M ϕ also present antigen to B cells in the LNs for their initial activation, so may have impacted B cells in this way. Alternately, ABX treatment may have impacted B cells directly. This is supported by previous studies showing that signalling of microbial components through TLRs on B cells is necessary to prompt effective B cell differentiation and antibody production^{69,158,234} and microbially-produced SCFAs directly provide B cells with substrates for metabolism and by inhibiting HDACs

enhance expression of genes required for PC differentiation that, in turn, boosts antibody production¹⁷⁵.

3.5 Conclusion

In this chapter, it has been demonstrated that short-term administration of broad-spectrum ABX alters the composition of the gut microbiota and severely reduces antibody response to influenza vaccination. This impairment of humoral immunity correlated with significant impairments in cellular compartments that are crucial to the humoral immune response. ABX treatment resulted in reduced frequency and activation of a number of myeloid cell subsets in the dLN, both prior to and post-vaccination. In addition, T_{FH} cell number were significantly reduced in the dLN following vaccination and this was concurrent with reduced frequencies of CS GC B cells and PCs.

As differentiation and function of T_{FH} and B cells are interdependent, these findings do not reveal if the defects induced by ABX treatment are intrinsic to T or B cells. Further investigation as to the involvement of microbial signals in the development of T and B cell responses to TIV vaccination are required to determine this. Additionally, it is clear that ABX treatment has significant effects on myeloid cells. Given their role in antigen transport and presentation to both T and B cell within the dLN, reduced number and activatory potential of myeloid cells following antibiotic treatment likely contributes to defects in these cellular responses, ultimately leading to poor antibody responses to vaccination.

This data suggests that the gut microbiota may be acting as an adjuvant in the immune response to TIV vaccination and presents the potential for beneficial modulation of gut microbial communities to enhance vaccine responsiveness.

4 The influence of functional foods on immune responses to influenza vaccination

4.1 Introduction

Recent alterations to human lifestyles have had significant effects on the structure and function of our gut microbiota and abnormal microbiome structures (i.e. dysbiosis) are associated with the steep rise in prevalence of many immunological diseases, such as inflammatory bowel diseases, diabetes, asthma and susceptibility to infection²³⁵. There is mounting evidence that dysbiosis has causative role in these diseases¹⁸⁵, as was shown in the previous chapter altering the microbiota with ABX has a detrimental impact on a number of immune cell populations at a systemic site and reduces the ability to mount protective antibody responses against influenza vaccination. Therefore, it is key that therapies are developed for the beneficial modulation of gut microbial communities to promote specific health outcomes. Diet has rapid and profound effects on microbiota community structure and function^{190,192}, and presents an exciting opportunity to improve health outcomes for dysbiosis-related disease in a safe and affordable manner.

In conjunction with the High-Value Nutrition Science Challenge, this thesis seeks to validate the beneficial effects of New Zealand food products to build immune defence against influenza. If successful, this study will lead to clinical trials that will investigate the benefit of high-value nutritional products in improving host-microbiota interactions and enhancing immune responses following vaccination. Four nutritional interventions were selected to be trialed in this thesis for their ability to influence the immune response to influenza vaccination in a pre-clinical mouse model. Candidates selected were the probiotic, *Bifidobacterium animalis* and potential prebiotics, Sungold kiwifruit, propolis with γ -cyclodextrin formulation and fibre-modified diets. Kiwifruit consumption has been linked to modulation of immune function, shown to

boosts baseline and vaccine-specific serum antibody responses in mice²³⁶⁻²³⁸, and there is some evidence of protection against upper respiratory tract infection in humans²³⁹. Kiwifruit is also rich in MACs, therefore is a purported prebiotic²⁴⁰. *B. animalis* is a probiotic bacterium and human intake has been shown to alter gut microbial communities, increasing butyrogenic bacteria and reducing pathobionts²⁴¹, as well as significantly enhancing antibody production to influenza vaccination in healthy adults²⁰⁵. Propolis is a bee product and a potential prebiotic, it is rich in polyphenolic compounds²⁴², many of which are substrates for bacterial fermentation to immunomodulatory compounds^{184,243} and propolis administration to rats has been shown to alter composition of the gut microbiota²⁴⁴. Increased dietary fibre intake has also been shown to enhance production of IgG both at steady-state and following bacterial infection by fueling the production of microbiota-produced SCFAs¹⁷⁵. Therefore, these foods have potential to alter the gut microbiota and improve influenza vaccine responses.

In the feeding experiments, mice are maintained on AIN93 rodent diet or this as the base for all dietary modifications. This was used for nutritional intervention studies because AIN93 is made from refined macronutrient sources and nutrient levels are more closely controlled compared to the standard cereal-based meat-free mouse diet. It also is lower in fibre and higher in fat, closer to the 'western' diet of the population we are modelling¹⁹¹.

In this chapter, mouse models of both human parenteral and mucosal vaccines are established. As discussed previously, the human parenteral influenza vaccination, TIV, induces a IgG antibody response, dominated by the IgG1 isotype, in the serum of recipients without inducing respiratory tract IgA^{107,108}. FluMist, the intranasal live-attenuated influenza vaccine, induces a strong IgA antibody response in the respiratory tract, as well as serum IgG¹¹⁰⁻¹¹². However,

neither vaccine induces complete protection against infection, with many individuals eliciting suboptimal antibody responses¹¹⁵. In this section, as well as quantifying antibody production to vaccination, we wanted to assess protection against influenza infection following administration of functional foods. This secondary measure will ensure that if functional foods improve protection against infection, but do not increase antibody titres, alterations to other immune parameters can be detected. Mice are resistant to human influenza strains²⁴⁵ and we do not have the laboratory facilities to challenge mice with human strains present in the human TIV vaccine. Therefore, it was decided that HKx31 influenza virus, a mouse-adapted influenza A strain, would be used to manufacture a murine influenza vaccine. Transient weight loss and viral load in the lung will be measured following HKx31 infection as a measure of viral control²⁴⁶.

4.2 Aims

For the murine influenza vaccine models to fulfil the requirements as a model for human TIV vaccination, it must induce the appropriate isotype of virus-specific antibody response, serum IgG by both the parenteral and mucosal vaccination route and respiratory tract IgA by mucosal vaccination. The virus-specific antibodies must be easily detectable by virus-specific ELISA. Antibody responses must also be suboptimal, below the maximal response in order for the response to be able to be improved through nutritional intervention.

Specific aims:

- 1) To establish mouse models of parenteral and mucosal influenza vaccination that model low-responding human populations, inducing antibody production and protection against subsequent infection that are suboptimal.
- 2) To assess the capacity of oral administered nutritional interventions with microbiota-altering potential to improve suboptimal immune responses in the influenza vaccination model.

4.3 Results

4.3.1 Formalin-inactivated influenza A vaccine does not induce strong antibody responses when administered SC or IN

To establish vaccination models, a whole inactivated virus vaccine was produced. HKx31 influenza A virus was killed by formalin inactivation according to methods described by Jonges and colleagues, who demonstrated complete viral inactivation²¹³. Mice were administered increasing doses of the vaccine or PBS as a control, SC or IN once a week for three weeks. Multiple doses were administered in order to ensure induction of robust antibody responses according to previous investigations^{247,248}. This also better models human vaccination, as pre-existing immunity to vaccine strains is common, due to previous vaccination or infection²⁴⁹. Mice were sacrificed one week following the final vaccination and serum, BAL and nasal lavage were collected to assess serum HKx31-specific total IgG and IgG1 antibody and HKx31-specific IgA antibody in the BAL and nasal lavage by ELISA assay (Figure 4.1A).

We have observed that 1 haemagglutination unit (HAU) – a unit of viral measurement - of live virus administered IN to naïve mice induces approximately 10% weight loss three days following infection (Section 4.3.3). Administration of inactivated virus did not induce weight-loss compared to PBS administered mice, regardless of the dose or route of administration (Figure 4.1B and C). This further confirms complete viral killing by this method. As was expected, SC vaccination did not elicit detectable levels of virus-specific IgA in the nasal lavage (Figure 4.1D) or BAL (Figure 4.1E). Mice vaccinated SC at any viral dose did not elicit high levels of virus-specific total IgG (Figure 4.1F) or IgG1 (Figure 4.1G) antibody. At doses of 10 or 100 HAU, vaccines elicited detectable virus-specific IgG, however these did not reach statistically significant

levels (Figure 4.1F). Low titres of IgA were detected in the nasal lavage (Figure 4.1H) and BAL (Figure 4.1I) of some mice IN vaccinated at the highest dose of 50 HAU. However, no virus-specific total IgG (Figure 4.1J) or IgG1 (Figure 4.1K) were detected in the serum at any dose.

This data demonstrates that neither vaccine route elicited consistent virus-specific IgG antibody in the serum or IgA in the respiratory tract detectable by ELISA at the doses tested. Therefore, SC nor IN administration of the formalin-inactivated influenza vaccine at the doses tested will not serve as effective models of either the parenteral or mucosal human influenza vaccines.

Figure 4.1: Formalin-inactivated influenza A vaccine does not induce sufficient antibody production when administered SC or IN. (A) Experimental outline for administration of formalin-inactivated HKx31 vaccine. C57BL/6 mice were administered either 200 μ L SC or 50 μ L IN of vaccine or PBS once a week for three weeks. Seven days following the final vaccination, mice were culled and serum, nasal lavage and BAL were collected for measurement of HKx31-specific antibodies by ELISA. Weight change from initial weight of mice vaccinated with PBS or increasing doses of vaccine via **(B)** SC or **(C)** IN route. Horizontal dotted lines indicate the day of vaccination. HKx31-specific IgA in the **(D)** nasal lavage and **(E)** BAL seven days following final SC vaccination. HKx31-specific serum **(F)** total IgG and **(G)** IgG1 seven days following final SC vaccination. HKx31-specific IgA in the **(H)** nasal lavage and **(I)** BAL seven days following final IN vaccination. HKx31-specific serum **(J)** total IgG or **(K)** IgG1 seven days following final IN vaccination. Data shown is n=5 per group. Statistical analysis of antibody titres was performed using One-Way ANOVA with Tukey's post-test showing mean $\pm$ SEM. *p $\leq$ 0.05.

4.3.2 UV-inactivated influenza A virus induces robust serum and mucosal antibody responses

Formalin inactivation utilised in the previous chapter required a dialysis step that likely resulted in loss of viral material, which may account for low antibody titres. To address this, UV inactivation was chosen as an alternative method as it allows for collection of all inactivated viral material and is a faster process. HKx31 virus was UV-inactivated according to methods described by Zou and colleagues, who demonstrated complete viral killing²¹⁴. Mice were administered increasing doses of UV-inactivated HKx31 influenza A vaccine either SC or IN, three times on days zero, seven and 14 of the experiment. Mice were sacrificed seven days later and serum, BAL and nasal lavage were collected. HKx31-specific total IgG and IgG1 antibody in the serum and HKx31-specific IgA antibody in the BAL and nasal lavage were assessed by ELISA assay (Figure 4.2A).

Administration of inactivated virus did not induce weight-loss compared to PBS controls when administered SC (Figure 4.2B) or IN (Figure 4.2C), again confirming complete viral killing by UV inactivation. As expected, SC vaccination did not elicit detectable virus-specific IgA in the nasal lavage (Figure 4.2D) or BAL (Figure 4.2E). Robust serum total IgG (Figure 4.2F) and IgG1 isotype (Figure 4.2G) antibodies were induced by SC vaccination at both doses tested, with lower levels of antibodies at the 200 HAU vaccine dose compared to 500 HAU, although these did not reach statistical significance. IN vaccination elicited strong virus-specific IgA antibody production in the nasal lavage (Figure 4.2H) and BAL (Figure 4.2I) at the doses tested. Virus-specific BAL IgA and serum IgG1 were significantly lower at the 200 HAU dose than 500 HAU, while nasal IgA and serum total IgG trended to lower levels.

This data demonstrates that the UV-inactivated 200 HAU vaccine dose, administered SC or IN, can serve as appropriate models for suboptimal parenteral and mucosal vaccination, respectively. Vaccinations induced appropriate antibody responses that were easily detectable by ELISA assay. Serum IgG, including of the IgG1 isotype, was elicited by both vaccine routes, while only IN vaccination induced respiratory tract IgA. Importantly, the chosen dosage does not induce a maximal antibody response, and therefore has the potential to be improved with nutritional interventions.

(A)

Figure 4.2: UV-inactivated influenza A virus induces strong serum and mucosal antibody responses. (A) Experimental outline for administration of UV-inactivated HKx31 vaccine. C57BL/6 mice were administered either 200 μ L SC or 50 μ L IN of vaccine or PBS once a week for three weeks. Seven days following the final vaccination, mice were culled and serum, nasal lavage and BAL were collected for measurement of HKx31-specific antibodies by ELISA. Weight change from initial weight throughout experiment for mice vaccinated via (B) SC or (C) IN route. Horizontal dotted lines indicate day of vaccination. HKx31-specific IgA in the (D) nasal lavage and (E) BAL seven days following final SC vaccination. HKx31-specific serum (F) total IgG and (G) IgG1 seven days following final SC vaccination. HKx31-specific IgA in the (H) nasal lavage and (I) BAL seven days following final IN vaccination. HKx31-specific serum (J) total IgG or (K) IgG1 seven days following final IN vaccination. Data shown is n=3-5 per group. Statistical analysis of antibody titres was performed using One-Way ANOVA with Tukey's post-test. All data is displayed as mean $\pm$ SEM. ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

4.3.3 Administration of inactivated influenza vaccines protect against infection

Next, the protection against subsequent infection with live HKx31 influenza virus induced by each vaccine was determined. To assess this, mice were administered increasing doses of UV-inactivated HKx31 influenza A vaccine either SC or IN, once a week for three weeks. Seven days following the final vaccination, mice were infected with 1 HAU of live HKx31 influenza virus. The ability to control infection was assessed by infection-induced weight loss and the quantity of viral RNA in the lung relative to that of mice administered PBS alone at three days post-infection (Figure 4.3A).

Although not statistically significant, SC vaccination of 200 HAU protected three of the four mice from infection-induced weight loss. In contrast, those administered lower doses lost approximately 10% of their body weight, comparable to weight loss observed in PBS control mice (Figure 4.3B). Despite no significant protection from weight loss, significantly less viral RNA was measured in the lungs of vaccinated mice at all doses tested compared to PBS control mice, however viral RNA was readily detectable by qPCR (Figure 4.3C), indicating incomplete protection. IN vaccination induced a far more potent protective effect at the same dose, with vaccine doses of 50 and 200 HAU inducing significant protection from the infection-induced weight loss observed in the PBS group, only one mouse exhibited weight loss at the lowest dose of 5 HAU (Figure 4.3D). Strikingly, IN vaccination resulted in viral RNA levels that were undetectable by qPCR at three days post-infection, except for one mouse administered the lowest dose (Figure 4.3E), the same individual that lost 10% of body weight following infection (Figure 4.3D).

This experiment demonstrates that both SC and IN administration of UV-inactivated influenza vaccine at 200 HAU elicits robust protection against subsequent viral infection. SC administration of 200 HAU elicited both suboptimal infection-induced weight loss and protection from infection. However, a much stronger protective response was elicited by IN vaccination, where 200 HAU dosage elicited complete protection against weight loss and no detectable viral RNA in the lung.

(A)

Figure 4.3: Parenteral and mucosal influenza vaccines induce protection against infection. (A)

Experimental outline for administration of UV-inactivated HKx31 vaccine followed by viral challenge. C57BL/6 mice were administered either 200 μ L SC or 50 μ L IN of vaccine or PBS once a week for three weeks. Seven days following the final vaccination, mice infected with 1 HAU of live HKx31 virus in 50 μ L IN. Three days following infection, mice were sacrificed and lungs were collected for quantitation of viral RNA by qPCR. Weight change was monitored following infection. **(B)** Weight change three days following infection for mice vaccinated SC. **(C)** Expression of viral RNA in the right lobe of the lung at three days following infection of mice vaccinated SC. Viral RNA levels are normalised to GAPDH and calculated relative to PBS. **(D)** Weight change three days following infection for mice vaccinated IN. **(E)** Expression of viral RNA in the right lobe of the lung at three days following infection of mice vaccinated IN. Viral RNA levels are normalised to GAPDH and calculated relative to PBS. N.D.= non-detectable. Data shown is $n=3-5$ per group. Statistical analysis was performed using One-Way ANOVA with Tukey's post-test showing mean $\pm$ SEM. * $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

4.3.4 Oral administration of kiwifruit does not improve antibody responses to influenza vaccination or protection from infection

SC or IN administration of UV-inactivated HKx31 influenza vaccine at 200 HAU dose induces robust antibody production and protection against infection, however, only SC administration elicits suboptimal responses. While IN administration at the same dose elicits suboptimal antibody responses, protection against infection is complete and no virus can be detected in the lung even at very low doses. Therefore, it would be impossible to determine an IN dose that would generate antibody readily detectable by ELISA assay, while still eliciting suboptimal response against infection. Furthermore, to assess mucosal antibody titres, mice must be sacrificed, therefore it is not possible to perform viral challenge simultaneously in the same mice. Based on this, it was decided to go forward only using SC administration of UV-inactivated virus at a 200 HAU dose to model the human parenteral vaccination in subsequent nutritional intervention studies.

To investigate the efficacy of kiwifruit to improve immune responses to influenza vaccination, mice were administered kiwifruit or control solution intragastrically (IG) once a day, for five days each week, from two weeks prior to vaccination and throughout the rest of the experiment whilst being maintained on AIN93 rodent diet. Mice were administered 200 HAU of UV-inactivated HKx31 influenza A vaccine SC once a week for three weeks. Seven days following the final vaccination, serum was collected for analysis of virus-specific antibody and mice were infected with 1 HAU of live HKx31 influenza virus. The ability to control infection was assessed three days post-infection by weight loss and the quantity of viral RNA in the lung, relative to that of mice administered PBS and control feed (Figure 4.4A).

Both influenza vaccine and PBS mice that received kiwifruit did not gain as much weight as their respective control groups (Figure 4.4B), and ate less food each week (Figure 4.4C). In response to influenza vaccination, mice fed kiwifruit had lower levels of virus-specific total IgG (Figure 4.4D) and IgG1 (Figure 4.4E) in the serum than control mice. Vaccination reduced viral RNA load in the lung three days following infection, however kiwifruit feeding did not improve viral control, with both the PBS and influenza vaccine groups exhibiting similar viral load between feeding regimes (Figure 4.4F). While vaccination protected mice against infection-induced weight loss, there was no difference in weight loss in the PBS groups, and influenza vaccinated mice receiving kiwifruit lost significantly more weight than control fed mice comparable to non-vaccinated mice (Figure 4.4G).

Collectively, these data demonstrate that influenza vaccinated mice fed with kiwifruit elicited lower levels of virus-specific antibody and, while they did not have significantly more viral RNA in the lung following infection, they were more susceptible to infection-induced weight loss. This coincided with reduced food intake in kiwifruit fed groups and subsequent failure to gain as much weight as control mice.

Figure 4.4: Administration of kiwifruit is detrimental to antibody responses to influenza vaccination and protection from infection. (A) Experimental outline for administration of kiwifruit and UV-inactivated HKx31 vaccine followed by viral challenge. C57BL/6 mice were vaccinated SC with 200 HAU of UV-inactivated influenza virus in 200 μ L or PBS once a week for three weeks. Mice were administered 300 to 500 μ L of kiwifruit IG five days per week from 14 days prior to initial vaccination and for the entirety of the experiment whilst being maintained on AIN93 rodent diet. Seven days following the final vaccination, mice were infected with 1 HAU of live HKx31 virus in 50 μ L IN. Three days following infection, weight loss was measured, mice were sacrificed and lungs were collected for quantitation of viral RNA by qPCR. (B) Weight change from initial weight of mice throughout the experiment. (C) Average weight of rodent diet eaten per mouse per week throughout experiment. HKx31-specific serum (D) total IgG and (E) IgG1 seven days following final vaccination. (F) Expression of viral RNA in the right lobe of the lung at three days following infection. Viral RNA levels are normalised to GAPDH and calculated relative to PBS. (G) Weight change three days following infection. Data shown is $n=5-10$ per group. Statistical analysis was performed using One-Way ANOVA with Tukey's post-test showing mean $\pm$ SEM. * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$.

4.3.5 Oral administration of probiotic *B. animalis* does not enhance antibody responses to influenza vaccination or protect from infection

Next, the impact of oral administration of the probiotic bacteria, *B. animalis*, to improve immune responses to influenza vaccination and subsequent infection was assessed. To test this, mice were administered *B. animalis* or control solution IG once a day, five days each week, from two weeks prior to vaccination and throughout the rest of the experiment whilst being maintained on AIN93 rodent diet. Mice were administered 200 HAU of UV-inactivated HKx31 influenza A vaccine SC once a week for three weeks. Seven days following the final vaccination, serum was collected for analysis of virus-specific antibody then mice were infected with 1 HAU live HKx31 influenza virus. The ability to control infection three days post-infection was assessed by infection-induced weight loss and the quantity of viral RNA in the lung, relative to that of mice administered PBS and control solution (Figure 4.5A).

Weight gain was similar between *B. animalis* and control fed groups (Figure 4.5B) and there were no significant differences in food intake between all groups (Figure 4.5C). Following influenza vaccination, *B. animalis* and control fed mice exhibited similar levels of virus-specific total IgG (Figure 4.5D) and IgG1 (Figure 4.5E). Following infection, *B. animalis* feeding did not improve control infection compared to those fed control solution, both in mice administered the influenza vaccine or PBS. Vaccination reduced viral load in the lung (Figure 4.5F) and protected mice against infection-induced weight loss (Figure 4.5G). *B. animalis* treated mice had comparable levels of viral RNA in the lung (Figure 4.5F) and exhibited similar weight change following infection (Figure 4.5G).

These data demonstrate that oral administration of *B. animalis* does not improve antibody production to an inactivated influenza virus vaccine or control of subsequent infection.

Figure 4.5: Oral administration of *B. animalis* elicits has no effect on vaccine-induced antibody production or protection against infection. (A) Experimental outline for administration of *B. animalis* and UV-inactivated HKx31 vaccine followed by viral challenge. C57BL/6 mice were vaccinated SC with 200 HAU of UV-inactivated influenza virus in 200 μ L or PBS once a week for three weeks. Mice were administered 1×10^9 CFU of *B. animalis* in 200 μ L IG five days per week from 14 days prior to initial vaccination and for the entirety of the experiment whilst being maintained on AIN93 rodent diet. Seven days following the final vaccination, mice were infected with 1 HAU of live HKx31 virus in 50 μ L IN. Three days following infection, weight loss was measured and mice were sacrificed and lungs were collected for quantitation of viral RNA by qPCR. (B) Weight change from initial weight of mice throughout the experiment. (C) Average weight of rodent diet eaten per mouse per week throughout experiment. HKx31-specific serum (D) total IgG and (E) IgG1 seven days following final vaccination. (F) Expression of viral RNA in the right lobe of the lung at three days following infection. Viral RNA levels are normalised to GAPDH and calculated relative to PBS. (G) Weight change three days following infection. Data shown is $n=5-10$ per group. Statistical analysis was performed using One-Way ANOVA with Tukey's post-test showing mean $\pm$ SEM. * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$.

4.3.6 Oral administration of propolis formulation improves protection against infection without altering antibody responses

Next, the effect of formulated propolis on immune responses to influenza vaccination and infection was investigated. To test this, mice were administered AIN93G rodent diet supplemented with 8% propolis formulated with cyclodextrin powder (propolis formulation) or AIN93G alone *ad libitum* from two weeks prior to vaccination and throughout the rest of the experiment. Mice were administered 200 HAU of UV-inactivated HKx31 influenza A vaccine once a week for three weeks. Seven days following the final vaccination, serum was collected for analysis of virus-specific antibody then mice were infected with 1 HAU live HKx31 influenza virus. Infection-induced weight loss and the quantity of viral RNA in the lung, relative to that of mice administered PBS and control feed, was assessed at three days post-infection (Figure 4.6A).

Influenza vaccinated mice administered the propolis formulation diet tended to gain less weight than those fed the control diet (Figure 4.6B), however apart from the first week of the experiment, mice consumed a similar amount of diet (Figure 4.6C). Both IgG and IgG1 antibody titres were comparable between mice fed the propolis formulation and control diets (Figures 5.6D and 5.6E). Vaccination reduced viral load in the lung (Figure 4.6F), however did not elicit protection against infection-induced weight loss compared to PBS controls (Figure 4.6G). Despite no differences in virus-specific antibody, there was a reduction in viral load in influenza vaccinated mice consuming the propolis formulation compared to those fed the control diet, with half of the individuals exhibiting no detectable viral RNA in the lung three days post infection (Figure 4.6G). Mice consuming propolis formulation and administered PBS also tended to have reduced viral load in the lung (Figure 4.6F). However, weight loss following

infection was comparable between propolis formulation and control groups (Figure 4.6G).

Collectively, this data suggests that oral administration of the propolis formulation may improve protective immunity against influenza infection following vaccination. However, this was not due to improving the production of virus-specific IgG antibodies elicited by the vaccination.

Figure 4.6: Propolis formulation intake elicits some protection against influenza infection without influencing vaccine-induced antibody production. (A) Experimental outline for administration of propolis formulation and UV-inactivated HKx31 vaccine followed by viral challenge. C57BL/6 mice were vaccinated SC with 200 HAU of UV-inactivated influenza virus in 200 μ L or PBS once a week for three weeks. Mice were administered modified AIN93G rodent diet with 8% propolis cyclodextrin powder (propolis formulation) *ad libitum* from 14 days prior to initial vaccination and for the entirety of the experiment. Seven days following the final vaccination, mice were infected with 1 HAU of live HKx31 virus in 50 μ L IN. Three days following infection, weight loss was measured, mice were sacrificed and lungs were collected for quantification of viral RNA by qPCR. **(B)** Weight change from initial weight of mice throughout the experiment. **(C)** Average weight of rodent diet eaten per mouse per week throughout experiment. HKx31-specific serum **(D)** total IgG and **(E)** IgG1 seven days following final vaccination. **(F)** Expression of viral RNA in the right lobe of the lung at three days following infection. Viral RNA levels are normalised to GAPDH and calculated relative to PBS. N.D.=non-detectable. **(G)** Weight change three days following infection. Data shown is n=5-10 per group. Statistical analysis was performed using One-Way ANOVA with Tukey's post-test showing mean $\pm$ SEM. * $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

4.3.7 Dietary fibre intake does not alter antibody responses to vaccination or prevent infection-induced weight loss

Next, alterations to fibre content of the murine diet were assessed for the influence on immune responses to influenza vaccination and infection. To test this, mice were administered either zero-fibre, zero-starch rodent diet (ZF) or a high-fibre equivalent diet formulated with 7.5% pectin and 7.5% inulin (HF) *ad libitum* from weaning (three weeks prior to vaccination) and throughout the rest of the experiment. Mice were administered 200 HAU of UV-inactivated HKx31 influenza A vaccine once a week for three weeks. Seven days following the final vaccination, serum was collected for analysis of virus-specific antibody then mice were infected with 1 HAU live HKx31 influenza virus. The ability to control infection was assessed by infection-induced weight loss and the quantity of viral RNA in the lung, relative to that of mice administered PBS and control feed, was assessed at three days post-infection (Figure 4.7A). Due to time constraints, the viral RNA load in the lung following infection could not be included in this thesis.

Weight gain throughout the experiment was comparable between mice fed ZF and HF diets (Figure 4.7B). Groups fed the ZF diet appeared to consume more food in the earlier weeks of the experiment, however as time went on food intake became equal between feeding groups (Figure 4.7C). Following vaccination, both ZF and HF fed mice mounted strong vaccine-specific IgG and IgG1 antibody responses, however fibre content of the diet had no impact on antibody responses to vaccination (Figure 4.7D and E). Moreover, following influenza infection, there was no difference in infection-induced weight loss between feeding groups (Figure 4.7F).

These data demonstrate that content of dietary fibre, specifically pectin and inulin, has no impact on antibody responses to influenza vaccination or protection against weight loss following infection.

Figure 4.7: Fibre intake does not influence antibody production to vaccination or protection against infection-induced weight loss. (A) Experimental outline for administration of fibre-modified diets and UV-inactivated HKx31 vaccine followed by viral challenge. C57BL/6 mice were vaccinated SC with 200 HAU of UV-inactivated influenza virus in 200 μ L or PBS once a week for three weeks. Mice were administered zero-fibre, zero-starch SF15-086 rodent (ZF) diet or modified SF15-086 with 7.5% pectin, 7.5% inulin (HF) diet *ad libitum* from weaning 21 days prior to initial vaccination and for the entirety of the experiment. Seven days following the final vaccination, mice were infected with 1 HAU of live HKx31 virus in 50 μ L IN. Three days following infection, weight loss was measured, mice were sacrificed. (B) Weight change from initial weight of mice throughout the experiment. (C) Average weight of rodent diet eaten per mouse per week throughout experiment. HKx31-specific serum (D) total IgG and (E) IgG1 seven days following final vaccination. (F) Weight change three days following infection. Data shown is $n=5-10$ per group. Statistical analysis was performed using One-Way ANOVA with Tukey's post-test showing mean $\pm$ SEM. ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

4.4 Discussion

The aim of this chapter was to establish mouse models of parenteral and mucosal influenza vaccination capable of inducing suboptimal antibody production and protection against subsequent infection, in order to model low-responding human populations. This model was used to determine if administration of potential microbiota-altering nutritional interventions improved immune responses to the vaccine. UV-inactivated live influenza virus induced potent antibody production and protection against infection when administered via parenteral and mucosal routes. However, mucosal vaccination gave full protection against infection, even at very low doses, meaning there was no opportunity to improve protection with nutritional interventions. Therefore, only the parenteral vaccine was taken forward for further studies. Administration of kiwifruit, *B. animalis* or a high fibre diet induced no discernible improvements in immune responses to vaccination or subsequent infection based on the parameters measured. In contrast, while there was no difference in vaccine-induced antibody production and weight change following infection upon administration of the propolis formulation, mice receiving this nutritional intervention tended to be better protected against infection, with a trend towards lower viral RNA loads in the lung.

When establishing the inactivated viral vaccine models, mice administered formalin-inactivated virus produced negligible antibody in response to vaccination. It has been observed that formalin inactivation may modify antigenic structures of vaccine proteins²⁵⁰, a potential contribution to observed low antibody titres. However, the dosages tested were also lower than those used for the UV-inactivated vaccine and this likely contributed to poor antibody

responses to the vaccine and there was insufficient time to perform another dose titration.

It should be noted that the virus-specific total IgG ELISA may not be an optimal assay. Naïve control mice are expected to have no virus-specific IgG in their serum, yet for these mice O.D. values are consistently above zero for the IgG ELISA, yet not for the IgG1 isotype ELISA. The difference between these assays is the secondary antibody, the IgG secondary antibody is likely binding non-specifically to other components of the serum bound to the plate. To rectify this issue in further investigations, O.D. values could be normalised to untreated samples. However, the same trends between groups would be expected. Alternatively, the ELISA could be optimised with different blocking and sample-dilution reagents to minimise background signal.

Previous studies have shown that kiwifruit administration was able to improve antibody responses to peptide vaccination in mice²³⁶. Surprisingly, in this model, mice administered kiwifruit exhibited a significant reduction in virus-specific antibodies and lost more weight following infection than controls, while influenza RNA load was unchanged. It should be noted that those fed kiwifruit also tended to eat less of their regular food and gain less weight throughout the experiment, likely because they were administered a large volume of kiwifruit daily. Decreased body weight and possible adverse effects from kiwifruit administration may have led to lower antibody responses. Further investigation with a lower dosage is required to determine the efficacy of kiwifruit in altering antibody responses to influenza vaccination.

Contrary to improvements in vaccine-induced antibody titres observed in a large human cohort administered *B. animalis*²⁰⁵, neither vaccine-specific antibody production nor protection against infection were improved in mice administered

B. animalis. Probiotics have been shown to successfully engraft in the microbiota of only certain individuals²⁵¹, suggesting that the microbiota of these mice may not have been permissive to colonisation by this strain. This could also be reflective of inherent differences in the physiology and microbiota between humans and mice²⁵².

It has been previously shown that consumption of a diet high in inulin and pectin fibre leads to significantly increased levels of circulating SCFAs compared to mice fed a zero-fibre diet and this boosted IgG production at baseline and following bacterial infection¹⁷⁵. Despite utilising the same dietary regime as this published investigation, no discernible difference in antibody production or protection against infection-induced weight loss between mice fed high or zero fibre diets was observed. However, the difference between mounting an antibody response to bacterial infection compared to influenza vaccination may account for this discrepancy. Additionally, differences in baseline microbiota composition between these investigations could have played a role, as it has been observed that the gut microbiota of mice significantly differs between research institutes, which can significantly impact outcomes²⁵³. Also, contrary to the investigation by Kim and colleagues, it has been observed that SCFAs can inhibit B cell CSR, SHM and PC differentiation and SCFAs from fermentation of a high fibre diet can decrease IgG titres in the blood²⁵⁴. In addition, SCFAs differentially affect immune responses depending on the context. Under steady-state conditions, SCFAs act directly on peripheral T cells to promote differentiation to IL-10⁺ regulatory cells, whereas inflammatory conditions prompt T cell polarization to a T_H1 and T_H17 effector phenotype¹⁸⁰. Analogously, it is possible SCFAs may promote the development of T follicular regulatory (T_{FR}) cells, which can develop from T_{REG} cells in the LN²⁵⁵ and limit the magnitude of the GC response²⁵⁶. However, the impact of SCFAs on T_{FH} cell development has not yet

been investigated. Thus, the immunomodulatory influences of SCFAs are complex and further investigation is required to elucidate their impact on antibody production. Further analysis of the viral RNA load following infection in this experiment is required and may reveal protection against infection with high fibre feeding.

In mice fed the propolis formulation, a clear trend towards reduced viral RNA was observed following infection, despite no improvements in virus-specific antibody production. Statistical analysis could not be performed as viral RNA was non-detectable in a number of mice. γ -cyclodextrin is used for microencapsulation of the propolis to enhance bioavailability and constitutes 75% of the propolis formulation. Cyclodextrins are oligosaccharides that are potential prebiotics and, as γ -cyclodextrin has been shown to alter faecal microbial populations²⁵⁷, it may be influencing the microbiota in this study. To determine if beneficial influence of the propolis formulation was due to the action of γ -cyclodextrin, future investigations should also analyse the effect of γ -cyclodextrin alone. This investigation does not demonstrate that the beneficial effect of propolis formulation on influenza vaccine responses is mediated through modulation of the microbiota community structure or its function. To prove a causative role of changes to the microbiota in improved immunity, it would be necessary to transfer the gut microbiota from propolis formulation treated mice to untreated mice and observe a transfer of the phenotype.

These experiments also demonstrate that serum IgG may not be a good correlate of protection against infection in this model. TIV vaccination induces virus-specific IgG in humans and this is a direct correlate of protection against infection⁹⁹. In contrast, mice administered propolis formulation exhibited increased protection from infection following vaccination despite no increase in vaccine-induced antibody, whilst mice administered kiwifruit had reduced

antibody production compared to controls yet protection against infection was comparable. Vaccination may induce other effector functions and immune cell populations to protect against infection, such as CD8⁺ T cells. Indeed, it has been demonstrated that whole inactivated influenza vaccines are able to induce CD8⁺ T cell responses in mice^{258,259}, although TIV lacks the ability to induce robust CD8⁺ T cell responses in humans. Despite the fact that correlates of protection against infection could be broader than those investigated currently, the measurement of virus-specific IgG allowed assessment of the efficacy of nutritional interventions to improve antibody responses. Influenza vaccine efficacy is currently measured by seroconversion, therefore a change in the definition of a correlate of protection to influenza vaccination would be required if health claims were to be made on data showing alteration of any other immune parameter.

4.5 Conclusions

In this chapter, a mouse model of human parenteral TIV vaccination and subsequent infection was established in which both antibody responses and protection against infection were suboptimal. Using this model, no appreciable improvement in antibody production to vaccination or protection against subsequent infection were observed with administration of kiwifruit, *B. animalis* or alterations to the fibre content of the murine diet. Interestingly, administration of a propolis formulation tended to improve protection against infection of vaccinated mice, with some mice exhibiting no detectable viral RNA in the lung, despite no enhancement in vaccine-induced IgG antibody.

Whilst administration of the propolis formulation improved immunity to influenza this investigation has not confirmed this was a consequence of changes

in the composition or function of the gut microbiota communities and further evaluation of the involvement of the microbiota in this phenotype are warranted.

5 General discussion

5.1 Summary of findings

The aim of this thesis was to examine the role of the microbiota in the humoral immune response to influenza vaccination and further investigate if microbiota-targeted nutritional interventions could enhance immune responses in a murine model.

Administration of broad-spectrum ABX was shown to alter the composition of the gut microbiota and lead to severely reduced antibody responses to influenza vaccination. Concurrently, ABX treatment led to significant impairments in many cell compartments crucial to the humoral immune response. The frequency and activation of a number of myeloid cell subsets in the dLN were reduced, both prior to and post-vaccination. In addition, there were significantly fewer T_{FH} cells in the dLN following vaccination in ABX-treated mice and generation of the GC B cell response was impaired at multiple stages. Collectively, this data demonstrates that altering microbial signals with ABX treatment leads to abnormalities in multiple cell compartments that are instrumental to the humoral immune response and, consequently, vaccine-induced antibody production.

Having established the importance of intact microbial signals in production of a robust antibody response to influenza vaccination, a murine model of a suboptimal response to human parenteral TIV vaccination was established to trial the benefit of potential microbiota-altering nutritional interventions to enhance immune responses in this model. Administration of kiwifruit, *B. animalis* or alterations to the fibre content of the murine diet did not improve immune responses to influenza vaccination or infection. Promisingly, an appreciable improvement was observed in viral control following

administration of a propolis formulation in vaccinated mice, despite antibody production to vaccination remaining unchanged.

5.2 The microbiota influences multiple cell populations in the influenza vaccine response

Whilst there is substantial evidence that the gut microbiota promotes systemic inflammatory responses in a number of contexts, the mechanism by which gut commensal-derived signals control innate and adaptive immunity at sites beyond the gut, such as in antibody responses to influenza vaccination, are incompletely understood.

In this investigation, administration of ABX to deplete the microbiota resulted in severe impairments in antibody production to TIV vaccination, in line with numerous reports showing robust immune responses to infection^{157,212,229,260,261} and vaccination^{127,158} are dependent on gut microbial signals. A major question that remains largely unexplained in this study and throughout the field is the mechanism by which the gut microbiota influences immune processes at gut-distal sites. One major theory that has been proposed is that, while it is unlikely that intact bacteria enter systemic circulation²⁶², microbial components or metabolites from the gut lumen may translocate across the mucosa into the circulation and effect distal immune cells. This has been directly demonstrated by Clarke and colleagues, who showed that peptidoglycan from the murine microbiota is able to enter the circulation, reaching the BM and enhancing the killing capacity of neutrophils²⁶⁰. Furthermore, this theory is supported by the observation that immune hyporesponsiveness in GF or ABX-treated mice can be rescued by infusion of heat-treated serum¹⁵⁹ or PRR ligands^{156,158}. In addition, gut microbiota-derived metabolites such as SCFAs are absorbed into the blood and contribute to many crucial immune processes, including haematopoiesis^{159,163} and directing T cell differentiation^{180,181,263}. An alternate theory is that immune cells are influenced by microbial products in the gut and migrate to the periphery where they prompt an augmented immune response. This is supported by

findings that in a model of autoimmune arthritis, the gut mucosa adherent microbes, segmented filamentous bacteria (SFB), promote differentiation of autoreactive T_{FH} in the Peyer's patches of the intestine, which are able to egress to systemic sites, such as the spleen and foot dLN, and are essential for the production of auto-reactive antibodies and the arthritic phenotype²⁶⁴. Indeed, evidence suggests that auto-reactive T cells specific for pancreatic islet cells in a model of type-1 diabetes originate in the gut and express gut-homing receptors^{265,266}. It has also been proposed that DCs and Mφ acquire microbial antigen from the gut and transport it to peripheral lymph nodes where it could influence immune cells²⁶⁷, although evidence for this hypothesis is lacking. Oh and colleagues confirmed that vaccine-specific B cells were not present in the gut or associated lymphoid tissues following influenza vaccination¹⁵⁸ and it has yet to be demonstrated if microbially-influenced immune cells from the gut migrate to the periphery to augment antibody production to influenza vaccination.

Notably, whilst this investigation focused on the gut-resident microbiota, it is likely that ABX treatment is altering microbial communities at other sites. Tissue-resident commensal microbes have been shown to influence immunity in proximal tissues. For example, skin microbes control differentiation of local T cells to protect against cutaneous parasitic infection²⁶⁸. Therefore, it is possible in this model that ABX are also modulating microbes at other sites, such as the skin, to influence the antibody response. To ensure this was a gut microbiota-specific effect, neomycin could be administered in isolation - an antibiotic not readily absorbed through the intestine, therefore only eliminating gut microbes^{157,269}.

Development of class-switched, high affinity antibody-secreting B cells to infection and vaccination is a 'multi-player' process in lymphoid organs. T_{FH} cells require concerted interactions with myeloid and B cells for their development, while signals from both myeloid cells and CD4⁺ T cells are required for B cell

expansion and differentiation. The data presented in this thesis suggests that reducing signals from the commensal microbiota with antibiotics leads to a reduction in the homeostatic frequency and activation status of many myeloid populations at gut-distal sites. This results in reduced quantity and quality of cells able to immediately respond upon vaccination, reflected in severely compromised myeloid populations three days following TIV vaccination. This is in agreement with a number of previous investigations showing the requirement for an intact microbiota in maintaining systemic immune homeostasis, maintaining steady-state haematopoiesis^{159,163}, and the constitutive production of chemokines to promote egress of myeloid cell precursors from the BM to sustain immune cell populations throughout the body²²⁸. Furthermore, deprivation of microbial signals impairs homeostatic DC distribution and activation, as well as their ability to migrate to the dLN following influenza infection¹⁵⁷ and M ϕ from ABX-treated mice have major defects in key antiviral transcriptional programmes and are less responsive to influenza virus infection²¹². Together with previous investigations, the data presented suggests that homeostatic signalling from an intact microbiota calibrates myeloid cells to respond effectively to vaccination and this likely is contributing to optimal humoral immunity to TIV.

In this investigation, ABX treatment also led to a reduction in the number of T_{FH} cells in the dLN following vaccination. Development of T_{FH} cells is dependent on signals from myeloid cells and B cells. These findings do not determine if T_{FH} cell development following TIV directly relies on microbiota signals or if reduced T_{FH} cell numbers are due to a lack of signals from myeloid or B cell populations. Given that microbiota-derived molecules are able to directly drive T cell polarisation¹⁸⁰ and that gut-primed T cells are able to migrate to systemic sites²⁶⁴⁻²⁶⁶, it is conceivable that defects may be T cell intrinsic. Nevertheless, because such profound changes are seen to affect dLN myeloid population number and

expression of costimulatory molecules, it is also highly likely that T cells are receiving reduced antigen-presentation, co-stimulation and cytokine signals from DCs and M ϕ , and this may account for reduced T_{FH} cells following vaccination.

B cell differentiation following vaccination was impaired at multiple stages in ABX-treated mice, with reductions in GC B cells, CS B cells and PCs and alterations to the microbiota may be directly signalling on B cells to account for this. Microbial components can influence B cells directly in a number of ways. B cells express TLR-1, 2, 4, 6, 7 and 9²⁷⁰ and ligation of TLRs by microbial products increases B cell survival, as well as antigen presentation and antibody production^{69,234}. At steady-state, binding of lipopolysaccharide (LPS) from the microbiota to B cells directly induces CSR to IgA in the gut²⁷¹. Bacterial metabolites, such as SCFAs also enhance B cell development and antibody production. Antibody production is an energy demanding process and SCFAs have been shown to be directly utilised by peripheral B cells to produce acetyl CoA for fatty acid synthesis, metabolically fueling antibody production, as well as simultaneously inhibiting HDACs for expression of genes enhancing CSR¹⁷⁵. Therefore, the detrimental effects of ABX treatment on B cell development following vaccination may be due to alterations to TLR signalling on B cells directly, or deprivation of certain microbial metabolites, such as SCFAs. Thus, whilst ABX treatment impacted multiple immune cell populations both at baseline and following vaccination, ultimately leading to defective B cell development and impaired antibody production, further investigation is required to elucidate the particular immune cell compartment alterations that are causative of this phenotype.

5.3 Enhancing influenza vaccine efficacy with functional foods

Antibody responses induced in individuals administered the influenza vaccine can vary up to 100-fold¹¹⁵, with particularly poor responses mounted in young children and the elderly⁸³, for reasons that have not been entirely elucidated. As discussed earlier and demonstrated in this thesis, there is emerging evidence of the pervasive influence of the gut microbiota on influenza vaccine responses^{115,158}. The microbiota of infants¹²⁵ and the elderly¹²⁴ are divergent from those of the general population and highly sensitive to change, which possibly contributes to the aberrant immune responses to influenza vaccination in these groups. Effective protection from influenza is particularly important in these populations and microbiota-targeted dietary interventions may be a safe and effective manner in which to improve vaccine responses in these vulnerable groups.

No improvement in antibody production to influenza vaccination nor protection from subsequent infection was observed following administration of kiwifruit, *B. animalis* or by altering the fibre content of the murine diet. The response to specific microbiota interventions may depend on the baseline microbiota composition. For example, following oral administration of the probiotic, *B. longum* AH1206, long-term incorporation of the bacteria into the host microbiota only occurred in individuals already harboring a lower abundance of the *B. longum* species²⁵¹. Furthermore, it has been found that when a low diversity microbiota from individuals consuming a typical 'western' diet was transplanted to GF mice, mice were only able to recover microbiota diversity if they were both cohoused with mice transplanted with microbiota from humans consuming a nutritious diet and simultaneously consuming the nutritious diet²⁷². This illustrates that dietary interventions or probiotic therapies may not be

effective therapies alone, certain species may have to be introduced with probiotics in conjunction with dietary interventions in order to beneficially modulate the microbiota and enhance immune responses to influenza vaccination. Of note, mice fed nutritional interventions were maintained on a lower fibre, higher fat and refined 'western' diet, which is known to reduce microbiota diversity²⁷³, and the baseline microbiota composition whilst on this diet may not have favoured beneficial changes in the microbiota. Furthermore, it would be interesting to test if these nutritional interventions were able to mediate the negative effects of ABX on antibody responses to influenza vaccination.

5.4 The future of microbiota-targeted therapies

Despite significant efforts to determine effective microbiota-targeted therapies to improve outcomes in immune-related diseases, effective therapies remain elusive for enhancing immune responses in human populations.

A major issue that remains is the high variability of gut microbiota composition that exists between individuals¹²⁸, and, in turn, the disparate responsiveness of microbial communities to microbiota-targeted interventions^{251,274}. The difficulty in identifying true probiotic or prebiotic candidates to improve disease outcomes may lie in effects being masked in trials by non-responders. Therefore, it is clear that population stratification based on structural or functional features of the microbiota will be necessary in order to predict the response to nutritional interventions and personalise microbiota-targeted therapies for enhancing immunity. Demonstrating the validity of this approach, Zeevi et al. observed high variability in glycaemic responses between individuals following intake of the same meals²⁷⁵. They showed that by integrating data on an individual's gut microbiota, genetics and environmental factors into a machine-learning algorithm, they were able to more accurately predict dietary recommendations

to lower postprandial glycaemic responses than a clinical dietician²⁷⁵. Approaches of this kind, focussed on the microbiome of the individual, may allow the design of personalised diets to augment gut microbiota functions to achieve optimal immune responses.

Another key issue is the lack of evidence of specific bacteria or metabolites that correspond to desired immune responses in human cohorts. Efforts to determine microbiota signatures that are associated with health and disease have up until recently been focussed on phylogenetic classification of the microbiota and have had limited success in translation to the clinic. Phylogenetic classification may be an irrelevant way to classify bacteria based on their immunomodulatory properties. A recent investigation analysed the effect of GF mice mono-colonised with different bacterial species on innate and adaptive immune responses, showing that there was no relationship between bacterial phylogeny and influences on the host immune system²⁷⁶. Integration of the range of microbiome analysis technologies, including metagenomics, metatranscriptomics and metabolomics will allow a more complete picture of both structure and function of microbial communities. This will allow classification of gut microbiota features in states of health and disease in order for rational design of effective microbiota-targeted therapies.

Continued efforts to understand what compositional and functional characteristics are associated with desired immune outcomes, as well as stratification of individuals, will lead to the development of microbiota-targeted therapies to improve disease outcomes, including the enhancement of vaccine responses.

5.5 Future directions

This report has focused on primary responses to influenza vaccination. It should be noted, however, that human recipients of the vaccine will often have had more than one influenza vaccination or been exposed naturally to multiple viral strains. Accordingly, although circulating viral strains vary seasonally, cross-reactive immunity can occur between similar strains and thus immune responses to vaccination may be secondary, or memory, responses¹²¹. Therefore, in order for this research to have greater translational impact in humans, future investigations should analyse secondary responses to vaccination to assess if the microbiota also influences recall of influenza-specific memory.

Deprivation of microbial signals with long-term ABX treatments has been shown to impact haematopoiesis^{159,229}. Flow cytometric analysis of BM precursors should be performed to determine if short-term ABX administration is having substantial effects on haematopoiesis, leading to the broad reductions in the frequency of myeloid cell populations that was observed.

In this thesis, the ABX treatment significantly altered gut microbiota communities and, in turn, impacted humoral immunity to vaccination. It would be interesting to determine if this phenotype was due to ABX-induced alterations to PRR ligands from the microbiota, or due to changes in microbiota-produced metabolites, such as SCFAs. To do so, alongside ABX-administration, mice should be orally administered SCFAs or autoclaved caecal contents from SPF mice in order to restore microbiota-derived MAMPS, and determining whether either of these stimuli restores antibody responses.

Following vaccination, mice orally administered the propolis formulation exhibited a reduction in viral RNA load independent of any improvement in virus-specific antibodies. To determine immunological parameters that may

have been altered in mice administered the propolis formulation, an array analysis of an extensive panel of cytokines should be performed on serum and lung homogenates following infection. This would give a broad indication of immune parameters stimulated by the propolis formulation that may be responsible for improved viral protection.

Furthermore, to ascertain if the improvements observed in anti-viral responses following propolis formulation administration were mediated by alterations to the microbiota, transferral of microbial communities from propolis formulation fed mice would need to transfer the phenotype. To test this, faecal transplantation experiments could be performed, where gut contents from propolis formulation fed mice are administered IG to control mice in order to transfer microbial communities. Alternately, as mice are coprophagic, propolis formulation fed mice could be cohoused with controls which would also result in transferal of microbial communities, albeit in a less controlled manner²⁷⁷.

5.6 Final conclusions

The evidence outlined in this thesis demonstrates the involvement of the gut microbiota in robust antibody responses to influenza vaccination in mice and that immune responses may be able to be improved through nutritional interventions. Considering the emerging links between the commensal microbiota and a wide range of immune-related diseases, this area of research is vital and microbiota-targeted therapies are likely to be key in combatting a range of diseases in the future.

To summarise:

- 1) Short-term administration of broad-spectrum ABX alters the composition of the gut microbiota and leads to severely reduced antibody responses to TIV vaccination.
- 2) ABX significantly reduce the number and activation status of myeloid cell subsets in the dLN at steady-state and following vaccination. This is concurrent with a reduction in T_{FH} cell numbers and a deficiency in B cells at multiple stages of differentiation following TIV vaccination
- 3) UV inactivation of a mouse-adaptive influenza virus serves as an appropriate model of a suboptimally-responding human population to parenteral TIV vaccination and infection.
- 4) Administration of kiwifruit, *B. animalis* or alterations to the fibre content of murine diet does not improve antibody production to vaccination or protection against subsequent infection. A propolis-cyclodextrin formulation leads to improved protection against infection in vaccinated mice following infection, without influencing vaccine-induced antibody production.

Supplemental figures

Supplementary table 1: qPCR primers

Target	Primer	Sequence (5' - 3')	Source	Supplier
Bacteroidetes	Bac960F	GTTTAATTCGATGATACGCGAG	215	Sigma
	Bac1100R	TTAAGCCGACACCTCACGG		
Firmicutes	Firm934F	GGAGCATGTGGTTTAATTCGAAGCA	215	Sigma
	Firm1060R	AGCTGACGACAACCATGCAC		
Actinobacteria	Act664F	TGTAGCGGTGGAATGCGC	215	Sigma
	Act941R	AATTAAGCCACATGCTCCGCT		
Candidatus Saccharibacteria	Sac1031F	AAGAGAACTGTGCCTTCGG	215	Sigma
	Sac1218R	GCGTAAGGGAAATACTGACC		
Deferribacteres	Defer1115F	CTATTTCAGTTGCTAACGG	215	Sigma
	Defer1265R	GAGATGCTTCCCTCTGATTATG		

Verrucomicrobia	Ver1165F	TCATGTCAGTATGGCCCTTAT	215	Sigma
	Ver1263R	CAGTTTTCAGGATTCCTCCGCC		
Tenericutes	Ten662F	ATGTGTAGCGGTAAAATGCGTAA	215	Sigma
	Ten862R	CATACTTGCGTACGTACTACT		
β proteobacteria	Beta979F	AACGCGAAAAACCTTACCTACC	215	Sigma
	Beta1130R	TGCCCTTTCGTAGCAACTAGTG		
ϵ proteobacteria	Epsilon940F	TAGGCTTGACATTGATAGAATC	215	Sigma
	Epsilon1129R	CTTACGAAGGCAGTCTCCTTA		
γ proteobacteria	Gamma877F	GCTAACGCATTAAGTACCCCG	215	Sigma
	Gamma1066R	GCCATGCAGCACCTGTCT		
Universal 16S	926F	AAACTCAAATGAATTGACGG	215	Sigma
	1062R	CTCACAACACGAGCTGAC

(B)

Time (days)	Sample	Nucleic acid (ng/ μ L)	A260/A280	A260/A230
D-3	1	424.237	2.013	2.101
	2	211.518	1.97	1.47
	3	239.099	1.952	1.792
	4	327.263	1.969	1.686
D0	1	31.202	1.815	0.514
	2	29.702	1.797	0.573
	3	40.018	1.793	0.423
D7	1	67.329	1.842	0.742
	2	23.867	1.976	0.367
	3	64.815	1.94	0.411
	4	57.212	1.81	0.454

Supplementary figure 1: qPCR analysis of faecal DNA in antibiotic-treated mice. C57BL/6 mice received ABX *ad libitum* in the drinking water for three days prior to initial vaccination until seven days following vaccination. **(A)** Faecal-resident bacterial frequencies within a cage of mice throughout ABX treatment was determined by qPCR using 16S rRNA gene-targeted group-specific primers for Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, Verrucomicrobia, Candidatus Saccharibacteria, Deferribacteres and Tenericutes, and proportions were determined against a universal 16S sequence. Proportions of bacteria are presented as the group mean. **(B)** Nanodrop quality analysis of faecal DNA. Data shown is n=3-5 per group.

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