Investigating a role for members of the respiratory tract microbiota in Th17 cell pathogenicity and CNS autoimmunity

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MSc Immunology

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Co-supervisor: Prof Rachel McLoughlin
School of Biochemistry and Immunology
Declaration of Authorship

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I consent to the examiner retaining a copy of the thesis beyond the examining period, should they so wish (EU GDPR May 2018).

Signed: ______________________ Date Submitted: 05/05/2022
Jenny Mannion
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Publications

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Clinical Reviews in Allergy and Immunology (2022)
DOI: https://doi.org/10.1007/s12016-022-08928-y

The respiratory symbionts Moraxella catarrhalis and Klebsiella pneumoniae promote pathogenicity in myelin-reactive Th17 cells
Mannion JM, Varadi K, McLoughlin RM, Lalor SJ. Manuscript in preparation
### Abbreviations

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<tr>
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<tbody>
<tr>
<td>Ahr</td>
<td>Aryl Hydrocarbon Receptor</td>
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<td>Alveolar Macrophage</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
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<td>ATCC</td>
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<td>B-CSF-B</td>
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<td>BMDC</td>
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<td>Conventional Dendritic Cells</td>
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<tr>
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<td>Dulbecco Modified Eagle Medium</td>
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<td>DMT</td>
<td>Disease Modifying Therapies</td>
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<td>ELISA</td>
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<tr>
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<td>GM-CSF</td>
<td>Granulocyte Macrophage-Colony Stimulating Factor</td>
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GWAS  Genome-Wide association Studies
HBSS  Hank’s Balanced Salt Solution
HK  Heat killed
HLA  Histocompatibility Leukocyte Antigen
HMP  Human Microbiome Project
HPRA  Health Protection Regulatory Authority
i.n  Intranasal
i.p  Intraperitoneal
i.v  Intravenous
IFNγ  Interferon γ
IgE  Immunoglobulin E
ILCs  Innate Lymphoid-Like Cells
IM  Interstitial Macrophages
iNKT  Invariant NK T
IPF  Idiopathic Pulmonary Fibrosis
iTreg  Inducible T regulatory
JC Virus  John Cunningham Virus
LDH  Lactate Dehydrogenase
LOS  Lipooligosaccharides
LPS  Lipopolysaccharide
LRT  Lower Respiratory Tract
M cells  Microfold Cells
M-CSF  Macrophage-Colony Stimulating Factor
MAMP  Microbe Associated Molecular Pattern
MBP  Myelin Basic Protein
MDSC  Myeloid Derived Suppressor Cell
MHC  Major Histocompatibility Complex
moDC  Monocyte-Derived Dendritic Cells
MOG  Myelin Oligodendrocyte Glycoprotein
MOI  Multiplicity of Infection
MRI  Magnetic Resonance Imaging
MS  Multiple Sclerosis
NALT  Nasal Associated Lymphoid Tissue
NLR  Nod-Like Receptor
nm  Nanometre
NO  Nitric oxide
NTCC  National Collection of Type Cultures
OD  Optical Density
OMP  Outer Membrane Proteins
OW  Oral Wash
PAMP  Pathogen Associated Molecular Pattern
PBS  Phosphate Buffered Saline
pDC  Plasmacytoid Dendritic Cells
PHE  Public Health England
<table>
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<td>PLP</td>
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<tr>
<td>PML</td>
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<td>Primary Progressive Multiple Sclerosis</td>
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<td>PRR</td>
<td>Pathogen Recognition Receptor</td>
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<td>Rheumatoid Arthritis</td>
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<td>RORyt</td>
<td>RAR-Related Orphan Receptor Gamma t</td>
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<td>Reactive Oxygen Species</td>
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<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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<td>Relapsing Remitting Multiple Sclerosis</td>
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<td>rRNA</td>
<td>Ribosomal Ribonucleic Acid</td>
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<tr>
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<td>Respiratory Tract</td>
</tr>
<tr>
<td>s.c</td>
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</tr>
<tr>
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<td>Segmented Filamentous Bacteria</td>
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<td>THYB</td>
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<td>Tumour Necrosis Factor-α</td>
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<td>T regulatory</td>
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<td>UPT</td>
<td>Upper Respiratory Tract</td>
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<tr>
<td>WT</td>
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<td>μg</td>
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The respiratory tract is home to a diverse microbial community whose influence on local and systemic immune responses is only beginning to be appreciated. Increasing reports have linked the airways with the trafficking of myelin-specific T cells in the pre-clinical stages of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS). Myelin-reactive Th17 cells are important pathogenic effectors in MS and EAE but are innocuous immediately following differentiation. IL-23 driven conversion to an ex-Th17 cell phenotype appears to be a critical step in their acquisition of pathogenic potential, but little is known about the mechanisms that mediate this process. We hypothesize that the airways are a critical site in the immunopathogenesis of EAE, where respiratory tract bacteria express crucial factors that promotes encephalogenicity in Th17 cells.

We exposed innate immune cells in vitro and ex vivo to a range of respiratory symbionts and examined IL-23 and related cytokine secretion. In vivo, we colonised the upper respiratory tract of mice with selected bacteria to determine expression of IL-23 and related cytokines in the airways. We transferred myelin-specific Th17 cells to congenic recipient mice exposed to a range of IL-23 inducing human respiratory symbionts and monitored disease severity and T cell trafficking. Disease was exacerbated in mice exposed to the IL-23 inducing Proteobacteria species Moraxella catarrhalis and Klebsiella pneumoniae, but not the Firmicute species Veillonella parvula (commonly associated with healthy human lungs), compared to PBS administered controls. Disease susceptibility was reduced in germ-free mice compared to conventionally housed mice but was partially restored in germ-free mice colonised with K. pneumoniae. In the pre-clinical stages of disease, we found a significant increase in the frequency of GM-CSF⁺ and GM-CSF⁺IFNγ⁺ double positive donor CD4 T cells in the lungs of mice exposed to M. catarrhalis or K. pneumoniae, compared to V. parvula-exposed mice or PBS controls. We also found elevated expression by donor Th17 cells of key trafficking molecules including CCR6 and CXCR6 in the lungs of these mice. In vitro, dendritic cells exposed to these respiratory bacteria secrete high concentrations of the critical pathogenic cytokine IL-23 and Th17-polarised cells co-cultured with these bacteria-stimulated dendritic cells also displayed a significant increase in GM-CSF and IFNg expression. Our data indicates that exposure to the respiratory symbionts M. catarrhalis and K. pneumoniae promotes expression of key pathogenic molecules in myelin-specific Th17 cells and supports the concept that perturbations in the respiratory microbiota may contribute to the pathophysiology of CNS autoimmune disease.
Chapter 1

General Introduction
Introduction

The human body is covered at all exposed and barrier surfaces with a panoply of bacteria, archaea, fungi, and viruses. These microbes coexist with the host as symbiotic commensals. The microbiota co-evolved with its host and colonises the host’s external barriers including the skin and mucosal surfaces of the oral cavity, respiratory tract, gastrointestinal tract and urogenital tract [1]. However, the true extent of the influence of these microbial communities on host health and pathology has only recently been appreciated. Bacteria are the most numerous microbial components of the commensal microbiota and research to date has primarily focused on the bacteria that colonize the gastrointestinal tract (GIT). Significant advances have been made in our understanding of the crucial role these microbes perform in the healthy host, from structural and metabolic function, regulating the development of the host immune system, to the normal wiring of the brain [2-6].

The respiratory tract (RT) too is colonized by commensal microorganisms that play critical roles in maintaining homeostasis and defence against pathobionts. Owing to the previous misconception that the lungs were sterile, the lower RT (LRT) was omitted from the Human Microbiome Project [7]. Recent advances using culture-independent technologies have identified several microbial species in healthy human lungs and it is now recognised that the RT microbiome is home to a diverse microbial community that forms distinct communities from site to site. Moreover, there is growing evidence that the upper and lower airways may be an important site for microbial regulation of immune responses, both locally and systemically. In the past decade, connections have been made between the composition of the RT microbiota and the pathophysiology of chronic airway diseases such as asthma [8], chronic obstructive pulmonary disease (COPD) [9], cystic fibrosis (CF) [10, 11] and idiopathic pulmonary fibrosis (IPF) [12]. In addition, the lung microbiota has been implicated in the early development of the systemic inflammatory disease rheumatoid arthritis (RA) [11, 13]. However, our understanding of how the RT microbiota influences local and peripheral immune responses is in its infancy and much remains to be learned about how perturbations in this bacterial community modulate the pathogenesis of a range of chronic inflammatory diseases.
1.1 The respiratory tract microbiota

The human airways are a highly vascularised, complex organ system, the structures of which provide distinct surfaces for bacterial growth [14]. The RT can broadly be divided into the upper (URT) and lower RT (LRT). The URT includes the anterior nares, nasal passages, paranasal sinuses, the nasopharynx, oropharynx and the portion of the larynx which is located above the vocal cords. The LRT includes the remainder of the larynx, the trachea, the bronchus, bronchi, and alveoli. The temperature of the epithelial surfaces of the URT and LRT exists in a gradient from ambient temperature on inhalation, 34°C in the nasopharynx to 37°C in the alveoli. The pH also increases along the RT from pH 6.3 in the nasal cavity to pH 7.5 in the alveoli, creating environmental niches at the different sites [15]. Bacterial biomass along the RT is relatively low when compared to other colonised anatomical sites and decreases in biomass from the upper to lower RT [16, 17].

After the GIT, the RT bears the largest exposure to the external environment. At 70 m², the surface area of the lungs alone is forty times that of the skin, owing to the physiological arrangement of the bronchi and alveoli [18]. We breath in approximately 11,000 L of air daily, containing both particulates and microorganisms [19]. As such, the RT is a major site of microbial exposure and colonisation. Indeed, the RT microbiota comprises over 600 individual bacterial species that form distinct communities from site-to-site, each adapting to the diverse surfaces and physiological conditions (e.g., temperature and pH) that exist along its length [7, 15].

In adults, the anterior nares are lined with keratinizing squamous epithelial cells and, due to the presence of sebum, host a lipophilic bacterial community that closely resembles that found on the skin, including Staphylococcus, Propionibacterium and Corynebacterium species [20]. Lined with columnar ciliated epithelial cells and non-ciliated mucus-secreting goblet cells, the nasopharynx comprises a diverse aero-anaerobic bacterial community comprising Streptococcus, Rothia, Veillonella, Prevotella and Neisseria [21]. The oral cavity is home to a varied microbial community in which Streptococcus, Haemophilus, Neisseria, Actinomyces, Prevotella and Veillonella species are commonly identified in healthy individuals [22]. The oropharynx is lined with a non-keratinizing squamous epithelium and
its bacterial community is characterised by the presence of *Neisseria, Rothia, Prevotella, Veillonella, Fusobacteria and Leptotrichia* species [15, 23-25].

Finally, the healthy lungs harbour a diverse microbial community [7]. The bronchial tree is lined with ciliated columnar epithelium that soon transitions to the low cuboidal epithelium of the respiratory bronchioles and, ultimately, alveolar epithelium that is specifically adapted for gas exchange [15]. A thin layer of surfactant contributes to alveolar integrity by reducing surface tension at the air-liquid interface. In addition, surfactant displays bacteriostatic effects and surfactant proteins bind to non-host oligosaccharides and promote leukocyte recruitment and phagocytosis [26]. Advanced sequencing technologies have identified *Prevotella* and *Veillonella* as the dominant genera in the trachea, bronchi and bronchioles [8]. *Streptococcus, Haemophilus, Moraxella, Pseudomonas, Fusobacterium* and *Porphyromonas* taxa are also commonly identified in this dynamic community that results from microaspiration of bacteria and other microbes in oropharyngeal secretions, and ongoing outward movement through mucociliary clearance and the cough reflex [8, 9, 27]. Immune surveillance by alveolar macrophages (AM) also contributes to the turn-over of pulmonary symbionts.

Despite a wide variation in lung environmental factors such as temperature, pH, relative alveolar ventilation, epithelial cell structure and the properties of inflammatory cells, spatial variation within the lungs of healthy individuals is minimal. This may be accounted for by the forceful act of coughing which is likely to homogenise the contents of the airway. Furthermore, spatial microbial variation is less significant than individual specific variation, highlighting the important balance between microbial immigration (which occurs via microaspiration, air inhalation and direct movement along mucosal surfaces) and elimination in shaping the healthy lung microbial community [16, 28, 29].

The development of the early RT microbiota is impacted by multiple factors including mode of delivery, feeding methods, crowding conditions such as day-care type and sibling numbers as well as antibiotic use [15]. Niche differentiation in the URT leads to high abundance of *Staphylococcus* species., which is followed by enrichment with *Corynebacterium* species as well as *Dolosigranulum* species, before *Moraxella* species dominate [30]. A correlation has been described between a stable microbiota in the first
2 years of life, predominated by *Corynebacterium* species *Dolosigranulum* species and *Moraxella* species, and respiratory health. This contrasted with less stable profiles which were characterised by high abundance of *Haemophilus* or *Streptococcus*, which have been linked to increased risk of developing childhood asthma [31-33].

The bacterial microbiota, although the most abundant microbial component of the RT microbiota, coexists alongside the many viruses and fungi that make up the virobiota and mycobiota, forming a polymicrobial community at this site. Metagenomic analysis has revealed that that the RT contains a diverse range of viruses, dominated in health by viruses from the Anellovirdae family which are abundant in the URT and LRT [34]. The interaction of viruses and bacteria in the RT is most well described in the context of respiratory pathologies. For example, *S. pneumoniae* infection is known to worsen disease outcomes during influenza virus co-infection [35]. Viruses act in a variety of ways to render the host more susceptible to bacterial infection such as by attenuating the ability of AM to phagocytose [36] or through the desensitisation of AM for TLR ligands [37]. However, the influence of the commensal virome on the overall microbial community structure in the RT is poorly understood.

The URT harbours a mycobiota which has been reported to include the species *Aspergillus, Penicillium, Candida* and *Alteraria* in healthy individuals [38, 39]. These URT fungi appear only at low abundance in the LRT which is dominated by members of the *Systenostreme, Eremothecium* and *Malassezia* genera as well as the Davidiellacea family [15]. A low burden of fungal DNA from the species *Aspergillus* and *Cladosporium* have also been identified in the LRT [38, 40]. Recently, a role for the RT mycobiome has been assigned to several chronic RT diseases. In asthma, for example, despite variability between individuals, those with severe asthma display a higher fungal burden than those with mild disease [41]. Additionally, *Malassezia pachydermatis* and *Cryptococcus pseudolongus* have been shown to occur at higher frequencies in the lungs of asthmatics than controls [40, 42]. Interestingly, in the GI tract, oral antibiotic use has been associated with fungal overgrowth which was shown to impair the recovery of the bacterial microbiota when antibiotic use has ceased [43]. There are limited studies documenting the relationship between bacterial and fungal species in the RT but synergistic interactions between *Candida* and *Streptococcus*, including the stimulation of the commensal oral
streptococcus strain *S. oralis* growth by *C. albicans* which promoted biofilm formation in the oropharynx. Additionally, an increase in *Candida* pathogenicity by *Streptococcus* have been described [44, 45]. However, the mechanisms by which the commensal mycobiome and bacterial microbiome interact with each other to influence the microbial composition of the healthy RT and contribute to human health is an area that requires significant research but will surely yield new insights into the chronicity of and susceptibility to chronic inflammatory diseases both in the lungs and at distant sites. Being the most numerous microbial components of the commensal microbiota, however, the current study focuses on the bacterial community in the RT, henceforth referred to as the RT microbiota.

The importance of the microbiota in lung development was revealed by the significantly reduced number and structure of alveoli, and decreased mucus production in germ-free (GF) mice compared to conventionally-housed mice or their wild counterparts [4]. Inoculation of GF mice with two Lactobacillus isolates from the murine lung restored normal alveolar development and mucus production [4]. This role of commensal bacteria in the structural development of the RT is akin to the reduced number and size of Peyer’s patches and mesenteric lymph nodes in the gut of GF mice, as well as impaired development and maturation of isolated lymphoid follicles, compared to conventional, specific pathogen-free (SPF) mice [46, 47]. In addition, the bacterial community in the airways regulates mucosal immune responses against respiratory infection and may have a role in the development of certain forms of lung cancer [48-51]. We are only beginning to understand, however, the impact of this airway microbiota on resident and circulating host cells, particularly T lymphocytes that orchestrate the pathophysiology of many chronic inflammatory diseases.

### 1.1.1 Immunomodulation by the respiratory microbiota

The RT epithelia are continuously exposed to airborne particles, allergens and microorganisms, as well as microbes contained in micro-aspirated fluids. It is crucial, therefore, that an appropriate immune response is mounted to eliminate any potential pathogen, but without overt inflammation and immunopathology. Lung homeostasis is
maintained by macrophages, dendritic cells (DC), regulatory T cells (Treg), B cells and epithelial cells [52-54]. The type of immune response generated in the airways is dependent on the microbe encountered and can range from benign tolerance to acute inflammation and immunopathology. The induction of tolerance contributes to microbiome community dynamics at this site. B cell-derived immunoglobulin A (IgA) prevents potential pathogens from infecting mucosal surfaces and acts to stimulate local epithelial cell receptors [55].

Lung macrophages are comprised of bronchial macrophages, AM and interstitial macrophages (IM). AM are an active phagocytic leucocyte population that reside on luminal surfaces of the alveoli and make up approximately 90% of the cellular context in the absence of infection [56]. AM eliminate extracellular bacteria through reactive oxygen species (ROS) production and neutrophil recruitment, and orchestrate lymphocyte responses via innate cytokine secretion [57]. Anti-inflammatory AM are also critical for lung immune homeostasis through their constitutive expression of TGFβ and retinaldehyde dehydrogenase (RALDH), which promote the expansion of inducible regulatory T cells (iTreg) that are crucial in maintaining a balance between pro- and anti-inflammatory responses [58, 59].

Lung DC comprise conventional DC (cDC), monocyte-derived DC (moDC) and plasmacytoid DC (pDC), each of which represent an independent developmental lineage and have distinct but overlapping functions. cDC populate the lungs in the absence of infection, whereas moDCs are recruited to the lungs following exposure to Toll Like Receptor (TLR) ligands, allergens or environmental pollutants [60]. Antigen sampling occurs in the alveolar lumen via extension of processes by DC or translocation of antigen or microorganisms to the lamina propria by microfold (M) cells, with DC stimulation occurring mainly at the basolateral surface of the epithelium [61]. Interestingly, DC-mediated immune-surveillance is accelerated at airway mucosal sites during acute inflammation [62]. Commensal and pathogenic microbes are also sampled at the luminal surface of pharyngeal and lung epithelial cells via pattern recognition receptors (PRRs), including TLRs and NOD-like receptors (NLRs) that contribute to downstream inflammatory signalling.
Akin to the immunomodulatory abilities of the GIT microbiota, recent investigations have begun to elucidate the potential of the RT microbiome to modulate host immunity. Larsen et al., compared the inflammatory properties of Prevotella species found in the healthy human lungs to several commensal and pathogenic Protebacteria also commonly identified in the human respiratory tract, but enriched in the airways of patients with asthma or COPD [63]. They found that, although all bacteria promoted DC maturation, Haemophilus influenzae and Moraxella catarrhalis were much more immunogenic in terms of their ability to stimulate production of inflammation-engendering cytokines including IL-23 and IL-12p70, compared to Prevotella species [63]. This demonstrates the potential for RT bacteria to regulate inflammation in the airways of otherwise healthy humans.

Naïve T cells differentiate along distinct lineages following recognition of their cognate antigen presented by professional antigen presenting cells (APCs) such as DC. For a long time, inflammatory diseases were thought to associate with either an IFNγ-driven Th1-type inflammatory response (e.g. multiple sclerosis) or a Th2-type response characterised by the expression of IL-4, IL-5 and IL-13 (e.g. asthma). However, the discovery in 2005 of Th17 cells, a distinct lineage of CD4 T helper cell that preferentially produced the inflammatory cytokine IL-17A, was a significant advance in our understanding of the pathophysiology of many chronic inflammatory diseases [64]. Since then, a central pathogenic role for IL-17-type immunity in the development or exacerbation of many chronic respiratory diseases has also become apparent, with a growing body of evidence linking activation of these cells with outgrowth of specific members of the airway microbiota.

1.2 Th17 cells

IL-17-producing CD4 T helper (Th17) cells were recognized as a discrete population of CD4+ T cells following the identification of their role in the pathogenesis of experimental autoimmune encephalomyelitis (EAE), a preclinical model of multiple sclerosis (MS) [64, 65]. Subsequently, these cells were found to be key orchestrators of numerous
autoimmune and chronic inflammatory diseases including Crohn’s disease [66], RA [67] and psoriasis [68]. In addition, several innate and adaptive immune cell populations were identified as sources of various IL-17 family members that play important roles in diverse inflammatory settings. These include CD4 T cells, γδ T cells, Natural Killer (NK) T cells, innate lymphoid cells, neutrophils, eosinophils, and AM [69-72].

Th17 cells develop from naïve precursors following recognition of their cognate antigen and are polarised along the Th17 lineage in response to signalling by the innate cytokines IL-1, IL-6 and TGFβ [73]. These cells are characterized by expression of the “master” transcription factor retinoic acid receptor-related orphan receptor gamma (RORγt) which, in turn, promotes expression of a number of key Th17 cell-associated receptors, including IL-23R and CCR6. Th17 cells produce IL-17A and IL-17F along with a range of other cytokines including IL-21, IL-22, TNFa and IL-10. IL-17A and IL-17F signal through a heterodimeric receptor complex composed of the ubiquitously expressed IL-17RA and the non-haematopoietic cell-restricted IL-17RC that, in the airways, is expressed on epithelial cells and fibroblasts [74]. TGFβ acts though SMAD family proteins early in Th17 cell differentiation to restrain Th1 and Th2 cell-associated factors that suppress Th17 cell development [75]. IL-6 signals through STAT3 to upregulate IL-1R expression and promotes RORγt expression via the IRF4 pathway [76]. Although required, expression of the master transcription factor for Th17 cells, RORγt, is not sufficient for Th17 cell differentiation, and factors such as BATF and IRF4 are also necessary [77]. Th17 cells express the chemokine receptor CCR6 which allows cells to cross endothelial barriers that express the corresponding ligand, CCL20 [78]. IL-17-receptor engagement induces epithelial cell expression of anti-microbial peptides (AMPs) and production of CXC chemokines and granulopoietic factors that are critical in the recruitment and activation of neutrophils which, collectively, contribute to protection against infection [79, 80].

Although Th17 cells have now been implicated in the pathogenicity of several autoimmune disorders, their primary function is apparently in protection against infection with fungi, parasites and extracellular bacteria, and the maintenance of the microbiota [81, 82]. Th17 cells are commonly found in the lamina propria of the small intestine and are rapidly induced at other mucosal sites, including the skin and RT, upon microbial
exposure and vaccination [81]. The importance of IL-17 in protection against *Klebsiella pneumoniae* pulmonary infection was highlighted in a study which demonstrated that IL-17R−/− mice displayed impaired neutrophil recruitment and failed to prevent bacterial dissemination [83]. Similarly, IL-17 has been shown to be required for *Staphylococcus aureus* clearance in the murine upper airways where IL-17-mediated recruitment of neutrophils and AMPs mediate bacterial clearance in the nasal cavity [84]. The importance of Th17 cells in *S. aureus* clearance in the lung and skin is further highlighted by the fact that patients with impaired Th17 cells due to Hyper IgE syndrome display increased incidence of *S. aureus* infection [85, 86]. Furthermore, Th17 cells were found to contribute significantly to tissue-resident memory elicited by mucosal immunisation against Gram-positive bacteria such as *S. pneumoniae*, Gram-negative bacteria such as *K. pneumoniae* and fungi [87, 88]. It is clear, that microbial stimulation and Th17 cell function are closely associated and, in fact, Th17 cell development is diminished in germ-free animals. Interestingly, researchers have identified a concomitant decrease in susceptibility of GF mice to many preclinical models of complex diseases such as MS, uveitis, RA, and psoriasis [89-92].

In humans, connections have recently been made between the composition of the RT microbiota and the pathophysiology of chronic inflammatory diseases such as asthma [8], chronic obstructive pulmonary disease (COPD) [9], cystic fibrosis (CF) [10] and RA [11]. In fact, changes in the relative abundance of only a small group of respiratory tract bacteria have repeatedly been associated with a range of chronic inflammatory conditions and a clear finding in many of these investigations is the centrality of IL-17 signalling in response to perturbations of this microbial community. A role for the respiratory microbiome in Th17 cell-mediated CNS autoimmunity remains completely unexplored.
Figure 1.1 Relative changes in bacterial taxa and inflammatory mediators during chronic inflammatory disease

Reduced microbial diversity in the lungs and outgrowth of specific taxa is associated with IL-17-type inflammation in the airways, and systemically. Relative changes in bacterial abundance and their association with cellular mediators of inflammation are indicated. Abbreviations: COPD, chronic obstructive pulmonary disease; IPF, idiopathic pulmonary fibrosis; ILC3, type-3 innate lymphoid cell; NKT, natural killer T cell; RA, rheumatoid arthritis.
1.3 Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic, progressive inflammatory disease of the central nervous system (CNS) [93, 94]. MS is instigated by the infiltration of autoreactive T cells and other immune cell subsets into the CNS, which attack the myelin sheath surrounding nerve axons, resulting in the formation of an inflammatory plaque and reduced signal conductance [95]. These focal demyelinating lesions are the pathological hallmark of MS and consist of T cells, B cells and macrophages, and are diagnosed by magnetic resonance imaging (MRI) or histopathological analysis [95]. Patients display clinical symptoms when signal conduction is blocked or reduced in a substantial number of fibres from one pathway concurrently [95, 96]. Axonal ensheathment and remyelination by oligodendrocytes contribute to the restoration of neuronal conduction which, alongside a reduction in oedema and inflammation, feature during clinical recovery [96]. Conversely, axonal loss is an irreversible process and is associated with impaired neurological function.

Aside from a clinically isolated syndrome (CIS), which refers to a single clinical attack of the CNS and is the first indicator of the onset of potential MS, there are three main forms of MS; relapsing-remitting MS (RRMS), secondary progressive MS (SPMS) and primary progressive MS (PPMS). RRMS presents clinically in approximately 85% of cases and is associated with acute episodes of neurological dysfunction followed by periods of functional recovery. Approximately 50% of RRMS patients go on to develop SPMS in which an accumulation of inflammatory insults results in gradual neurological decline and ascending paralysis, without intermittent disease-free periods. In approximately 10% of cases, patients follow a progressive disease course from first diagnosis, in the absence of any remission. The onset of this PPMS generally occurs at a later age than in RRMS, although cases of childhood PPMS are encountered [97, 98].

MS patients suffer from substantial disability due to impaired sensation and loss of motor, autonomic and neuro-cognitive function. Clinically, patients present with a wide range of symptoms including monocular visual loss resulting from optic neuritis, loss of strength in the lower and/or upper limbs, paresthesia or a decline in senses in the limbs or trunk, sensory or cerebellar ataxia, cranial nerve symptoms including facial sensory disturbance,
diplopia (double vision), oscillopsia (swinging vision) and nystagmus (rapid, rhythmic and repetitious involuntary eye movements), which is associated with brain stem inflammation [99]. Clinical symptoms can also include disruption of bladder and bowel control as well as loss of memory and cognition. MS is the main cause of neurological disability in young adults, with disease onset typically between 20 and 40 years of age, although it can occur at any age [99]. The global incidence of MS ranges from 50 to 300 per 100,000, afflicting over 2.5 million people worldwide and approximately 9,000 people in Ireland, significantly impacting a wide swathe of society in the prime of their lives [93].

1.3.1 MS pathophysiology

The etiology of MS remains elusive, but it is thought that genetic, epigenetic and environmental factors play a role in disease development. It is evident that genetics play an important role in disease development. Approximately 200 genes have been associated with MS susceptibility, the majority of which have a known immunologic function [100]. MHC class II region histocompatibility leukocyte antigen (HLA) genes and T cell receptor (TCR) genes, appear to exert the strongest individual effect on disease risk [97]. The HLA locus is responsible for 20-30% of genetic predisposition to MS [101]. Carriers of the HLAnDRB1*15:01 allele have been shown to have risk of MS development three times greater than non-carriers, while other HLA alleles have also been associated with risk and protection [102]. Genome wide association studies (GWAS) and meta-analyses have been utilised to identify non-HLA associated genetic variants such as genes in \textit{IL2RA}, \textit{IL17RA}, \textit{CD58}, \textit{STAT3} and \textit{TNFRSF1A} [103]. MS susceptibility appears to be influenced by the combination of sequence allelic variation in more than one gene [104]. Furthermore, there is evidence to support the interaction of environmental factors loci such as smoking and obesity with HLA genetic risk loci [105]. In fact, high rates of discordance (up to 70%) in monozygotic twins highlight the importance of environmental factors in disease progression [106]. Factors such as vitamin D deficiency, cigarette smoking, diet, childhood obesity, shift work at a young age and infection have all been implicated in the development of MS, without significant progression of our understanding of disease development or opportunities for therapeutic intervention. Like
other autoimmune disorders, there is a preponderance of females to males of approximately 3:1, suggesting that hormones may also impact disease development [107-109].

The prevalence of MS generally increases with distance from the equator raising questions such as exposure to sunlight and the role of vitamin D in disease development. However, a recent Cochrane Collaboration systematic review found no effect of Vitamin D supplementation in MS patients on relapse rate, worsening of disability or new MRI lesions [110]. MS occurs more frequently amongst Northern European populations. Migration studies have shown that disease susceptibility is determined by where individuals live in early life. By moving from a region of high susceptibility to a region of low susceptibility during childhood (before 15 years of age), an individual will lower their risk of developing disease. Conversely, migration from a region of low susceptibility to a region of high susceptibility results in an increased risk of disease development [111, 112].

Cigarette smoking has been investigated as a risk factor for the development of MS. Smokers develop more severe clinical symptoms and more aggressive secondary progression [113]. It appears that the risk associated with tobacco smoke is due to lung irritation and inflammation [114, 115]. Similarly, air pollution has been associated with increased risk of relapse or MRI activity in MS patients [116-119].

Epidemiological studies have long associated relapses in MS patients with systemic infection. Correale and colleagues demonstrated that bystander activation and increased sensitisation of autoreactive myelin-specific T cells resulted in elevated numbers of circulating IFNγ+ Th1 cells and exacerbated disease in RRMS patients shortly following systemic infection [120]. Notably, it appears that upper airway infections are primarily responsible for enhanced disease activity in MS patients. Viral infections of the URT have been strongly linked with relapses in MS. Moreover, there is strong epidemiological evidence that past Epstein-Barr virus (EBV) infection, the causative agent of mononucleosis, is associated with increased risk of MS [121]. The LRT is considered a reservoir of EBV [122]. Despite intense investigations for many decades, a potential mechanism by which EBV might influence disease risk remains unclear.
In the past two decades major advances have been made in the treatment of many autoimmune conditions, including MS [123]. However, there is still no cure for MS and no broadly effective treatment. Most current disease modifying therapies (DMTs) are efficacious in less than 55% of patients. The gold standard treatment for MS, Natalizumab (Tysabri) reduces relapse rates at 1 year by approximately 65% and reduces the risk of sustained progression of disability by 42% over 2 years compared to placebo [124]. Natalizumab is a monoclonal antibody that targets α4β1 integrin and prevents binding with vascular cell adhesion molecule 1, a receptor on the surface of vascular endothelial cells in the CNS. Blockade of this interaction acts by reducing leukocyte migration into areas of inflammation [125]. However, Natalizumab is associated with a risk of developing progressive multifocal leukoencephalopathy (PML), a fatal demyelinating disease caused by JC virus infection of oligodendrocytes [126]. Antibodies that block IL-17 or its receptor have shown high efficacy in the treatment of psoriasis, [127], as well as in psoriatic arthritis, and rheumatoid arthritis [128, 129] which share commonalities in their IL-17 associated pathophysiology with MS, thus validating the IL-17A pathway as an important therapeutic target in T cell-mediated autoimmunity. There have been some promising indications from a phase IIa clinical trial with the anti-IL-17A antibody, Secukinumab in patients with the RRMS, although this trial did not meet its primary endpoint, blocking IL-17A reduced MRI lesion activity in MS patients without causing adverse effects [130]. Additionally, multiple case reports have described the successful treatment of comorbid MS and psoriasis and comorbid MS and ankylosing spondylitis with Secukinumab [131-133]. Hence, more advanced monoclonal antibodies targeting IL-17 with improved pharmacodynamics might be considered for application in MS in the future.

As the crucial roles for IL-23 and GM-CSF in the pathogenesis of EAE have emerged, there has been increasing efforts to target these molecules in the treatment of MS. Ustekinumab is a fully human monoclonal antibody that targets the p40 subunit shared by IL-12 and IL-23 [134, 135]. Ustekinumab has displayed clinical efficacy in IBD and psoriasis [136, 137]. However, despite efficacy in the EAE model [138], Ustekinumab failed to substantially improve clinical disease in a phase II trial in patients with RRMS [139]. However, at the induction of this study, the patients had already progressed to a chronic disease course with long disease duration. Given that data from the EAE model suggests
that IL-23 exerts its greatest importance in the initial generation or expansion of autoreactive T cells it might be considered that the window of opportunity for the effective treatment with anti-IL-12/23p40 might have passed for the patients enrolled in this study [139, 140]. Another explanation for the lack of efficacy of Ustekinumab in MS could be that the antibody failed to cross the BBB efficiently and neutralize the production of IL-23 by CNS resident cells that might have a role in promoting inflammation at this site [139, 141]. Based on preclinical studies in EAE, an antibody targeting GM-CSF, Otilimab, was enrolled in a phase I clinical trial for the treatment of MS [142, 143]. Moreover, Otilimab, and other monoclonal antibodies targeting GM-CSF, have now been enrolled in a phase III trial for the treatment of RA, highlighting the validity of targeting this pathway for the treatment of chronic inflammatory diseases [144, 145].

There remains an urgent need to find new treatments for MS. Given that MS lesions accumulate in the CNS in the absence of clinical symptoms or signs and that diagnosis is made following multiple episodes of neurological dysfunction, prophylactic treatment is not an option for patients with this disease. Genetic predisposition means CNS-antigen specific T cells are present in the circulation long before the onset of clinical symptoms [146-148]. A crucial goal therefore is to prevent activation of autoantigen-specific T cells that mediate relapses and disease progression. To achieve this, we need a more complete understanding of the pathogenesis of MS and the factors that instigate disease development and relapse.

1.3.2 Experimental Autoimmune Encephalomyelitis

Much of our understanding of the pathophysiology of MS has arisen from studies of experimental autoimmune encephalomyelitis (EAE), an animal model that recapitulates many of the clinical and pathological features of the human disease. EAE is a chronic demyelinating disease of the CNS in which animals experience attacks of inflammation in the brain and spinal cord, with consequent ascending flaccid paralysis [149]. EAE can follow an acute, chronic progressive or relapsing-remitting course, dependent on the immunisation strategy and genetic background of the animal used, mirroring the typical clinical course of most patients with MS [150].
EAE can be actively induced via immunisation of susceptible animals with spinal cord homogenate, individual myelin antigens such as myelin basic protein (MBP) and proteolipid protein (PLP), or peptides corresponding to the major encephalitogenic regions of myelin proteins (e.g. myelin oligodendrocyte glycoprotein (MOG)), all typically emulsified in complete Freund’s adjuvant (CFA). This results in T cell-mediated immunity against myelin and high incidence of autoimmunity [151]. After differentiation in the periphery, myelin-specific T cells translocate to the CNS where they are reactivated by local and infiltrating APC, leading to inflammation, demyelination and axonal damage.

Alternatively, EAE can be passively induced by the adoptive transfer of in vitro-polarised myelin-specific T cells to naïve syngeneic hosts, a method which has been critical for establishing the nature of pathogenic T cells in the development of EAE. Here, T cells are isolated from myelin-primed donors and stimulated in vitro with their cognate antigen in the presence of combinations of recombinant cytokines that polarize CD4 T cells towards a specific effector lineage [149]. This allows for the in vitro manipulation of encephalitogenic T cells, such as the labelling of myelin-reactive T cells with a fluorescent dye that permits characterization T cell trafficking in vivo following transfer [149, 152].

Given the limited availability of afflicted tissues during the early stages of MS pathogenesis, the EAE model has been fundamental in identifying the critical immune cell subsets that orchestrate the inflammatory process in the CNS. In addition to their role in antigen presentation, PRRs on innate immune cells such as DC recognise microbe-associated molecular patterns (MAMPs) or danger-associated molecular patterns (DAMPs), resulting in the production of cytokines that polarize naïve CD4 T cells towards a Th1, Th2, Th17 or Treg cell phenotype. Activated T cells can enter the CNS via the blood-brain barrier (BBB) or blood-cerebrospinal fluid barrier, where they are re-activated and proliferate following recognition of their cognate antigen presented by resident microglia as well as monocytes and inflammatory DC that traffic to the site of inflammation [153]. Inflammatory cytokines such as IL-17A, IFNγ and TNF and granulocyte-macrophage colony-stimulating factor (GM-CSF) are secreted by infiltrating T cells and instigate a neuroinflammatory cascade that ultimately results in demyelination, axonal damage and the clinical manifestations of EAE [96, 149-151].
Studies in EAE were imperative in shifting the previously accepted paradigm that Th1 cells were the main pathogenic effectors in MS to the current understanding that Th17 cells are in fact the critical subset for disease development. The original theory was largely based on the observation that mice lacking IL-12p40, one of two subunits that dimerise to form the functional IL-23 cytokine and which is shared with the Th1 cell-promoting cytokine IL-12, were resistant to EAE [154]. This was corroborated by a clinical trial outcome in which MS patients administrated the prototypical Th1 cytokine, IFNγ, developed severe relapses [155]. Surprisingly, it was found that STAT1−/− and IFNγ−/− mice, which lack Th1 cells, developed exacerbated EAE [156]. The discovery of the role played by IL-23 in EAE development marked a paradigm shift in our understanding of the T cell axis in EAE. Cua et al utilised the EAE model to show that mice lacking the IL-23p19 subunit or the shared IL-12p40 chain of IL-23, but not the IL-12p35 chain, were resistant to EAE, indicating that IL-23 but not IL-12 is required for CNS autoimmune inflammation [154]. These findings in EAE have shaped our current understanding that IL-23-driven Th17 cells, and not IL-12-driven Th1 cells, are the pathogenic effectors in EAE and MS [154, 157, 158].

The EAE model allows for the study of the inflammatory processes underlying disease development in MS, including T cell activation in response to self-molecules and immune cell trafficking across the BBB. Additionally, the use of genetically engineered animals in the EAE model has provided a useful mechanism with which to identify key inflammatory mediators in MS and develop novel therapeutics against these targets [96]. Indeed, much of the progress made on the treatment of MS has arisen from studies EAE, exemplified by the fact that the majority of currently licensed DMTs, which have only become available in the past 20 years or so, were developed or tested in the EAE model [159].

1.4 IL-23 and Th17 cell pathogenicity

The discovery that IL-23 signalling is crucially required for Th17 cell pathogenicity, marked a significant milestone in our understanding of the pathogenesis of CNS autoimmune inflammation [73, 154, 157]. IL-23 is a member of the IL-12 superfamily of heterodimeric cytokines that also includes IL-12, IL-27 and IL-35. IL-12 family members are typically
produced by activated APCs such as DC and macrophages (MΦ) and play important roles in innate and adaptive immune modulation, primarily through the coordination of T cell responses [160]. All four IL-12 family cytokines are dimeric proteins made up of an alpha subunit (p19, p28 or p35) and a beta subunit (p40 or EBi3) [161]. Given the structural similarity between all three α-subunits, they have potential to pair with either of the structurally homologous β subunits, thus allowing for shared IL-12p40 by IL-12 and IL-23 and shared Ebi3 by IL-27 and IL-35 [161, 162]. IL-12 family cytokines initiate their effects through high-affinity engagement of their cognate receptor (IL-12Rβ1, IL-12Rβ2, IL-23R, IL-27Rα, or gp130), the individual chains of which are also shared among family members. For example, the IL-23 receptor (IL-23R) and IL-12R share the common IL-12Rβ1 subunit, but IL-23 uses the unique IL-23R chain, whereas IL-12R comprises IL-12Rβ2 [160]. IL-12 family receptor engagement activates downstream Janus kinases (JAKs) by transphosphorylation and results in the recruitment of specific STAT family transcription factors [160, 163].

Despite the cross-utilisation of cytokine subunits and common signalling receptors, IL-12 family members play immunologically divergent roles. IL-27 and IL-35 are generally immunoregulatory. IL-27 can display pro- or anti-inflammatory properties through the induction of Th1 cell master transcription factor Tbet and via inhibition of Th17 cell differentiation in an IL-10-dependent fashion [164], whereas IL-35 functions to induce regulatory T and B cells [165]. IL-12 is important for the differentiation of IFNγ-producing Th1 cells whereas IL-23 is thought to be required for the survival, expansion, and maintenance of Th17 cells, but not for initial differentiation [166, 167]. In fact, naïve CD4 T cells do not express the IL-23R, which is only upregulated following expression of the transcription factor RORγt [168]. How IL-23 promotes pathogenesis in self-reactive Th17 cells remains unknown.

McGeachy and colleagues attempted to answer this conundrum by suggesting that T cells lacking IL-23R followed normal early developmental patterns but failed to downregulate IL-2 and IL-27, or upregulate IL-17RA expression, thereby implicating IL-23 signalling in the expansion and stabilisation of Th17 cells, but not in their initial lineage commitment [168]. Th17 cells that differentiate in the presence of IL-6 and TGFβ secrete IL-17A, but also high
levels of IL-10, and fail to induce EAE following transfer to naïve syngeneic hosts [76]. Becher and colleagues further demonstrated that, while proliferation and activation of myelin-reactive Th17 cells is not dependent on IL-23, it’s signalling is required for their accumulation in the CNS [169]. It appears, therefore, that exposure of recently polarized Th17 cells to the cytokine IL-23 is a critical step in their acquisition of pathogenic potential.

A caveat in our understanding of Th17 cell pathogenicity in EAE arises from the high level of context-dependent plasticity displayed by Th17 cells. Depending on their environmental niche, Th17 cells can acquire characteristics associated with Th1 cells – a feature which contributes to the role played by Th17 cells in both protection against pathogens and in autoimmune disease (Figure 2). For example, IFNγ production by Th17 cells has been reported during intestinal infection with Helicobacter hepaticus and Citrobacter rodentium as well as in a Th17 cell transfer model of colitis [170-172]. Conversely, Th17 cells do not require IL-23 signalling or conversion to an ex-Th17 phenotype to be protective against Candida albicans or Salmonella enterica infection, signifying the extent to which different environmental stimuli can shape Th17 cell effector function [152, 173, 174].

Th17 cell plasticity plays an important role in the pathogenesis of MS and EAE too. Using fate-mapping IL-17-reporter mice, it was demonstrated that the majority of IFNg+ Th1 cells found in the CNS at the height of the acute phase of disease in EAE are actually derived from so-called ‘ex-Th17’ cells (lately referred to as Th17.1 cells in human studies) [152]. It appears that these Th17 cells transition through an IL-17A/IFNg double-positive phenotype, before completely downregulating RORγt and IL-17 expression. Crucially, IL-23p19-deficient mice lacked the IL-17A+/IFNg+ double positive and IFNg single positive ex-Th17 cells observed in their WT counterparts, which was associated with impaired Tbet upregulation. In contrast, IL-12 signalling was redundant for the production of IFNg by ex-Th17 cells in vivo [152]. This study reinforced previous findings implicating IL-23 signalling in Th17 cell pathogenicity and demonstrated that, while IL-17-producing T cells may initiate immune cell mobilization during an inflammatory response that does not result in autoimmunity, conversion of autoantigen-specific Th17 cells to a Th1-like phenotype is a critical encephalitogenic event in EAE.
Although these IFNγ+ Th17 cells are now accepted as the critical pathogenic effects in MS and EAE, expression of this Th1 signature cytokine is dispensable for the development of autoimmune encephalomyelitis in mice [154]. Rather, the cytokine GM-CSF has been demonstrated to be the critical pathogenic signal induced by IL-23, with GM-CSF-deficient mice being completely resistant to EAE [142, 156, 175]. GM-CSF is a heterodimer composed of α and common β subunit that is shared with IL-3 and IL-5 [176]. Although first described as a growth factor, given its ability to induce the proliferation and differentiation of myeloid progenitors in the bone marrow, GM-CSF also plays a key role in orchestrating the cross talk between lymphocytes and tissue-invading myeloid cells at sites of inflammation [177, 178]. Mice deficient in GM-CSF are resistant to EAE, displaying an inability to sustain immune cell infiltrates in the CNS as well as a reduction in antigen-specific T cell proliferation. Recipients of GM-CSF-deficient myelin-specific CD4+ T cells are resistant to EAE while antibody-mediated blockade of GM-CSF has been shown to effectively ameliorate disease [142, 179]. Interestingly, there was no deficiency in disease development when WT CD4+ T cells were transferred to GM-CSF−/− mice, highlighting the non-redundant role of T cell-derived GM-CSF in the induction of EAE. This supports previous findings that T cell-derived GM-CSF is required for microglial activation during EAE [179]. In fact, T cells do not express the GM-CSF receptor and it is now clear that GM-CSF acts on CNS infiltrating myeloid cells such as DCs, monocytes and macrophages to amplify immunopathology in the CNS [156, 179-182].

Other factors too have been implicated in promoting pathogenicity in CD4+ Th17 cells. CCR6 is regulated by the Th17 master transcription factor RORγt and has been implicated in promoting trafficking of myelin reactive Th17 cells to the CNS in the early stages of EAE [78, 183]. CCL20, the ligand for CCR6, is constitutively expressed on epithelial cells of the choroid plexus in mice and humans, thus representing a potential first entry point for myelin-specific Th17 cells [78]. Analysis of inflamed CNS tissue at the peak of EAE revealed that GM-CSF producing T cells are characterised by expression of the chemokine CXCR6 that is involved in the trafficking of lymphocytes to non-lymphoid tissues [184-186]. CXCR6 plays an important role in pathogenic Th17 cell recruitment and its blockade ameliorates the development of EAE [185]. Expression of CXCL16, the ligand for CXCR6, is upregulated in the CNS during EAE [187]. Finally, SerpinB1 is a protease inhibitor and
neutrophil survival factor that is upregulated in effector T cells during EAE and is required for the development of clinical disease [185].

In MS, the dominant cerebrospinal fluid (CSF)-associated CD4+ T cell subset also express the Th17-associated chemokine receptor CCR6 and bear a ‘non-classical’ Th17.1 cell phenotype expressing high levels of IFNγ and GM-CSF [158, 188]. Epigenetic analysis indicates that these cells develop from Th17 cells that have downregulated their IL-17-producing capacity [189].

IL-23 signalling is critical for the expression of GM-CSF, SerpinB1 and CXCR6 that appear to confer pathogenicity upon self-antigen specific Th17 cells. However, because Th17 cells only upregulate IL-23R following differentiation in peripheral lymphoid tissues, it remains unclear where or when exposure to this pathogenic signal occurs during the development of EAE.

![Image: Th17 cell development and effector function](image_url)

**Figure 1.2 Th17 cell development and effector function**

Th17 cells differentiate in the presence on IL-1, IL-6 and TGFβ. Th17 cells produce the cytokines IL-17A, IL-17F, IL-21, IL-22 and IL-10, express the chemokine receptor CCR6 and the transcription factor RORγT. Th17 cells are innocuous immediately following differentiation and conversion to an ex-Th17 cell phenotype is a critical step in their acquisition of pathogenic potential. For the conversion to a pathogenic GM-CSF and IFNγ producing ex-Th17 cell phenotype, Th17 cells must be exposed to the cytokine IL-23. Th17 cells only upregulate the IL-23R following differentiation. Where or when Th17 cells are exposed to this pathogenic signal is unknown.
1.5 The respiratory microbiota and MS/EAE

Because Th17 cells only upregulate IL-23R following differentiation in peripheral lymphoid tissues, it remains unclear where or when myelin-specific Th17 cells are exposed to this pathogenic signal during the development of EAE. In fact, very little is known about the trafficking of myelin-reactive Th17 cells prior to the onset of clinical disease. Previous work in our lab monitored T cell trafficking every day following adoptive transfer to naïve recipient mice and found that myelin-reactive Th17 cells traffic to both the mucosa-associated gut-draining mesenteric lymph nodes and the lungs in the time between their transfer and the onset of neurological deficits (Lalor, S.J. Unpublished data). It was found, however, that antibody-mediated blockade of α4β7-MAdCAM-1 interactions, preventing transferred Th17 cells from trafficking through the mesenteric lymph nodes, Peyer’s patches and small intestinal lamina propria, had no impact on donor Th17 cell accumulation in the CNS and failed to attenuate disease. Similarly, genetic insufficiency of β7 integrin in donor Th17 cells preventing trafficking to these gut associated lymphoid tissues, or surgical resection of the mesenteric lymph nodes, had no effect on EAE development.

Recently, research has focused on microbial dysbiosis in the gut of MS patients and in EAE, sometimes with conflicting outcomes. As mentioned previously, GF or antibiotic-treated mice are relatively resistant to EAE, associated with lower infiltration of leukocytes into the CNS compared to their conventionally-housed, SPF controls [190, 191]. Reconstitution of these GF mice with the Clostridial species segmented filamentous bacteria (SFB), which is known to promote Th17 cells in the murine gut, partially restored susceptibility to EAE [190, 191]. Several studies have demonstrated that changes in the composition of the gut microbiota can alter EAE disease course. For example, a number of studies have described attenuated EAE upon administration of Bifidobacterium [192], Bacteroides fragilis [193], and Prevotella histicola [194]. It was suggested that these bacteria reduce EAE disease severity through the induction of FoxP3+ T regulatory cells in the gut and may exert their effects by dampening inflammatory responses [190].

Transfer of a fecal microbiota from RRMS patients, but not the microbiota from a paired healthy twin, was sufficient to enhance the incidence of EAE in mice, indicating that the
gut microbiota of MS patients contains factors that regulate the autoimmune response in MS [195]. It was reported that patients with inactive RRMS display greater microbial diversity (a hallmark of a healthy microbiome), compared to patients with active disease [196]. More specifically, the relative abundance of Euryarchaeota and Verrucomicrobia were increased in faecal samples of RRMS patents, compared to healthy controls [197]. Conversely, bacteria from the phyla Actinobacteria and Bacteroidetes, including Prevotella species, were significantly reduced in untreated MS patients, but increased in abundance following treatment with IFNβ or glatiramer acetate [197]. Separate studies have identified higher relative abundance of Streptococcus, Akkermansia and Methanobrevibacter taxa and a lower relative abundance of Prevotella, Bacteroides (B. coprophilus and B. fragilis) and Faecalibacterium prausnitzii for MS cases relative to controls [198, 199]. Collectively, these findings suggest that the commensal gut microbiota have the potential to regulate the pathogenicity of EAE and MS. However, a recent systematic review of relevant human studies failed to find major differences in the overall composition of the gut microbiomes of MS patients and healthy non-MS controls [199].

A role for the lungs in the development pathogenic effector ex-Th17 cells that mediate neuro-inflammation and demyelination in EAE has not been explored. Nevertheless, this concept has recently garnered some support. A number of studies have now identified the lungs as an important staging point in the preclinical stages of EAE [200-203]. Odoradi et al employed intra-vital microscopy in a rat model of disease to show that transferred myelin-reactive CD4 T cells migrate to the lungs and bronchus-associated lymphoid tissue, prior to their appearance in the CNS and the onset of neurological deficits [200]. It was shown that these cells transiently reside in the lungs where they under-go functional reprogramming that allows them to enter the CNS and instigate neuroinflammation. Although not specifically assessing Th17 cells, Odoardi et al demonstrated that donor CD4 T cells upregulated a migratory programme in the lungs including expression of the Th1-associated chemokine receptors CXCR3 and CCR5 [200]. Another report by Glenn et al described the IL-6-dependent promotion of Th17 cell pathogenicity in the lungs by a population of granulocytic myeloid derived suppressor cells (MDSCs) which expand in the lungs during the development of EAE [202].
The potential importance of the lungs in the pathogenesis of CNS inflammation was further highlighted in two studies that showed that pulmonary inflammation can attenuate EAE disease severity. RT infection with the bacterium *Bordetella pertussis* prior to the induction of EAE resulted in attenuated disease severity, associated with increased IL-10-mediated Treg responses in the lungs [201]. Kanayama and colleagues also found that subclinical pulmonary inflammation in mice lacking the protein Autophagy Related 7 (ATG7) also led to the delayed onset of EAE [203]. In this setting, it appears that lung inflammation stalled the trafficking of Th17 cells to the CNS, and holds true following adoptive transfer of Th17 cells from WT mice to Atg7/- hosts. Taken together, these studies support the concept that myelin-reactive Th17 cells traffic to the lungs in the preclinical stages of disease and suggest that the lungs may be a critical site for the conversion of Th17 cells into pathogenic effector ex-Th17 cells that mediate neuroinflammation and demyelination in EAE. How T cell encephalitogenicity might be licensed in the lungs remains to be established.
1.6 Project Aims

Th17 cells are critical pathogenic effectors in MS but are innocuous following differentiation. The exposure of recently polarized Th17 cells to the cytokine IL-23 appears to be a critical step in their acquisition of pathogenic potential and may correlate with their development of Th1-like properties. Where and when Th17 cells are exposed to this pathogenic signal during disease development is unknown. Our preliminary data indicates that myelin-reactive Th17 cells traffic to the lungs in the preclinical stages of the animal model, EAE. We hypothesise that CD4 T cells that have invoked the Th17 genetic programme are initially attracted to the airways where putative RT bacteria induce expression of IL-23 by innate immune cells that licenses encephalitogenic Th17 cells to infiltrate the CNS and mediate neuroinflammation in EAE. We suggest that a paucity of information about interactions between the mucosal immune system and bacteria that colonize the RT has hindered our understanding of the fundamental pathways that mediate this complex autoimmune disease. Hence, the goal of this research programme is to elucidate local and systemic immune responses to human RT bacteria, and how this impacts disease exacerbation in EAE and MS. Specifically, we aimed to:

1. Investigate the immunogenic potential, in the context of Th17 cell pathogenicity, of a wide range of bacteria that commonly colonise the human RT, using in vitro culture systems.
2. Examine the effect of RT bacteria on immune responses locally in the lungs, and systemically, in novel models of airway colonisation in mice.
3. Determine if human RT bacteria can impact the effector function of myelin antigen-specific Th17 cells during the development of EAE.
4. Assess the ability of selected human RT bacteria to promote conversion of donor Th17 cells to an ex-Th17 cell phenotype in vitro and in vivo.
Chapter 2

Materials and Methods
2. Methods

2.1 Bacterial culture

2.1.1 Bacterial growth conditions

Stocks of all bacterial strains were prepared in 70% glycerol and stored at -80°C. Bacteria was grown over-night on tryptic soy agar (TSA), blood agar (BA) (prepared by supplementing TSA with 4% defibrinated sheep’s blood (Sparks Labs)), chocolate agar (prepared by supplementing TSA with 4% lysed defibrinated sheep’s blood (ThermoFisher)) or anaerobic agar. Liquid cultures were grown in tryptic soy broth (TSB), Muller-Hinton broth (MHB), Todd-Hewitt yeast broth (THYB) or anaerobic broth. Anaerobic conditions were created using an Aerogen gas pack (ThermoFisher) and an anaerobic chamber (Oxoid). Optimised growth conditions for all bacterial strains are listed in Table 2.1.

2.1.2 Preparation of bacterial inocula

Bacterial strains were streaked on appropriate agar for the indicated times (Table 2.1) before being collected from the plate and suspended in 1 ml sterile PBS. The OD$_{600}$ of the suspension was adjusted to 0.45 (containing 4 X 10$^8$ Colony Forming Units (CFU)/ml) or OD$_{600}$ of 1.0 (containing 1 X 10$^9$ CFU). Dilutions in PBS were performed to generate a multiplicity of infection (MOI) of 0.1, 1, 10 and 100. For i.n. administration, a 1:4 dilution in PBS was performed such that 20 μl contained 1 X 10$^6$ CFU. To confirm the CFU count, serial dilutions of all inocula were spread on the appropriate agar (Table 2.1) and grown at 37°C in the appropriate conditions for the indicated times (Table 2.1). Heat killed bacteria was prepared by heating prepared bacterial suspension at desired OD$_{600}$ on a heat block at 70°C for 30 min before centrifuging at 10,000 RPM for 10 min and resuspending in sPBS. Bacterial cell death was confirmed by plating 100 μl heat-killed stock on appropriate agar for 5 d (Table 2.1).

2.1.3 Bacterial growth assay

Overnight liquid cultures were diluted to an OD$_{600}$ of 0.1 in appropriate broth (Table 2.1). 200 μl of diluted culture was added to triplicate wells in a flat-bottom 96 well microtiter plate sealed with breathable or airtight seals for aerobic or anaerobic conditions. The
plate was incubated at 37 °C for 10 h in a spectrophotometer (Synergy H1 Plate Reader, BioTek) and absorbance values at 600 nm were recorded at 10 min intervals.
<table>
<thead>
<tr>
<th>Species</th>
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<th>Growth Conditions</th>
<th>Growth Media (Agar/Broth)</th>
<th>Growth Duration (h)</th>
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<td>ATCC 25586</td>
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</tbody>
</table>
2.2 Mice

Wild-type C57BL/6J mice were bred in-house under specific pathogen free (SPF) or germ free (GF) conditions in the TCD Comparative Medicine Unit (CMU). The sterility of the GF mice was confirmed by PCR for bacterial DNA in fresh fecal samples as well as routine microbiological screening of animals and isolators using aerobic and anaerobic culture methods. All mice were 6-12 weeks old at the initiation of experiments. All animal experiments were conducted under licence (AE19136/P098) in accordance with the recommendations and guidelines of the Health Products Regulatory Authority (HPRA), the competent authority in Ireland.

2.3 Cell culture

Cells were cultured in an incubator at 37°C with an atmosphere of 95% humidity and 5% CO₂.

2.3.1 Generation of murine bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BMDC) were generated from C57BL/6 mice, using a method similar to that described by Lutz et al [204]. Mice were euthanized, and their femurs and tibiae removed and dissected from the surrounding muscle tissue. The bone marrow was flushed out with complete Roswell Park Memorial Institute-1640 medium (cRPMI; Table 2.3) using a 27 G needle attached to a 20 ml syringe. The cell aggregates were dissociated using a 19 G needle attached to a 20 ml syringe. The cell suspension was pelleted by centrifugation (300 x g for 5 min at 4°C) and cells were resuspended in 2 ml of ACK lysis buffer (warmed to 37°C; Invitrogen) for 2 min, to lyse red blood cells. The cells were washed in cRPMI medium, then pelleted by centrifugation (300 x g for 5 min at 4°C) and resuspended in 10 ml cRPMI medium. Cell viability was assessed by Trypan Blue staining (section 2.4). BMDC precursor cells were cultured at 4 x 10⁵ / ml in cRPMI medium containing 20 ng/ml of GM-CSF in the form of supernatant from a GM-CSF expressing J558 cell line in T75 tissue culture flasks. After 3 d incubation, 20 ml of fresh cRPMI medium containing 20 ng/ml GM-CSF was added to each culture flask. 3 d later, the flasks were removed from the incubator and the cell culture supernatant was decanted to eliminate
contaminating cells (e.g. granulocytes) from the culture. 25 ml of sterile PBS (Sigma), warmed to 37°C, was added to each flask and the flasks were gently agitated before the PBS suspension was transferred to 50 ml tubes containing 10 ml cRPMI medium. 20 ml of sterile Trypsin-EDTA Solution (0.02%; Sigma), warmed to 37°C, was added to each culture flask before returning the flask to the incubator for 10 min. The cells removed in the PBS step were pelleted by centrifugation (300 x g for 5 min at 4°C) and resuspended in cRPMI medium. Culture flasks were removed from the incubator and the EDTA solution repeated-pipetted over the layer of remaining cells, before being added to 10 ml fresh cRPMI medium warmed to 37°C, and centrifuged at 300 x g for 5 min. The cell pellet was resuspended in cRPMI medium and pooled with the pellet obtained in the PBS step. Cells were counted in Trypan Blue and re-cultured at 4 x 10^5 cells/ml in cRPMI medium supplemented with 20 ng/ml GM-CSF. After a further 2 d of incubation, 20 ml cRPMI medium containing 20 ng/ml GM-CSF was added to each flask of cells. After a further 2 d in culture (d 10), the loosely adherent cells were harvested by gentle repeat pipetting of the culture medium. Viability of BMDC was assessed and the cells were cultured at required concentrations (e.g. 1 x 10^6/ml) in antibiotic-free media in tissue culture plates. BMDCs were rested at 37°C / 5% CO₂ for 3 h before stimulation.

**2.3.2 Generation of murine bone marrow-derived macrophages**

Bone marrow-derived macrophages (BMM) were generated as above. Immature BMMs were plated in petri dishes in Dulbecco Modified Eagle Medium (cDMEM; Table 2.3) containing M-CSF in the form of 20% L929-conditioned media. After 3 d incubation, 1 ml of fresh cDMEM containing 20% L929-conditioned media was added to each culture dish. 3 d later, the petri dishes were gently removed from the incubator and the cell culture supernatant was carefully decanted. 5 ml antibiotic-free DMEM was added to each culture dish and cells harvested using a cell scraper to remove adherent cells. Viability of BMM was assessed and the cells were cultured at required concentrations (e.g. 1 x 10^6/ml) in antibiotic-free media in 96 well cell culture plates. BMM were rested at 37°C / 5% CO₂ for 3 h before stimulation.
2.3.3 Cell isolation from lung and spleen tissue

The lungs of naïve mice or mice that had been administered 5µg Flt3 ligand (Invitrogen) in 200µl PBS i.p. every 2 d for 10 d were harvested and dissociated using a scalpel and incubated in HBSS containing 1µg/ml Collagenase IV (Sigma) and 100ng/ml DNAse I (Applichem Lifesciences) for 50 min at 37°C, shaking regularly. Lung digest was passed through a 70μm cell strainer. Spleens were dissociated through a 70μm cell strainer. Cells were centrifuged at 300 X g for 5 min, red blood cells lysed with ACK lysis buffer (Invitrogen) and washed in cRPMI. Cells were resuspended at 1 x 10^6/ml and 200µl of the cell suspension was added to wells of a 96-well ‘U-bottomed’ tissue culture plate. For antigen-recall experiments, cells were stimulated with media, heat killed *K. pneumoniae* at MOI 10 and 100, heat killed *S. aureus* MOI 10 or PMA (1µg/ml) and αCD3 (1µg/ml) and supernatants collected 72 h later.

2.3.4 Purification of lung cell populations ex vivo

After digest (2.3.3) lung cells were resuspended in sterile FACS buffer (2% FCS in PBS) and stained with sterile fluorochrome-conjugated antibodies against CD11b, CD11c, MHCII, F4/80 and Siglec F (Table 2.3) for 15 min before washing and resuspending in sterile FACS buffer. AM and lung DC were sorted using an Aria Fusion Cell Sorter (BD) and resuspended in cDMEM (AM) or cRPMI (DC) for use in bacterial stimulation assays (2.6).

2.3.5 Mononuclear Cell Isolation from CNS Tissue

Mice were anaesthetized and perfused intracardially with 20 ml ice-cold PBS and brain and spinal cord isolated to Hanks Balanced Salt Solution (HBSS) containing 5% FBS. Tissue was dissociated through a 70 µm cell strainer, washed through with HBSS/FCS, centrifuged at 170 x g for 10 min at 18°C, and enzymatically digested in Collagenase D (1 mg/ml) and DNase 1 (50 µg/ml) for 1 h at 37°C, with gentle agitation. Percoll gradients were prepared by layering 4 ml 70% isotonic percoll on 4ml 30% isotonic percoll. After digest, cells were washed in HBSS/FCS and resuspended in 4 ml HBSS before layering on percoll gradients. Percoll gradients were centrifuged at 1250 x g with brake off, for 20 min at 18 ºC. Myelin debris was aspirated off and cells recovered from the 30 % : 70 % interface. Cells were washed twice in HBSS/FCS and resuspended in complete 250 ul medium for counting, restimulation or flow cytometric analysis.
2.4 Bacterial Stimulation

2.4.1 Bacterial stimulation of innate immune cells in vitro

BMDC, BMM, AM or lung DC that had been rested in antibiotic-free media for 3 h were exposed to bacterial inocula at an MOI 0.1, 1, 10, 100, or PBS alone, in triplicate and incubated at 37 °C / 5 % CO\textsubscript{2} for 24 h. After 1 h or 3 h, some cultures were centrifuged for 5 mins at 300 X g at room temperature (RT) and supernatants collected and stored at -20°C. Cells were washed once with media containing gentamicin (100 μg/ml) and re-cultured in fresh media containing gentamicin (100 μg/ml) for a further 23 or 21 h, respectively. 24 h post-exposure, plates were centrifuged for 5 mins at 300 X g at RT, and supernatants collected and stored at -20°C.

2.4.2 Local immune response to bacterial exposure in the airways

20 μl of bacterial suspension (1 or 2 X 10\textsuperscript{6} CFU) in PBS was administered i.n. to conscious SPF or GF mice using a P20 pipette (10μl per nostril). At 4 and 24 h, or 7 d post-administration, bronchoalveolar lavage (BAL) was performed with 1 ml of PBS, supplemented with 50mM EDTA, using a 20 gauge catheter attached to a 1 mL syringe. Lungs and nasal associated lymphoid tissue (NALT) were harvested to 1 ml or 0.5 ml PBS, respectively. For RNA extraction, 2 lung lobes and the NALT were homogenized using a Polytron tissue homogeniser (Kinematica) in 1 ml or 0.5 ml Trizol reagent, respectively. For FACS analysis, 2 lobes were transferred to cRPMI containing 1μg/ml Collagenase IV and 100ng/ml DNAse I for 50 min at 37°C. Lung digest was passed through a cell strainer, centrifuged at 300 X g for 5 min, red blood cells lysed with ACK lysis buffer (Invitrogen) and washed in cRPMI. Cells were resuspended in cRPMI prior to staining for FACS analysis. For CFU enumeration, 1 lung lobe was homogenized in PBS for and plated on appropriate agar. Fecal pellets were collected from all GF mice and cultured in a range of medium.

2.4.3 Long-term monocolonisation of germ-free mice

1 X 10\textsuperscript{2} CFU K. pneumoniae was introduced in the drinking water to mice in a GF isolator under negative pressure. Colonisation was monitored through the collection of fecal samples at regular intervals. Bacterial burden was monitored for 7 months and CFU
enumerated in NALT, lungs, small intestine, and fecal material of 2 wk old mice (pre-weaning), 4 wk old mice (post-weaning) and adult mice. Samples were weighed, homogenised, serial diluted and plated on TSA. Immunophenotyping was carried out on the lungs and spleen of 8 wk old mice that had been colonized from birth.

2.5 Induction of Experimental Autoimmune Encephalomyelitis

2.5.1 Active EAE
EAE was induced in C57BL/6 mice by s.c. injection at two sites on the back of 100 µg of myelin oligodendrocyte glycoprotein (MOG)_{35-55} (GenScript) emulsified 1:1 in 100 µl complete Freund’s adjuvant (CFA; Chondrex Inc.), containing 4 mg/ml H37 Ra Mycobacterium tuberculosis (Mtb). Mice were also injected i.p. with 500 ng pertussis toxin (PT; Kaketsuken, Japan) on d 0 and 2.

2.5.2 Adoptive Transfer EAE
C57BL/6 mice were primed by s.c. injection at two sites on the back (one between the shoulder blades and one between the hips) with 100 µg of MOG_{35-55} (GenScript) emulsified 1:1 in 100 µl CFA (Chondrex Inc.), containing 4 mg/ml H37 Ra Mtb. Auxiliary, brachial, inguinal lymph nodes and spleens were harvested between 10 and 13 d post-immunization and a single cell suspension prepared in cRPMI media by passing lymph nodes and spleens through a 70mm cell strainer. Cells were centrifuged at 300 X g for 5 min, red blood cells lysed with ACK lysis buffer (Invitrogen) and washed in cRPMI. Cells were cultured at 10 x 10^6 /ml containing 30% lymph node cells and 70% splenocytes in cRPMI containing β-mercaptoethanol (55 μM), MOG (100 ug/ml), IL-23 (10ng/ml) IL-1β (10ng/ml), αIL-4 (10ug/ml) and αIFNg (10ug/ml). Cells were cultured in a 6 well plate (5ml/well). After 72 h, cells were collected by repeat pipetting, washed, resuspended in MACS buffer and magnetically labelled with sterile CD4 (L3T4) MACS beads (Miltenyi Biotec). CD4 positive cells were magnetically separated by positive selection, as per manufacturer’s instructions. Sorted CD4^+ cells were washed twice and resuspended in sterile PBS. 1 X 10^6 CD4^+ cells were transferred to naïve mice by i.p. injection. An aliquot
of sorted cells was analysed by flow cytometry after staining with fluorochrome-conjugated antibodies against CD3 and CD4 (Table 2.3).

Animals were monitored daily for weight and signs of clinical disease. Disease severity was graded as follows: grade 0 - normal; grade 1 - flaccid tail; grade 2 - wobbly gait; grade 3 - hind limb weakness; grade 4 - hind limb paralysis; grade 5 - tetraparalysis/death.

### 2.5.3 MOG-specific Th17 cell co-culture with respiratory bacteria-stimulated DC

After Th17 cell polarisation and MACS-sorting, as described in 2.5.2, cells were rested at 4 X 10^6/ml in a 48 well plate for 72 h. At this point, cells were collected and pelleted by centrifugation (300 x g for 5 min at 4°C), counted and co-cultured at a 1:1 ratio with DC that had been exposed to selected bacteria at MOI 100 for 1 h (as described in 2.6). For IL-23 blocking experiments, cells were treated with an αIL-23 (10 µg/ml) monoclonal antibody, or isotype control (10 µg/ml), at the same time as T cells were added to DCs. Alternatively, 1x10^5 T cells were co-cultured with supernatants recovered from DC exposed to selected bacteria at MOI 100. Cells were cultured at 37°C / 5% CO₂ for 24 h. Following culture, cells were pelleted by centrifugation at 300 x g for 5 min at RT and supernatant collected and stored at -20°C. Cells were resuspended in media containing brefeldin A for 4 h prior to intracellular staining and flow cytometric analysis (2.9).

### 2.6 ELISA

ELISAs for IL-23, IL-12p70, TNF-α IL-17A, IFNg, IL-10 and GM-CSF (all R&D), were performed on cell culture supernatants, as per the manufacturer’s instructions. Absorbance was measured at 450nm using a BMG Labtech Spectrostar Nano plate reader, and assay data analysed by BMG Spectrostar MARS software.

### 2.7 RNA isolation and RT-PCR

Tissues were isolated to RNA later (Ambion) and cultured cells resuspended in TRIzol reagent, and both stored at -20°C until analysis. Total ribonucleic acid (RNA) was extracted from cells or tissue using the TRIzol/chloroform method. Fixed tissue was transferred to 1 ml TRIzol reagent in sterile RNase-free tubes and homogenized using a Polytron tissue
homogenizer (Kinematica). Cells were transferred to sterile RNase-free tubes and disrupted by repeat pipetting in TRIzol. 200 µl chloroform was added to each tube, vortex mixed, stood at room temperature for 3 min and then centrifuged at 12,000 x g for 15 min at 4°C. The resulting upper aqueous phase was removed to a sterile RNase-free tube, and 500 µl isopropanol added, mixed vigorously and stood at room temperature for 15 min, before centrifugation at 12,000 x g for 15 min at 4°C. Supernatants were decanted and the pellet washed twice (7,600 x g for 3 min) in 1 ml 75% ethanol in nuclease-free H2O. Remaining ethanol was removed with a pipette and the tubes inverted and allowed to air-dry for 10 min. RNA was resuspended in 1 µl nuclease-free H2O / mg original tissue sample or 20 µl nuclease-free H2O for cells, incubated on ice for 10 min, heated to 65°C for 10 min and then stored on ice for 20 min. RNA concentration was determined spectrophotometrically using an Eppendorf BioPhotometer 6131 (Eppendorf) by diluting 2 µl RNA in 98 µl nuclease-free H2O and reading the absorbance at 260 nm and determining the 260/280 nm ratio. 300 ng - 1 µg of each RNA sample was reversed transcribed into cDNA using the Applied Biosystems High-Capacity cDNA Reverse Transcription kit, according to the manufacturer’s protocol, and the cDNA reverse transcription product diluted 1:8 with nuclease-free H2O. Transcripts were quantified by real time semi-quantitative PCR on an ABI 7500 Fast Real Time PCR System with Applied Biosystems predesigned Taqman Gene Expression Assays and reagents according to the manufacturer’s instructions, or an iCycler PCR machine (Bio-Rad Laboratories). For each sample, mRNA concentration was normalised to 18S ribosomal RNA (rRNA) and expressed as fold difference compared to controls.
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2.8 LDH Assay

Lactate dehydrogenase (LDH) assay reaction mixture was prepared as per manufacturer’s instructions. Briefly, 25 μl cell supernatant was mixed with 25 μl reaction mixture in a flat bottom 96 well plate. After 30 minutes incubation at RT, reactions were stopped by adding 25 μl stop solution. 100 % cell death controls were prepared by adding 15 % Triton X-100 to cells 1 h prior to supernatant collection in bacterial exposure assays. Absorbance was read at 490 nm and 680 nm using a BMG Labtech Spectrostar Nano plate reader. To determine LDH activity, the 680nm absorbance value was subtracted from the 490nm absorbance and presented as percentage of total cell death in the positive controls.

\[
\frac{\text{Unknown 490nm} - \text{680nm}}{\text{Control 490nm} - \text{680nm}} \times 100 = \% \text{ cytotoxicity}
\]

2.9 Flow cytometry

For intracellular cytokine staining, T cells were stimulated with PMA (50 ng/ml) and ionomycin (0.5 μg/ml), and cytokine secretion blocked with brefeldin A (5 μg/ml), or treated with brefeldin A (BFA 5μg/ml) alone, for the final 4 h of culture at 37°C / 5% CO₂. Cell samples were washed in 2 ml PBS and centrifuged at 300 x g for 5 min at RT. Cells were resuspended in 1 ml PBS containing a fixable LIVE/DEAD Aqua stain (1:1000) for 20 min at RT. Cells were washed in FACS buffer (2 % FCS in PBS) and resuspended in FACS buffer containing 1 μg/ml anti-CD16/CD32 (Fcγ Block; BD Pharmingen) for 15 min to block non-specific binding. Cells were stained with fluorochrome-conjugated antibodies against various surface markers (Table 2.2) and incubated at 4 °C for 15 min in the dark.

For intracellular cytokine staining, T cells were fixed and permeabilized with the Fix and Perm Cell Permeabilisation Kit (Life Technologies) and stained with fluorochrome-conjugated antibodies against intracellular cytokines (Table 2.2). Samples were washed in and resuspended in FACS buffer for flow cytometric analysis. For intranuclear staining, cells were fixed and permeabilised with Foxp3 Fixation/Permeabilisation solution and incubated at RT for 30 minutes in the dark. Cells were washed in 1X permeabilization buffer before staining for intranuclear antibodies in 1X permeabilization buffer. Samples were washed and resuspended in FACS buffer for flow cytometric analysis. Flow
cytometric analysis was carried out on a BD FACS Canto II or a BD Fortessa and analysed using FlowJo software (Treestar, Inc). Gating was determined using the fluorescence-minus-one (FMO) technique, where a tube of cells contains antibodies conjugated to fluorochromes for all molecules being examined, except the antibody of interest.
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2.9.1 Flow Cytometry Gating Strategies

Groups of SPF or GF mice were exposed to individual RT bacterial species via i.n. administration of 20 μl PBS containing 1x10^6 CFU or PBS alone. At selected timepoints, the lungs were harvested and digested using Collagenase D and DNAse1. Cells were stimulated with PMA and ionomycin with brefeldin A for 4 h and stained for surface CD3, CD4 and γδTCR and intracellular IL-17A and IFNγ. Representative FACS plots from one individual experiment showing gating strategy applied for generation of data in Figures 3.21, 3.22 and 3.26.
Figure 2.2 Representative gating strategy for assessment of cytokine production by lung T cells in response to monocolonisation with K. pneumoniae

*K. pneumoniae* was introduced in the drinking water to mice in a GF isolator. Mice were bred and maintained under monocolonised conditions. Lungs were harvested from 8 wk old adult mice that had been colonised from birth, and GF and SPF mice at the same age, digested using Collagenase D and DNAse1. Cells were stimulated with PMA and ionomycin with brefeldin A for 4 h and cells stained for surface CD3, CD4, CD8, γδTCR, CCR6 and CXCR3 and intracellular IL-17A, IFNγ and TNFα. Representative FACS plots from one individual experiment showing gating strategy applied for generation of data in Figures 3.29-3.31.
Figure 2.3 Representative gating strategy for assessment of cytokine production by lung T cells in response to monocolonisation with *K. pneumoniae*

*K. pneumoniae* was introduced in the drinking water to mice in a GF isolator. Mice were bred and maintained under monocolonised conditions. Lungs were harvested from 8 wk old adult mice that had been colonised from birth, and GF and SPF mice at the same age, digested using Collagenase D and DNAse1. Cells were stimulated with PMA and ionomycin with brefeldin A for 4 h and cells stained for surface CD3, CD4, CD25 and intracellular IL-5, IL-13, IL-4 and IL-10. Representative FACS plots from one individual experiment showing gating strategy applied for generation of data in Figures 3.29-3.31.
CD45.1 congenic donor Th17-polarized MOG-specific T cells were adoptively transferred to CD45.2 SPF mice via i.p. injection (1x10^6 CD4^+ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to bacteria via i.n. administration of 20 μl PBS containing 2x10^6 CFU M. catarrhalis, 1x10^6 CFU K. pneumoniae or PBS alone. At peak disease, mice were perfused intracardially and mononuclear cells isolated from the spinal cord and brain. Cells were stimulated with PMA and ionomycin with brefeldin A for 4 h, stained for surface CD3, CD4, CD45.1, CCR6, CXCR3 and CXCR6 and intracellular IFNγ, IL-17A and GM-CSF and analysed by flow cytometry.

Representative FACS plots from one individual experiment (spinal cord cells) showing gating strategy applied for generation of data in Figures 4.9 – 4.16. Gates for CCR6, CXCR3 and CXCR6 and intracellular IFNγ, IL-17A and GM-CSF were also applied to host CD45.1^+ cells.
Figure 2.5 Representative gating strategy for assessment of cytokine production by donor CD4 T cells in the lungs of mice exposed to individual RT bacteria during the preclinical stages of Th17 cell-mediated EAE

CD45.1 congenic donor Th17-polarized MOG-specific T cells were adoptively transferred to CD45.2 SPF mice via i.p. injection (1X10^6 CD4^+ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to bacteria via i.n. administration of 20 μl PBS containing 2x10^6 CFU V. parvula, 2x10^6 CFU M. catarrhalis, 1x10^6 CFU K. pnumoniae or PBS alone. During the pre-clinical stage of EAE (6 d post-transfer) lungs and spleens were harvested and digested using Collagenase D and DNAse1. Cells were stimulated with PMA and ionomycin with brefeldin A for 4 h, stained for surface CD3, CD4 and CD45.1 and intracellular IL-17A, IFNγ and GM-CSF and analysed by flow cytometry. Representative FACS plots from one individual experiment (lung cells) showing gating strategy applied for generation of data in Figures 4.17 – 4.20. Gates for IFNγ, IL-17A and GM-CSF were also applied to host CD45.1 cells.
Figure 2.6 Representative gating strategy for assessment of chemokine expression by host and donor CD4 T cells in the lungs and spleens of mice exposed to individual RT bacteria during the preclinical stages of Th17 cell-mediated EAE

CD45.1 congenic donor Th17-polarized MOG-specific T cells were adoptively transferred to CD45.2 SPF mice via i.p. injection (1X10^6 CD4^+ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to bacteria via i.n. administration of 20 μl PBS containing 2x10^6 CFU V. parvula, 2x10^6 CFU M. catarrhalis, 1x10^6 CFU K. pneumoniae or PBS alone. During the pre-clinical stage of EAE (6 d post-transfer) lungs were harvested and digested using Collagenase D and DNase1 and a single cell suspension was prepared from spleens. Cells were stained for surface CD3, CD4, CD45.1, CCR6, CXCR3 and CXCR6 and analysed by flow cytometry. Representative FACS plots from one individual experiment (lung cells) showing gating strategy applied for generation of data in Figures 4.22 and 4.23.
2.10 Statistics

For all data sets, statistical analysis was performed by first analysing the data for normal distribution (Shapiro–Wilk test), subsequently, for the analysis of the difference between two groups, the data was analysed by unpaired two-tailed student’s t test for parametric data or Mann–Whitney U test for nonparametric data, as appropriate. EAE clinical scores and mouse weight over the course of disease were analysed by two-way ANOVA with multiple comparisons. Statistical analyses and graph representation was performed using GraphPad Prism (v9) statistical analysis software. Data are shown as means ± SEM. P-values of 0.05 or less were considered significant. Statistical details for each figure can be found in the figure legends.
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Chapter 3

Investigating the immunogenicity of human respiratory tract bacteria
3.1 Introduction

The development of culture-independent methods to identify commensal taxa has contributed to a revolution in microbiome research since the turn of the century [205]. Since the initiation of the human microbiome project (HMP) in 2007, we have gained vast amounts of knowledge on the influence of the microbiota in so many areas of human health, from inflammatory bowel disease (IBD) to obesity and autism [206]. Most research has focused on the microbial community in the GIT. In fact, the LRT was omitted from the HMP due to a misconception at that time that the lungs were sterile in the absence of infection. Therefore, our knowledge on the influence of the RT microbiota on health and disease is lagging far behind that of the gut. We now know that the RT is home to a diverse microbial community and a growing literature supports a role for dysbiosis of this microbial community in the pathogenesis of a number of chronic inflammatory diseases, both within the RT and at distant sites. Our understanding on how individual respiratory tract bacteria modulate pathogenicity of immune cells that orchestrate site-specific inflammation remains sparse.

3.1.1 The Respiratory Tract Microbiota

Advances in culture-independent techniques such as I6S rRNA sequencing, have revealed that the RT is colonised by a vast number of microbes both in the healthy state and during disease [7]. The bacterial biomass of the respiratory tract is relatively low when compared to other colonised anatomical sites and decreases from upper to lower respiratory tract [16, 17]. Bacteria from the phyla Bacteroidetes, Firmicutes, Proteobacteria, Fusobacteria and Actinobacteria dominate the airways of healthy individuals [8, 9, 17, 20, 18-21]. Owing to the diverse surfaces and physiological conditions that exist along its length the composition of the RT microbiota varies from site to site. The anterior nares are home to Staphylococcus, Propionibacterium and Corynebacterium species [20]. The nasopharynx is dominated by Streptococcus, Rothia, Veillonella, Prevotella and Neisseria species [21]. The oropharyngeal microbiome is characterised by the presence of Neisseria, Rothia, Prevotella, Veillonella, Fusobacteria and Leptotrichia species [15, 23-25]. The adult lower airway microbiome is dominated by Prevotella and Veillonella [8]. Streptococcus, Haemophilus, Moraxella, Pseudomonas, Fusobacterium, Rothia and Porphyromonas taxa are also commonly identified.
in this dynamic community that results from microaspiration of bacteria and other microbes
in oropharyngeal secretions, and ongoing outward movement through mucociliary clearance
and the cough reflex [8, 9, 27].

### 3.1.2 Immune Surveillance of the RT

The primary function of the RT is gaseous exchange which leaves the lungs continuously
exposed to a vast array of inhaled particles and microorganisms. This constant exposure to
exogenous stimuli requires a finely tuned immune system which relies on multiple innate and
adaptive immune cells including macrophages, DC, regulatory T (Treg) cells and epithelial cells
for the maintenance of homeostasis [52-54]. Pathogen associated molecular patterns
(PAMPs) derived from commensal or pathogenic microbes or danger associated molecular
patterns (DAMPs) generated as a result of host cell stress or death are recognised via pattern
recognition receptors (PRR) on innate immune cells including respiratory epithelial cells, AM
and lung DCs. PRRs are a family of germline encoded receptors which encompasses the Toll-
like receptors (TLRs), the C-type lectin receptors (CLR), the cytoplasmic proteins retinoic acid-
inducible gene-i-like receptors (RLRs) and the NOD-like receptors (NLRs) [211]. TLRs allow the
host to recognise a range of PAMPs including viral RNA, bacterial lipopolysaccharides (LPS),
CpG-containing DNA and flagellin, amongst others. Ligation of these receptors triggers the
transcriptional expression of inflammatory mediators including cytokines and chemokines
that recruit and activate innate and adaptive immune cells [211-213]. For example, TLR4,
together with the myeloid differentiation factor 2 (MD-2), binds LPS expressed by Gram-
negative bacteria and can recognise a common pattern in structurally diverse LPS molecules
[4]. Interestingly, although TLRs are expressed on alveolar and airway epithelial cells, low
expression of MD-2 renders these cells less responsive to LPS stimulation [214].

The surface of the lung harbors a continuous layer of epithelial cells that are at the interface
between the external and internal milieu. The composition of this epithelial layer varies from
site to site reflecting the distinct functions of the airway epithelium lining the conducting
airways and the alveolar regions of the lungs [215]. The RT epithelium performs its barrier
function through intracellular tight junctions that regulate epithelial paracellular permeability
and mucociliary escalators that trap and remove inhaled microbes and noxious stimuli from
the airways. The airways epithelium also contributes to RT defenses through the production of antimicrobial peptides (AMPs) and proteins, reactive oxygen species (ROS), reactive nitrogen species (RNS) and various growth factors [216]. Under steady state conditions, actively phagocytic AM account for the majority of airspace leukocytes in the lung [217]. AM also produce ROS and recruit neutrophils to the site of inflammatory insult [57]. Lung DCs are found in fewer numbers than AM but play a key role in maintaining lung homeostasis as APCs surveilling the respiratory mucosa and alveoli. Lung DCs comprise different subsets that actively present antigen at the interface between innate and adaptive immune responses [62, 218]. Pulmonary homeostasis is maintained by various DC populations, resident AMs and lung epithelial cells which in tandem regulate inflammation and the recruitment of lymphocytes via innate cytokine and chemokine secretion.

Additionally, T cells play a critical role in pulmonary host defenses. γδ T cells, invariant NK T (iNKT) cells and innate lymphoid-like cells (ILCs) play important roles in the early responses to pulmonary infection [219]. CD8⁺ T cells contribute to pulmonary immunity though the production of cytokines and the direct killing of virally-infected cells [220]. CD4⁺ T cells are central mediators of host immunity by stimulating B cell maturation and antibody production, providing feedback to DCs via costimulatory molecules and cytokine production and enhancing and maintaining the cytotoxic function of CD8⁺ T cells. CD4⁺ T cells can also display direct effector activity through the activation of macrophages, and the upregulation of genes in mucosal tissues that contribute to host defense. CD4⁺ T cells are classified by their effector function and cytokine production. IFNγ-producing Th1 cells are driven by IL-12 signalling and play a role in the protection against intracellular bacterial infection, whereas Th2 cells that express IL-4, IL-5 and IL-13 protect against parasites and contribute to the regulation of humoral immunity and allergy. Treg cells express the transcription factor FoxP3 and the surface marker CD25 and produce IL-10. These suppressive cells are critical in the maintenance of tolerance [221]. Th17 cells protect against extracellular bacteria and fungi and contribute to the maintenance of barrier function at mucosal sites [222]. Th17 cells develop from naïve precursors following recognition of their cognate antigen and are polarised along the Th17 lineage in response to signalling by the innate cytokines IL-1, IL-6 and TGFβ [73]. Th17 cells are characterized by the expression of the transcription factor RORγt, the chemokine receptor CCR6 and the cytokines IL-17A, IL-17F, IL-22 and IL-21.
Numerous studies have assigned a role for the prototypical Th17 cytokine IL-17A in protection against pulmonary infections, including against bacteria such as *K. pneumoniae* [223]. However, exposure of Th17 cells to IL-23 has been shown to drive a pathogenic phenotype in these cells which has been implicated in CNS, skin, joint and intestinal autoimmune inflammation [152]. Microbial stimulation and Th17 cell function are closely connected and, in fact, Th17 cell development is diminished in GF animals, with a concomitant decrease in susceptibility to many preclinical models of complex diseases such as MS, uveitis, RA and psoriasis [89-92].

### 3.1.3 Lung Microbiome in Health and Disease

The RT microbiota has been shown to play an important role in respiratory homeostasis and is contrasted to both the acute and chronically diseased lung in which bacterial dysbiosis is characterised by decreased diversity, increased bacterial burden and overt inflammation [224]. A prominent feature of chronic lung diseases such as cystic fibrosis (CF) and idiopathic pulmonary fibrosis (IPF) is recurrent bacterial infection which contributes to disease morbidity and mortality [10, 225, 226]. In the chronically diseased lung, the main factor influencing microbial colonisation shifts from immigration and elimination to regional growth conditions and the relative reproductive rate. Changes in the composition of the RT microbiota have also been implicated in the pathogenesis of other chronic respiratory diseases including chronic obstructive pulmonary disorder (COPD) and asthma [8] and even inflammatory diseases distant from the lung, such as RA[11].

In COPD patients, notwithstanding inter- and intra-subject heterogeneity, even when clinically stable, the RT microbiome lacks the diversity and richness of that in a healthy individual [8, 227]. A reduction in *Prevotella* and *Veillonella* and an overrepresentation of *Actinobacteria* and *Proteobacteria* species, in particular *Haemophilus, Moraxella* and *Pseudomonas*, have been reported in COPD patients [9, 228, 229]. These shifts in the composition of the microbiome appear to be strongly associated with COPD exacerbation [227, 230, 231]. Perturbations in the RT microbiota have also been associated with asthma, a chronic inflammatory disease associated with airway hyperresponsiveness (AHR), bronchoconstriction and airflow obstruction. The adult human asthmatic airway has been
associated with increased burden of *Haemophilus, Moraxella, Neisseria, Staphylococcus* and *Streptococcus* taxa, and a reduction in *Veillonella* and *Prevotella* species [8, 232-237].

A common feature in diseased airways is a shift from dominant Bacteroidetes and Firmicutes species to pathogenic Gammaproteobacteria species such as *Pseudomonas aeruginosa*. This change in microbial community structure is due to an altered metabolic environment at the mucosa, associated with increased production of inducible nitric oxide synthase (iNOS) and RNS by inflammatory cells. Some facultative anaerobes, particularly members of the Gammaproteobacteria, encode the metabolic capacity to utilise by-products of inflammation, such as extracellular nitrate, as a nutrient source [238]. The availability of specific terminal electron acceptors confers a selective pressure and allows for ROS and RNS to be used for anaerobic respiration. Gammaproteobacteria perpetuate inflammation through the interaction of PAMPs and PRRs on recruited immune cells. This leads to the formation of a feedback loop in which immunostimulatory bacteria benefit from the creation of an inflammatory environment, with increased bacterial dysbiosis and decreased diversity [239].

Much of our knowledge of the lung microbiota in disease is correlative. Recently, however, research has shifted focus from defining the membership of this bacterial community in health and disease to understanding the role of the RT microbiome in the modulation of host defenses. Larsen *et al.*, examined immune cell activation by several commensal and pathogenic Proteobacteria which are commonly found in the RT of both healthy individuals and in patients with asthma and COPD. They found that, although all bacteria stimulated DC maturation characterised by comparable surface expression of CD83, CD40 and CD86, the cytokine profile and resulting helper T cell responses differed. *Hemophilus* and *Moraxella* species strongly induced IL-23 and IL-12p70 when compared to *Prevotella* species associated with healthy lungs (*Prevotella melaninogenica*, *Prevotella nanceiensis* and *Prevotella salivae*) or a combination of *Veillonella* species and *Actinomyces* species, suggesting that T cells are skewed towards a Th17 and Th1 phenotype in response to pathogenic airway bacteria [63]. A separate study also found reduced IL-8, TNFα and TSLP production by lung stromal cells and reduced neutrophilic airway inflammation in response to *Prevotella* species compared to that induced by *Haemophilus influenzae* [240]. Conversely, Segal and colleagues reported that the enrichment of the LRT with oral taxa including *Prevotella* and *Veillonella* was associated with heightened subclinical lung inflammation, characterised by increased neutrophils and
lymphocytes in BAL samples and an increase in exhaled nitric oxide [241]. Healthy individuals in which BAL samples were enriched with oral-derived bacteria displayed a distinct metabolic profile, enhanced expression of inflammatory cytokines such as IL-1 and IL-6, and increased frequency of Th17 cells. This data indicates a role for orally-derived bacteria as a regulator of airway inflammation and Th17 cell responses. [242]. Collectively, these findings have begun to address the regulation of lung-tropic T cells and pulmonary inflammation by members of the RT microbiota.

Studies of the GIT demonstrate the strong influence that various mucosal symbionts exert over Th17 cell development and effector function. Yet we have scant understanding of the role played by individual RT bacteria in shaping Th17-mediated chronic inflammation. One condition in which Th17 cells play a critical role in orchestrating pathogenicity is MS. Exposure of Th17 cells to the cytokine IL-23 is a critical step in their acquisition of pathogenic potential, but where and when this occurs is unknown. Although the etiology of MS remains elusive, epidemiological studies and research using GF mice in the preclinical model, EAE, suggest a role for the microbiota in disease development. It also appears now that the lungs might be a crucial staging site in the preclinical stages of disease. Here, we examined the immunogenic potential of a range of bacteria that commonly colonise the human RT microbiota, with a focus on their ability to promote secretion of IL-23 and associated cytokines.
3.1.4 Aims and rationale for this study

Our knowledge of how the RT microbiota influences local and systemic immune responses remains in its infancy. Recent efforts to characterise the microbial community at this site have revealed that the lungs are home to a diverse bacterial community that is dominated by species from the phyla Bacteroidetes, Firmicutes, Proteobacteria, Fusobacteria and Actinobacteria. However, there remains very little knowledge of the immunogenic properties of specific members of this microbial community. The goal of this research programme is to elucidate local and systemic immune responses to human RT bacteria, and how this impacts Th17 cell pathogenicity in EAE, a preclinical animal model that mimics many of the features of MS.

Hence, we aimed to:

1. Determine the immunomodulatory capacity of the most commonly encountered RT bacteria in vitro.

2. Examine the effect of human RT bacteria on immune responses locally in the lungs, and systemically, in conventionally housed SPF mice.

3. Define the discrete effects of each bacterial species on innate and adaptive immunity in vivo using novel models of short- and long-term monocolonisation of mice with selected bacteria that commonly colonise the healthy human RT.
3.2 Results

3.2.1 Optimisation of bacterial growth conditions for representative species from the five dominant phyla

To investigate the immunogenicity of the RT microbiome we first needed to build a strain collection that would represent the diversity of this microbial community. A comprehensive literature review was performed, and the key members of the RT microbiota identified. Based largely on metagenomic and 16S rRNA gene sequencing data, it appears the RT microbiota consists of a dynamic population that displays intra-individual variability and can alter significantly in a diseased and healthy state. However, a core RT microbiota has been described in which Prevotella species from the phyla Bacteroidetes and Veillonella, a member of the Firmicutes phyla dominate [8, 243]. The anterior nares are commonly colonised by Staphylococcus, Propionibacterium, Corynebacterium and Streptococcus species, [244-246]. There is significant overlap between nasal colonisers and bacteria found in the nasopharynx, however the nasopharynx has been found to be enriched with Haemophilus, Neisseria, Moraxella, Dolosigranulum and Streptococcus spp. [243]. The bacterial community of the oropharynx is characterised by the presence of Streptococcal species as well as Neisseria, Rothia, Prevotella, Veillonella and Leptotrichia spp [15, 23-25]. The lower airways are dominated by bacteria from the Firmicutes phyla such as Streptococcus and Veillonella spp. and Prevotella species from the phyla Bacteroidetes [23, 28, 210, 241]. Based on these studies we selected 28 strains of bacteria, representative of 18 species and 5 different phyla (Figure 3.1). We built an extensive strain collection by acquiring bacterial species from colleagues here at TCD and other academic institutions, as well as from the Culture Collection, University of Gothenburg, the American Type Culture Collection (ATCC) and the National Collection of Type Cultures (NTCC) at Public Health England (PHE). (Appendix 1). Optimum growth conditions for each bacterial strain in this library were established (Table 2.1) and bacterial stocks prepared in 70% glycerol and stored at -80°C.

We also needed to confirm that all bacteria were viable and capable of proliferation. To measure bacterial cell growth, overnight liquid cultures of each species were diluted to an OD$_{600}$ of 0.1 in the appropriate nutrient broth and OD$_{600}$ values measured at 10-minute intervals for 10 h. Some strains, including P. aeruginosa, K. pneumoniae and the Firmicutes
strains *Streptococcus mitis*, *S. aureus* Newman and *S. aureus* 1108 as well as the Actinobacteria strain *Rothia mucilaginosa* proliferated vigorously over this period (Figure 3.2). In the case of other strains including the Bacteroidetes species we did not see any change in OD$_{600}$ reading. It should be noted that, although growth was assessed in an airtight system using an unbreathable seal on a 96 well plate, oxygen levels were not depleted and may account for the lack of proliferation observed in readings from the anaerobic species (outlined in table 2.1).
A comprehensive review of the literature was performed to identify the most prevalent members of the respiratory tract microbiota. Based on this information we selected 28 species representative of 13 genera and 5 phyla that cover all levels of the respiratory tract. An extensive collection of these bacterial strains was acquired, and stocks prepared in 70% glycerol and stored at -80°C. Figure adapted from Man et al 2017.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Genus</th>
<th>Representative Species</th>
<th>Strain</th>
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<td><strong>Fusobacteria</strong></td>
<td><em>F. nucleatum</em></td>
<td>ATCC 25586</td>
</tr>
</tbody>
</table>

**Figure 3.1 Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria and Fusobacteria dominate the respiratory tract microbiome**

Nasal Cavity
- *Staphylococcus* spp.
- *Propionibacterium* spp.
- *Moraxella* spp.
- *Streptococcus* spp.

Nasopharynx
- *Moraxella* spp.
- *Streptococcus* spp.
- *Neisseria* spp.
- *Haemophilus* spp.
- *Streptococcus* spp.

Oropharynx
- *Streptococcus* spp.
- *Prevotella* spp.
- *Veillonella* spp.
- *Prevotella* spp.
- *Fusobacteria* spp.
- *Gemella* spp.
- *Klebsiella* spp.

Lungs
- *Prevotella* spp.
- *Veillonella* spp.
- *Streptococcus* spp.
Figure 3.2 Respiratory tract bacteria were viable for at least 10 h post-culture

Overnight cultures of each bacterial species were diluted to an OD$_{600}$ of 0.1 in the appropriate nutrient broth and 200 μl added to triplicate wells in a flat bottom 96 well microtiter plate or 1.5 ml Eppendorf tubes. Bacteria were incubated at 37°C for 10 h and OD$_{600}$ values measured at 10 min intervals.
3.2.2 Dendritic cell and macrophage culture and phenotyping

We wanted to determine the ability of RT bacteria to induce cytokine secretion in innate immune cells present in the airways. Given the low number of immune cells found in the lungs of naïve, specific pathogen-free (SPF) mice, we took advantage of the fact that the majority of these myeloid cell populations originate from a common progenitor in the bone marrow [247]. We began, therefore, by measuring cytokine responses to RT bacteria in bone marrow-derived dendritic cells (BMDC) and bone marrow-derived macrophages (BMM). Primary DC were cultured from bone marrow-derived progenitors in the presence of J558-conditioned media containing the lineage specific growth factor GM-CSF. Primary macrophages were cultured from bone marrow-derived progenitors in the presence of L929-conditioned media containing the lineage specific growth factor Macrophage Colony Stimulating Factor (M-CSF). In these conditions, the bone marrow-derived progenitors proliferate and differentiate into a homogenous population of DC and MΦ, respectively. Following culture, we assessed the efficiency of immune cell differentiation by flow cytometry. We found that DCs were consistently viable (> 95%), with at least 90% CD11c+, the majority of which were also MHCII hi indicating a consistent DC lineage (Figure 3.3 A). After 6 d culture, MΦ too were consistently viable (> 95%), the vast majority being CD11b+F4/80+ double positive, indicating a definite MΦ lineage (Figure 3.3 B).
Figure 3.3 BMDC and BMM purity and phenotyping

Representative FACS plots of (A) BMDC and (B) BMM after 10 or 6 d culture, respectively. Differentiated cells were stained with fluorochrome-conjugated antibodies against CD11b, CD11c, MHCII and F4/80 and analysed by flow cytometry. Numbers on plots represent percentage of a) viable cells and b) CD11b$^+$ gate.
3.2.3 Optimising the experimental design for innate immune cell exposure to human RT bacteria

At the outset, it was important to establish a robust and reproducible *in vitro* assay system which could be applied to the diverse bacterial species selected and to identify the culture conditions under which innate immune cells were sufficiently stimulated by bacteria, but at which the bacterial burden did not lead to adverse cytotoxicity.

Following differentiation, BMDC were plated in triplicate at 2 x 10⁵ cells/well in a 96 well plate and rested for 3 h before stimulation with three different bacterial species, representatives of two dominant phyla; *Streptococcus pneumoniae* (strain 6301) and *S. aureus* (strain Newman), both Gram positive members of the Firmicute phyla and *P. aeruginosa* (strain PAO1), a Gram negative Proteobacteria. BMDC were exposed to bacteria at MOI 1, 10 and 100 for 24 h at 37°C/5% CO₂. In order to prevent the potential overgrowth of the cell culture by bacteria, some wells were washed at 1 and 3 h post-stimulation with media containing the broad-spectrum bactericidal antibiotic gentamicin, and re-cultured in media containing gentamicin for the remaining 23 or 21 h, respectively. Supernatants were collected prior to gentamicin treatment at 1 h and 3 h, and again at 24 h post-initial exposure to bacteria. Figure 3.4 shows representative data from optimization experiments and from which we established the optimal exposure assay system as shown in Figure 3.5.

The pro-inflammatory cytokine TNFα is readily released by innate immune cells in response to bacterial stimuli. Hence, the concentration of TNFα in cell culture supernatants was measured by ELISA as a read-out of BMDC activation. TNFα secretion into the supernatant by 1 h post-stimulation was negligible. However, TNFα concentration in the supernatant had increased significantly by 3 h post-stimulation, and which would be unaccounted for if gentamicin treatment was performed at this timepoint (Figure 3.4 A). When BMDC were treated with gentamicin 1 h post-stimulation, we found a dose-dependent TNFα response to all bacterial species tested after 24 h culture. This response was lost in cells exposed to *P. aeruginosa* for 3 h or 24 h (Figure 3.4 B), potentially indicating cytotoxicity in the BMDC at the higher MOIs if not treated at the earlier time point.

Lactate dehydrogenase (LDH) is a cytosolic enzyme released by eukaryotic cells when membrane integrity is compromised. The presence of LDH in culture supernatants can thus
be measured as an indicator of cytotoxicity. LDH data is presented as percentage cytotoxicity compared to BMDC lysed with the detergent Triton X-100. We found that exposure to live bacteria for more than 1 h before gentamicin treatment led to increased toxicity in BMDC (Figure 3.4 C). Taken together, these results indicate that treatment with gentamicin at 1 h following exposure to bacteria, and culture for a further 23 h in media containing gentamicin were the optimal culture conditions to assess immune cell stimulation by commensal RT bacteria.

Having optimised our in vitro assay system, we set about investigating the immunomodulatory capacity of 28 strains of bacteria, representative of 18 different species and 5 bacterial phyla, to induce cytokine production in DC and MΦ. Given the critical role played by IL-23 in the acquisition of pathogenicity by Th17 cells, we measured IL-23 secretion as a read out of immunogenicity. IL-23 shares the common p40 subunit with the Th1-promoting cytokine IL-12. Hence, we also measured functional IL-12p70 in these assays. Following differentiation, BMDC and BMM were plated in triplicate at $2 \times 10^5$ cells/well in a 96 well plate and rested for 3 h. Cells were then exposed to live bacteria at MOI 0.1, 1, 10 and 100 for 1 h before washing once in media containing 100 μg/ml gentamicin and re-culturing cells in media containing gentamicin. Supernatants were collected 23 h later and IL-23, IL-12p70 and TNFα (as a positive control for cell activation) measured by ELISA. LDH activity in cell culture supernatants was also measured as an indicator of cytotoxicity (Figure 3.5).
Differentiated BMDC were seeded in triplicate at $2 \times 10^5$ cells/well in a 96 well plate and rested for 3 h. Cells were exposed to live bacteria at MOI 1, 10 and 100 for 24 h, with or without gentamicin treatment at 1 h and 3 h. Supernatants were collected before gentamicin treatment at each timepoint (A) and at 24 h post-initial exposure (B), and the concentration of TNFα quantified by ELISA and cell viability determined by measuring LDH activity in the supernatants (C). Results are expressed as mean ± SEM ($n = 5-6$ group).
Figure 3.5 Schematic of experimental setup for *in vitro* exposure assay

BMDC and BMM were cultured for 10 and 6 d, respectively. Cells were collected and seeded @ $2 \times 10^5$ cells/well in a 96 well plate. Bacterial inocula was prepared and cells exposed to bacteria at MOI 0.1, 1, 10 and 100 for 1 h. Cells were treated with gentamicin (100 μg/ml) after 1 h and supernatants collected 23 h later. Cytokine secretion was measured by ELISA and cytotoxicity was determined by LDH activity.
3.2.4 Species from the phyla Bacteroidetes, Proteobacteria and Actinobacteria strongly induce IL-23 secretion by BMDC *in vitro*

*Prevotella melaninogenica* and *Prevotella nigrescens* are two species from the Bacteroidetes phyla commonly identified in the RT of healthy individuals. *Prevotella* species are strictly anaerobic, Gram-negative bacteria that are considered human commensals given their abundance in the healthy lung and oropharyngeal microbiotas. We found that both *P. melaninogenica* and *P. nigrescens* strongly induced IL-23 (Figure 3.6 A) and IL-12p70 (Figure 3.6 B) secretion by BMDC at MOI 10 and 100. Neither IL-23 nor IL-12p70 were detected in BMM exposed to these bacterial species (Figure 3.6 A & B). However, *P. melaninogenica* and *P. nigrescens* induced significant TNFα production in both BMDC and BMM *in vitro* (Figure 3.6 C).

Alongside Bacteroidetes, species from the phylum Firmicutes dominate the airway microbiota of healthy individuals. Firmicutes species are generally Gram-positive bacteria, although there exist a number of Gram-negative exceptions in this phylum, including LRT-associated species *Veillonella parvula*. We investigated the ability of *S. pneumoniae* (D39), *S. pneumoniae* (6301), *S. mitis*, *Gemella morbillorum*, *V. parvula*, *S. aureus* (Newman) and *S. aureus* (1108) to induce IL-23 production by BMDC and BMM *in vitro*. Amazingly, we found that not one of these bacteria were capable of inducing IL-23 production in DC or MΦ in this assay system (Figure 3.7 A). IL-12p70 secretion was induced in BMDC cultures by *S. pneumoniae* strain 6301, while small but detectable concentrations of IL-12p70 were also found in supernatants from BMDC stimulated with *S. pneumoniae* (D39), *S. mitis*, *G. morbillorum* and *V. parvula* (Figure 3.7 B). Indeed, *S. mitis* also induced a small IL-12p70 response in BMM during 24 h in culture. All Firmicutes species tested in this assay induced a robust TNFα response in BMDC and BMM, with the exception of *S. pneumoniae* strain D39 (Figure 3.7 C).

Proteobacteria are a major phylum of Gram-negative bacteria whose members are commonly identified in the RT of healthy individuals. Representative strains including *Neisseria meningitidis*, *Neisseria lactamica*, *Neisseria cinerea*, *M. catarrhalis*, *K. pneumoniae* (BAA-2146), *K. pneumoniae* (43816), *P. aeruginosa* (PAO1), *P. aeruginosa* (NCIMB950), *H. influenza* Type B (Hib) and Non-typable *H. influenza* (NTHi) all induced IL-23 production in BMDC, but not in BMM, in our *in vitro* assay system (Figure 3.8 A). *K. pneumoniae*, although often
considered a pathogen, also commonly colonises both the healthy URT and the gut of both humans and mice [248-250]. Notably, *Neisseria*, *Moraxella* and *Klebsiella* species strongly induced IL-23 secretion by BMDC. In addition, IL-12p70 was detected in supernatants from all BMDC cultures stimulated with Proteobacteria species in this assay. Surprisingly, none of these strains stimulated the release of IL-23 or IL-12p70 by BMM (Figure 3.8 A & B). Nevertheless, BMDC and BMM secreted comparable levels of TNFα upon exposure to each strain of Proteobacteria (Figure 3.8 C).

*Fusobacterium nucleatum* is a member of the Gram-negative bacterial phyla Fusobacteria which are frequently identified in the oropharyngeal microbiota of healthy individuals. The Actinobacteria are a phylum of Gram-positive bacteria that are also often identified in the URT of healthy individuals. We found that *F. nucleatum*, but not the Actinobacteria species *Propionibacterium acnes* or *R. mucilaginosa*, induced IL-23 secretion in BMDC (Figure 3.9 A). However, *F. nucleatum* failed to induce IL-23 secretion by BMM or IL-12p70 from either BMDC or BMM. IL-12p70 was not detectable in supernatants from *P. acnes* or *R. mucilaginosa*-stimulated BMDC or BMM either (Figure 3.7 B), despite equal concentrations of TNFα being secreted by both cell types in response to all bacteria (Figure 3.9 C).

Having observed significantly different cytokine secretion levels by BMDC in response to bacteria across each phylum, we wished to determine if the bacterial challenge was resulting in significant cell death as a result of exposure to certain species. We measured accumulation of the cytosolic enzyme LDH in supernatants collected after 24 h culture and expressed results as percentage cytotoxicity compared to cells lysed with the detergent Triton X-100. Although there was some variability in cytotoxicity across species and phyla, no significant increase in cell death was observed in BMDC (Figures 3.10) or BMM (Figures 3.11) that had been exposed to any RT bacteria, at any MOI, compared to cells cultured in media alone.

We collated the data from figures 3.4 - 3.9 in order to build a clear and easily referenceable graphical representation of the relative ability of individual RT species to stimulate an IL-23 response in each immune cell subset, in addition to overall cell responsiveness to bacterial stimulation (TNFα expression) and cytotoxicity in the presence of each bacterial species (Figures 3.12 & 3.13). These heat maps clearly demonstrate that DC, but not MΦ, are an
important source of IL-23 and IL-12p70 *in vitro* following stimulation by species from the phyla Bacteroidetes, Proteobacteria and Fusobacteria.

This data demonstrates that innate immune cells respond differentially to stimulation by discrete bacterial species and that, amongst the selected members of the RT microbiota, the Bacteroidetes, Proteobacteria and Actinobacteria are strong inducers of IL-23 secretion by DC. This data also indicates that DC but not MΦ are important sources of IL-23 in response to stimulation by RT symbionts. We chose the strongest IL-23-stimulating species from each phyla to take forward into T cell co-culture and *in vivo* colonisation studies. These included *P. melaninogenica, N. cinerea, M. catarrhalis, K. pneumoniae* and *F. nucleatum*. Given the abundance of *V. parvula* in the LRT of healthy humans, this species was also selected as representative Firmicutes species, and negative control for future studies. Based on the observed cytokine responses, MOI 100 was selected as the optimal bacteria to immune cell ratio for the induction of IL-23 responses in future assays.
Figure 3.6 Species from the phylum Bacteroidetes induce IL-23 and IL-12p70 secretion by DC in vitro

Cells were seeded in triplicate at $2 \times 10^5$ cells/well in a 96 well plate and rested for 3 h before exposure to *P. melaninogenica* and *P. nigrescens* at MOI 0.1, 1, 10 and 100. Cells were treated with gentamicin (100 μg/ml) after 1 h and supernatants collected 23 h later. The concentration of IL-23 (A), IL-12p70 (B) and TNFα (C) was quantified by ELISA. Results are expressed as mean ± SEM (n = 4-6/group).
Cells were seeded in triplicate at 2 X 10^5 cells/well in a 96 well plate and rested for 3 h. Cells were exposed to S. pneumoniae, S. mitis, G. morbillorum, V. parvula and S. aureus at MOI 0.1, 1, 10 and 100. Cells were treated with gentamicin (100 μg/ml) after 1 h and supernatants collected 23 h later. The concentration of IL-23 (A), IL-12p70 (B) and TNFα (C) was quantified by ELISA. Results are expressed as mean ± SEM (n = 4-8/group).
Species from the phylum Proteobacteria strongly induce IL-23 and IL-12p70 secretion by DC in vitro.

Cells were seeded in triplicate at 2 × 10^5 cells/well in a 96 well plate and rested for 3 h, before exposure to *N. meningitidis*, *N. lactamica*, *M. catarrhalis*, *K. pneumoniae*, *P. aeruginosa*, *H. influenzae* Type B and Non-typeable *H. influenzae* at MOI 0.1, 1, 10 and 100. Cells were treated with Gentamicin (100 μg/ml) after 1 h and supernatants collected 23 h later. The concentration of IL-23 (A), IL-12p70 (B) and TNFα (C) in the supernatants was quantified by ELISA. Results are expressed as mean ± SEM (n = 6-9/group).

Figure 3.8 Species from the phylum Proteobacteria strongly induce IL-23 and IL-12p70 secretion by DC in vitro.
Figure 3.9 *Fusobacterium nucleatum*, but not Actinobacteria species, can induce IL-23 secretion by DC *in vitro*

Cells were seeded in triplicate at 2 X 10⁵ cells/well in a 96 well plate and rested for 3 h, before exposure to *F. nucleatum*, *P. acnes* and *R. mucilaginosa* at MOI 0.1, 1, 10 and 100. Cells were treated with gentamicin (100 μg/ml) after 1 h and supernatants collected 23 h later. The concentration of IL-23 (A), IL-12p70 (B) and TNFα (C) in the supernatants was quantified by ELISA. Results are expressed as mean ± SEM (n = 4-6/group).
Figure 3.10 Exposure to respiratory tract bacteria was not cytotoxic to DC during 24 h culture

DC were seeded in triplicate at 2 X 10^5 cells/well in a 96 well plate and rested for 3 h, before exposure to bacteria from the phyla Bacteroidetes (A), Firmicutes (B), Proteobacteria (C), Actinobacteria and Fusobacteria (D) at MOI 0.1, 1, 10 and 100. Cells were treated with gentamicin (100 μg/ml) after 1 h and supernatants collected 23 h later and cytotoxicity determined based on LDH activity in supernatants. Results are expressed as mean ± SEM (n = 4-6/group).
**Figure 3.11** Exposure to respiratory tract bacteria was not cytotoxic to MΦ during 24 h culture

MΦ were seeded in triplicate at $2 \times 10^5$ cells/well in a 96 well plate and rested for 3 h, before exposure to bacteria from the phyla Bacteroidetes (A), Firmicutes (B), Proteobacteria (C), Actinobacteria and Fusobacteria (D) at MOI 0.1, 1, 10 and 100. Cells were treated with gentamicin (100 μg/ml) after 1 h and supernatants collected 23 h later and cytotoxicity determined based on LDH activity in supernatants. Results are expressed as mean ± SEM (n = 4-6/group).
Figure 3.12 Proteobacteria, Bacteroidetes and Fusobacteria strongly induce IL-23 secretion by DC in vitro

Heat map representing collated data from figures 3.4-3.9 indicating average cytokine concentration ml⁻¹ (A-C) or percentage cytotoxicity at the indicated MOI (D). DC were exposed in vitro to 31 strains of bacteria – representative of 18 species and 5 different phyla. Concentrations of the cytokines IL-23 (A), IL-12p70 (B) and TNFα (C) in culture supernatants were quantified by ELISA. Heat maps were generated using Graphpad Prism (v9) based on the average cytokine concentration ml⁻¹ measured in cell culture supernatant upon exposure to indicated bacteria (indicated by row) at MOI 1, 10 and 100 (indicated by column) as shown in Figures 3.4-3.9, and the average % cytotoxicity based on lactate dehydrogenase (LDH) activity assessed to determine the cytotoxicity of each bacteria on DC (D). The colour scale corresponds to the cytokine concentration for the minimum (white) and the maximum (dark blue) for all values where the maximum concentration in A and B is 1200 pg/ml and for C is 20,000 pg/ml.
Figure 3.13 Representative respiratory tract bacteria do not induce IL-23 secretion by ΦΦ in vitro

Heat map representing collated data from figures 3.4-3.9 indicating average cytokine concentration ml⁻¹ (A-C) or percentage cytotoxicity at the indicated MOI (D). DC were exposed in vitro to 31 strains of bacteria – representative of 18 species and 5 different phyla. Concentrations of the cytokines IL-23 (A), IL-12p70 (B) and TNFα (C) in culture supernatants were quantified by ELISA. Heat maps were generated using Graphpad Prism (v9) based on the average cytokine concentration ml⁻¹ measured in cell culture supernatant upon exposure to indicated bacteria (as indicated by row) at MOI 1, 10 and 100 (as indicated by column) as shown in Figures 3.4-3.9 and the average % cytotoxicity based on lactate dehydrogenase (LDH) activity assessed to determine the cytotoxicity of each bacteria on DC (D). The colour scale corresponds to the cytokine concentration for the minimum (white) and the maximum (dark blue) for all values where the maximum concentration in A and B is 1200 pg/ml and for C is 20,000 pg/ml.
3.2.5 Alveolar macrophages do not secrete IL-23 or IL-12p70 following stimulation with selected RT bacteria *ex vivo*

It was surprising that BMM were unresponsive to stimulation with RT bacteria – from any phyla – in terms of IL-23 secretion *in vitro*. Although the majority of MΦ derive from bone marrow derived precursors, and primary BMM ought to be a faithful representation of their *in vivo* counterparts, we wanted to test if tissue-resident AM from the lungs of naïve mice would respond differently to primary, *in vitro*-cultured BMM [247]. AM are located in the alveolar spaces under steady state conditions and are the dominant myeloid population in the naïve lung. Hence, we isolated AM from the pooled lungs of naïve mice based on relative expression of the following cell surface markers: CD11c<sup>hi</sup>CD11b<sup>lo</sup>F4/80<sup>hi</sup>SiglecF<sup>+</sup>MHCII<sup>+</sup> (Figures 3.14 and 3.15A) [251].

FACS-sorted AM were rested for 3 h in cDMEM before exposure to selected bacterial species at MOI 100. AM were washed with media containing gentamicin at 1 h post-stimulation, and re-cultured in media containing gentamicin for a further 23 h. Supernatants were collected and IL-23, IL-12p70 and TNFα concentrations measured by ELISA. Similar to the response observed in BMM, we could not detect IL-23 or IL-12p70 in supernatants from AM cultured with the selected bacteria (Figure 3.15B). TNFα was secreted by AM in response to stimulation with each bacterial species, again replicating the response of RT bacteria-stimulated BMM *in vitro* (Figure 3.15B).

BMDC responded robustly to stimulation by several human RT bacteria *in vitro* and we wanted to see if lung DC would respond similarly. DC represent an incredibly small population of immune cells in the healthy lungs. Nevertheless, we isolated lung DC from the pooled lungs of naïve mice based on the following surface phenotype: CD11c<sup>hi</sup>CD11b<sup>+/−</sup>F4/80<sup>−</sup>SiglecF<sup>−</sup>MHCII<sup>hi</sup> (Figure 3.14). Sorted lung DC were approx. 85% pure in each of 6 separate experiments. However, given the low numbers of DC in the healthy murine lung, we did not obtain enough lung DC to effectively measure cytokine secretion in response to RT bacteria.

To address this issue, we attempted to expand lung DC *in vivo*. Administration the Flt3-ligand has been shown to expand both mature lymphoid and myeloid-related DC subsets in mice. Flt-3 ligand is a growth factor that binds to cells expressing the tyrosine kinase receptor and regulates proliferation of early hematopoietic
cells [252]. We administered 10µg Flt-3 ligand i.p. daily to naïve mice for 10 d. To recover as many DC as possible, we FACS-sorted CD11c‘autofluorescence’ cells from the lungs of pooled naïve or Flt3-ligand-treated mice [252]. A representative gating strategy for FACS-sorted lung DC is presented (Figure 3.16 A). Unfortunately, the repeated administration of Flt3-ligand did not significantly increase the number of lung DC recovered, compared to those isolated from naïve mice (Figure 3.16 B).

FACS-sorted lung DC were plated at 5 X 10^4 and rested for 3 h in cRPMI before stimulation with selected bacterial species at MOI 100. Lung DC were washed with media containing gentamicin at 1 h post-stimulation, and re-cultured in media containing gentamicin for a further 23 h. Supernatants were collected and IL-23, IL-12p70 and TNFα concentrations measured by ELISA. We detected a small amount of IL-23 in response to stimulation with M. catarrhalis, but no IL-12p70, in supernatants from lung DC cultured with selected bacteria (Figure 3.16 C). TNFα was secreted by lung DC in response to stimulation with each species. This data indicates that our observation that BMM do not produce a strong IL-23 response upon stimulation with selected RT bacteria holds true in the more biologically relevant AM and, considering the strong IL-23 by BMDC, and the potential replication of that response by lung DC, we focused on the ability of DC to respond to RT bugs in future experiments.
Figure 3.14 Gating strategy for FACS-sorting of AM and DC populations from the lungs of naïve mice

Lungs were harvested from naïve C57BL/6J mice and digested using Collagenase D and DNAse I. Cells were stained with sterile fluorochrome-conjugated antibodies against CD11b, CD11c, MHCII, F4/80 and Siglec F and sorted by flow cytometry. Representative FACS plots for one individual experiment.
Figure 3.15 AM do not secrete IL-23 or IL-12p70 following exposure to selected respiratory tract bacteria ex vivo

After sorting (A), AM were resuspended in cDMEM and seeded at a concentration of 5 X 10^4 cells/well in a 96 well plate and rested for 3 h. Cells were stimulated with selected RT bacteria at MOI 100 for 1 h before treatment with Gentamicin (100 μg/ml), and supernatants collected 23 h later. Concentrations of the cytokines IL-23, IL-12p70 and TNFα were quantified by ELISA (B). Results are expressed as mean ± SEM (n = 4-6/group).
Figure 3.16 Lung DC secrete IL-23 following exposure to *M. catarrhalis* ex vivo

Flt3 ligand (5µg) was administered to naïve mice every 2 d for 10 d via i.p. administration. Lungs were harvested and lung DC sorted based on autofluorescence and CD11c expression (A & B). After sorting, DC were resuspended in cRPMI and seeded at a concentration of 5 X 10⁴ cells/well in a 96 well plate and rested for 3 h. DC were exposed to selected RT bacteria at MOI 100 for 1 h before treatment with Gentamicin (100 µg/ml), and supernatants collected 23 h later. Concentrations of the cytokines IL-23, IL-12p70 and TNFα were quantified by ELISA (C). Results are expressed as mean ± SEM (n = 2/group).
3.2.6 Proteobacteria species stimulate IL-23 and IL-17 expression in murine lungs

We next questioned if the cytokine responses to selected RT bacteria we have observed in vitro are recapitulated in vivo. To test this, we established a model of airway exposure via intra-nasal (i.n.) administration of bacterial suspension and examined cytokine production in both the URT (nasopharynx) and LRT (lungs) at defined timepoints following exposure (Figure 3.17).

Mice are not natural hosts for the majority of human colonizing bacteria. In conventional mice we can achieve short term colonization of the nasopharynx and lungs. For example, a model designed to mimic S. aureus nasal colonisation was already in use in our lab but focused on bacterial persistence in the anterior nares and URT in the days and weeks following administration [253]. As the strains of S. aureus used are not mouse-adapted, the bacterium is cleared from the animal over the course of approximately one week. As such, high CFUs of S. aureus are generally well tolerated in mice. Other RT bacteria can have more severe adverse effects when administered to live animals. Alnahas et al. found, for example, that i.n. challenge with $2 \times 10^8$ CFU M. catarrhalis gave rise to 50 % mortality within 3 d of administration [254]. Galvao and colleagues found that $1 \times 10^6$ CFU K. pneumoniae lead to 40 % mortality by 5 d post-i.n. administration [255]. Similarly, it was reported that intra-tracheal (i.t.) administration of as low as $1 \times 10^4$ CFU K. pneumoniae leads to 40% mortality in WT mice within 6 d [256].

In order to accurately compare the local immune response to a number of different bacterial species in our airway exposure assays, without triggering morbidity or mortality in the host mice, we attempted to administer the same number of CFU for all bacteria tested. Hence, groups of mice exposed i.n. to 20 μl bacterial suspension containing $1 \times 10^6$ CFU. At 4 h, 24 h, 3 d and 7 d post-administration, CFUs were quantified in the NALT and lungs, and IL-23 and related cytokine expression analysed by PCR and flow cytometry.

At 4 h post administration, we detected a significant increase in Il23a gene expression in the lungs of mice exposed to P. melaninogenica (P<0.05), N. cinerea (P<0.01), M. catarrhalis (P<0.05) and K. pneumoniae (P<0.05), compared to PBS-administered controls (Figure 3.18 A). Il23a gene expression was still elevated in the lungs of mice exposed to these bacteria at 24 h post-exposure, although this was only significant in mice exposed to N. cinerea (P<0.001)
and *K. pneumoniae* (*P*<0.01) at this time (Figure 3.18 A). This coincided with a significant increase in expression of mRNA for the other IL-23 subunit, *Il12b*, in response to *P. melaninogenica* (*P*<0.001), *N. cinerea* (*P* ≤ 0.0001) and *K. pneumoniae* (*P*<0.05) at 4 h and *P. melaninogenica* (*P*<0.01) and *M. catarrhalis* (*P*<0.05) as 24 h post-exposure (3.18 B). We did not detect a significant increase in *Il23a* or *Il12b* gene expression in the lungs of mice exposed to *V. parvula* or *F. nucleatum* at either timepoint (3.18 A and B). *P. melaninogenica* (*P*<0.001 at 4 h and *P* ≤ 0.0001 at 24 h) and *M. catarrhalis* (*P*<0.05) elicited a significant increase in *Il12a* mRNA expression at both 4 and 24 h after exposure, a response that was also detected in mice exposed to *V. parvula* (*P*<0.05) and *N. cinerea* (*P*<0.001) after 4 h exposure but had waned when samples were analysed at 24 h post exposure (Figure 3.18 C). No significant changes in *Il17a* gene expression were detected in response to any of the RT bacteria tested at these timepoints (Figure 3.18 D). *K. pneumoniae* was the only bacterium that elicited a significant increase in *Il1b* gene expression after 4 h (*P*<0.05) (Figure 3.18 E).

We next assessed changes in gene expression in the NALT at 4 h and 24 h post-administration of selected RT bacteria. We found a significant increase in *Il23a* gene expression in response to *N. cinerea* at 24 h post-exposure (*P*<0.05). Exposure to both *P. melaninogenica* and *M. catarrhalis* resulted in a significant increase in *Il12a* gene expression (*P*<0.05 and *P*<0.01 respectively). However, we didn’t detect any consistent changes in of *Il12b, Il17a* or *Il1b* gene expression in the upper airways in response to any RT bacteria tested in this assay (Figure 3.19).

We next questioned whether the elevated *Il23a* gene expression in the lungs of mice exposed to the Proteobacterial species *N. cinerea, M. catarrhalis* and *K. pneumoniae* translated to a downstream increase in IL-23-driven IL-17A or IL-12p70-driven IFNγ protein expression by T cells in the lungs. Groups of SPF mice were exposed to individual bacterial species via intranasal administration of 20 μl PBS containing 1x10⁶ CFU bacteria or PBS alone. At 3 d and 7 d post-administration, lungs were harvested, bacterial burden determined, and intracellular cytokine expression analysed by flow cytometry. At each time point, a lobe of one lung from each mouse was homogenised and cultured on appropriate media to determine bacterial burden. 3 d following exposure, we detected an average log₁₀CFU of 0.78, 0.69 and 1.84 for *N. cinerea, M. catarrhalis* and *K. pneumoniae* respectively. At 7 d, no bacteria were detected
in the lungs of mice in any group (Figure 3.20). We next measured changes in IL-17A and IFNγ production by CD4+ T cells and γδ T cells in the lungs by flow cytometry. We detected a significant increase in IL-17A production by γδ T cells (P<0.01) at 3 d post-administration, and an increase, although not significant, in IL-17A production by CD4+ T cells in the lungs of mice exposed to *K. pneumoniae* (Figure 3.21 A and B). At 7 d, a small increase in IL-17A production by CD4+ and γδ T cells was detected in response to *K. pneumoniae* (Figure 3.21 A & Figure 3.22). No significant change in IFNγ production by CD4+ T cells or γδ T cells was detected in response to *K. pneumoniae* (Figures 3.21 and 3.22). Neither was a significant change in the expression of IL-17A or IFNγ by CD4+ T or γδ T cells measured in the lungs of mice in response to *N. cinerea* or *M. catarrhalis* at 3 or 7 d (Figure 3.21 & Figure 3.22). This data indicates that the RT bacteria *N. cinerea*, *M. catarrhalis* and *K. pneumoniae* drive early IL-23 gene expression in the lungs of SPF mice and this is associated with elevated IL-17A production in the lungs of *K. pneumoniae*-exposed mice 3 d later.
Figure 3.17 Schematic of acute intranasal exposure and long-term colonisation models

To assess changes in gene and cytokine expression in response to acute colonisation with RT bacteria, groups of SPF or GF mice were exposed to bacteria via i.n. administration of 20 μl PBS containing bacteria or PBS alone. At 4 and 24 h post-administration, RNA was extracted from lungs and gene expression for \( \text{Il}23 \), \( \text{Il}12a \), \( \text{Il}12b \), \( \text{Il}1\beta \), and \( \text{Il}17 \) assessed by RT-PCR. At 24 h, 3 and 7 d post-administration, lungs and spleens were harvested and cells analysed by flow cytometry.

To assess the impact of long-term monocolonisation, \( K. \ pneumoniae \) was introduced in the drinking water to mice in a GF isolator. Mice were maintained under monocolonised conditions for 7 months and bacterial burden in fecal samples quantified at regular intervals. Lungs and spleens were harvested from 8 wk old mice that had been colonised from birth and gene expression for \( \text{Il}23 \), \( \text{Il}12a \), \( \text{Il}12b \), \( \text{Il}1\beta \), and \( \text{Il}17 \) assessed by RT-PCR. Cell populations from the lung and spleen were analysed by flow cytometry and compared to those harvested from GF and SPF mice.
Figure 3.18 Proteobacteria species stimulate IL-23 and IL-17 expression in the lungs of SPF mice in vivo

Groups of SPF mice were exposed to the indicated bacteria via i.n. administration of 20 μl PBS containing 1x10⁶ CFU or PBS alone. 4 and 24 h post-administration, RNA was extracted from the lungs and gene expression for (A) Il23a, (B) Il12b, (C) Il12a, (D) Il17a and (E) Il1β assessed by RT-PCR. mRNA values are expressed as mean fold change ± SEM, compared to PBS-administered controls, after normalising to a housekeeping control gene (18S rRNA)(n=4-8/group from 2-3 independent experiments). Statistical analysis performed by student’s t test, *P<0.05, ** P<0.01, *** P<0.001, **** P ≤ 0.0001 vs PBS administered controls.
Figure 3.19 Proteobacteria species stimulate IL-23 and IL-17 expression in the NALT of SPF mice in vivo

Groups of SPF mice were exposed to the indicated bacteria via i.n. administration of 20 μl PBS containing 1x10^6 CFU or PBS alone. At 4 and 24 h post-administration, RNA was extracted from the NALT and gene expression for (A) Il23a, (B) Il12b, (C) Il12a, (D) Il17a and (E) Il1b assessed by RT-PCR. mRNA values are expressed as mean fold change ± SEM, compared to PBS-administered controls, after normalising to a housekeeping control gene (18S rRNA)(n=4-8/group from 2-3 independent experiments). Statistical analysis performed by student’s t test, *P<0.05, ** P<0.01 vs PBS administered controls.
Figure 3.20 Proteobacteria species persist in the lungs of SPF mice at 3 days but are cleared by day 7 post-exposure.

Groups of SPF mice were exposed to the indicated bacteria via i.n. administration of 20 μl PBS containing 1x10⁵ CFU or PBS alone. 3 and 7 d post-administration, the lungs were harvested and one lobe homogenised and serial dilutions in PBS cultured on appropriate growth medium for 24 h. Bacterial CFU were enumerated and results expressed as mean CFU/lobe ± SEM (n = 6-9/group from 3 independent experiments).
Figure 3.21 *K. pneumoniae* stimulates early IL-17 expression by T cells in the lungs of SPF mice

Groups of SPF mice were exposed to the indicated bacteria via i.n. administration of 20 μl PBS containing 1x10⁶ CFU or PBS alone. 3 d post-administration, the lungs were harvested and digested using Collagenase D and DNase1. Cells were stimulated with PMA and ionomycin with brefeldin A for 4 h and stained for surface CD3, CD4 and γδTCR and intracellular L-17A and IFNγ. Results are mean percentage of IL-17⁺ and IFNγ⁺ cells amongst total CD4⁺ cells (A) or IL-17⁺ and IFNγ⁺ cells amongst total γδTCR⁺ cells (B) ± SEM (n = 6-9/group from 3 independent experiments). Statistical analysis performed by Students T Test **P<0.01 vs PBS-administered controls. Representative FACS plots for one individual experiment (C).
Figure 3.22 No difference in T cell-expressed IL-17 in the lungs 7 d post-exposure to selected Proteobacteria species

Groups of SPF mice were exposed to the indicated bacteria via i.n. administration of 20 μl PBS containing 1x10⁶ CFU or PBS alone. 7 d post-administration, the lungs were harvested and digested using Collagenase D and DNase1. Cells were stimulated with PMA and ionomycin with brefeldin A for 4 h, stained for surface CD3, CD4 and γδTCR and intracellular IL-17A and IFNγ. Results are mean percentage of IL-17⁺ and IFNγ⁺ cells amongst total CD4⁺ cells (A) or IL-17⁺ and IFNγ⁺ cells amongst total γδTCR⁺ cells (B) ± SEM (n = 6-9/group from 3 independent experiments). Representative FACS plots for one individual experiment (C).
3.2.7 Individual Proteobacteria species can directly promote IL-23/IL-17 type responses in the lungs of monocolonised mice

The rapid clearance of bacteria in SPF mice (Figure 3.20) emphasises how acute colonisation likely fails to replicate the lifetime’s exposure that a human immune system has to various RT bacteria. Additionally, conventionally housed mice already harbor a complex microbiota that might be influence, or be influenced by, exposure to human RT bacteria and affect the immune response observed in the RT of exposed mice. Therefore, we monocolonised GF mice with selected RT bacteria which allowed for the defined assessment of the influence on local and systemic immunity of each bacterium in isolation. Groups of GF mice were exposed to individual bacterial species via i.n. administration of 20 μl PBS containing 1x10^6 CFU bacteria or PBS alone. At 24 h and 7 d post-administration, lungs were harvested, and changes in gene expression compared to PBS-administered controls analysed by PCR. At 24 h post-administration, we detected a significant increase in Il23a gene expression in mice exposed to *M. catarrhalis* (P<0.01) and an increase, although not significant, in Il23a gene expression in response to *K. pneumoniae* (Figure 3.23). At 7 d, we observed a significant increase in Il23a gene expression in the lungs of mice exposed to *K. pneumoniae*, but not *M. catarrhalis* (P<0.05; Figure 3.23). *N. cinerea* did not significantly increase Il23a gene expression at either 24 h or 7 d timepoints (Figure 3.23 A). However, *N. cinerea*, as well as *M. catarrhalis*, promoted a significant increase in Il17a gene expression at both 24 h and 7 d post bacterial exposure whereas neither bacterium significantly altered Il12b or Il1b gene expression in the lungs (Figures 3.23 B, D & E). Additionally, we detected a significant upregulation of Il17a gene expression in the lungs in response to *K. pneumoniae* which coincided with a small but not significant Il12a gene expression and no notable changes in Il12b or Il1b gene expression (Figures 3.23 B – E). In the NALT, we detected an increase, although not significant in Il23a, Il17, Il12b gene expression at 7 d and in Il12a gene expression at 24 h in response to *N. cinerea* (Figures 3.24 A – D). *K. pneumoniae* promoted elevated gene expression of Il1b in the NALT at 24 h whereas *M. catarrhalis* exposure did not drive increased expression of the genes for Il23a, Il12b, Il12a, Il1b or Il17 (Figures 3.24 A – E). When we looked in the small intestine, we detected an increase, although not significant, in Il23a and Il12a gene expression in response to *N. cinerea*, *M. catarrhalis* at 24 h and 7 d and *K. pneumoniae* at 24 h (Figures 3.25 A & C). The increase in Il12a gene expression was most pronounced in response to *M. catarrhalis* at
7 d (Figure 3.25 C). At this timepoint we also detected a slight increase in *Il17* gene expression in mice colonised with *M. catarrhalis* and *K. pneumoniae* and a small increase in expression of the *Il12b* gene in response to *N. cinerea* (Figures 3.25 B & D). No notable change in *Il1b* was detected in the small intestine in response to any of the bacteria measured here (Figure 3.25 D). These results indicate that intranasal exposure of GF mice to these strains of RT bacteria preferentially promotes an IL-23 type response in the lungs.

To assess if changes in gene expression detected in the lung translated to significant changes in cytokine production, we measured IL-17A and IFNγ production by T cell subsets in the lungs in response to each bacterial species. No significant change in cytokine expression was detected in response to any bacteria at 24 h, compared to PBS-administered controls (Figures 3.26 A). 7 d after bacterial exposure, we detected a significant increase in IL-17A production by both CD4+ T cells and γδ T cells in response to *K. pneumoniae* (P<0.01) (Figures 3.26 A & B). Although not significant, we did detect an increase in IFNγ production by CD4+ T cells in mice exposed to *K. pneumoniae* and an increase in IFNγ production by γδ T cells in response to *N. cinerea* at 7 d post-exposure. No change in cytokine production was detected in response to *M. catarrhalis* at either time point (Figures 3.26 A & B).
Figure 3.23 *M. catarrhalis* and *K. pneumoniae* can directly stimulate IL-23 and IL-17 expression in the lungs of monoclonised mice

Groups of GF mice were exposed to the indicated bacteria via i.n. administration of 20 μl PBS containing 1x10^6 CFU or PBS alone. 24 h and 7 d post-administration, RNA was extracted from the lungs and gene expression for (A) *Il23a*, (B) *Il12b*, (C) *Il12a*, (D) *Il17a* and (E) *Il1b* assessed by RT-PCR. mRNA values are expressed as mean fold change ± SEM, compared to PBS-administered controls, after normalising to a housekeeping control gene (18S rRNA)(n=6-8 per group representative of 2-3 independent experiments). Statistical analysis performed by Mann–Whitney U Test or Students T Test, * P<0.05 ** P<0.01 vs PBS controls.
**Figure 3.24** *N. cinerea* can directly stimulate IL-23 and IL-17 expression in the NALT of monocolonised mice

Groups of GF mice were exposed to the indicated bacteria via i.n. administration of 20 μl PBS containing $1 \times 10^6$ CFU or PBS alone. 24 h and 7 d post-administration, RNA was extracted from the NALT and gene expression for (A) *Il23a*, (B) *Il12b*, (C) *Il12a*, (D) *Il17a* and (E) *Il1b* assessed by RT-PCR. mRNA values are expressed as mean fold change ± SEM, compared to PBS-administered controls, after normalising to a housekeeping control gene (18S rRNA) (*n*=6-8 per group representative of 2-3 independent experiments).
Figure 3.25 Multiple Proteobacteria species can directly stimulate IL-23 expression in the small intestine of monocolonised mice

Groups of GF mice were exposed to the indicated bacteria via i.n. administration of 20 μl PBS containing 1x10^6 CFU or PBS alone. 24 h and 7 d post-administration, RNA was extracted from the small intestine and gene expression for (A) Il23a, (B) Il12b, (C) Il12a, (D) Il17a and (E) Il1b assessed by RT-PCR. mRNA values are expressed as mean fold change ± SEM, compared to PBS-administered controls, after normalising to a housekeeping control gene. (18S rRNA) (n=6-8 per group representative of 2-3 independent experiments).
Figure 3.26 *K. pneumoniae* can directly promote IL-23/IL-17-type responses in the airways of monocolonised mice

Groups of GF mice were exposed to the indicated bacteria via i.n. administration of 20 μl PBS containing 1x10^6 CFU or PBS alone. 24 h and 7 d post-administration, the lungs were harvested and digested using Collagenase D and DNase1. Cells were stimulated with PMA and ionomycin with brefeldin A for 4 h and stained for surface CD3, CD4 and γδTCR and intracellular L-17A and IFNg. Results are mean percentage of IL-17+ and IFNg+ cells amongst total CD4+ cells (A) or IL-17+ and IFNg+ cells amongst total γδTCR+ cells (B) ± SEM (n=6-9 per group representative of 2-3 independent experiments). Statistical analysis performed by student’s t test, ** P<0.01 vs PBS controls. Representative FACS plots for one individual experiment (C).
3.2.8 Gnotobiotic mice maintain *K. pneumoniae* monocolonisation in the airways and gut which promotes chronic IL-17 type responses in the lungs

Having identified acute Th17 associated host immune responses following i.n. administration of a number of RT bacteria, we wanted to investigate the impact of long-term monocolonisation of mice with *K. pneumoniae* to assess how a lifetime exposure to the bacterium can influence innate and adaptive immunity.

To achieve long-term colonisation, *K. pneumoniae* was introduced to GF mice via the drinking water in a negative pressure isolator, and bacterial burden in fecal samples and various tissues quantified at regular intervals. We consistently detected the presence of *K. pneumoniae* in fecal samples, averaging between 8.35 and 10.48 log_{10}CFU/g fecal matter, over the course of 7 months (Figure 3.27 A). Groups of mice were sacrificed at 3 wk old (pre-weaning), 5 wk (post-weaning) or 8 wk old (adult) and bacterial burden in the NALT, lung, liver, small intestine, and fecal pellet derived from the distal colon quantified. The URT of all mice was consistently colonised with *K. pneumoniae*, although adult mice displayed a higher bacterial burden (average log_{10}CFU/sample = 5.58) than pre-weaning (average log_{10}CFU/sample = 1.55) and post-weaning pups (average log_{10}CFU/sample = 0.73) (Figure 3.27 B). *K. pneumoniae* was detected in the lungs of mice at each stage of development with pre-weaning mice displaying an average log_{10}CFU/sample of 1.26, post-weaning mice displaying an average of log_{10}CFU/sample of 0.07 and adult mice displaying an average log_{10}CFU/sample of 0.83. *K. pneumoniae* was detected in the liver of adult and post-weaning mice, but not in mice that had yet to be weaned. All mice maintained a high level of *K. pneumoniae* colonisation in the small intestine and colon (Figure 3.27 B).

In order to examine the impact of long-term monocolonisation with *K. pneumoniae* on innate and adaptive immune responses in the airways, we harvested the lungs from 8 wk old mice that had been colonised from birth, GF and SPF mice. *K. pneumoniae* monocolonisation resulted in slightly elevated *Il23a* (Figure 3.28 A) but no change in *Il12b* (Figure 3.28 B) or *Il12a* (Figure 3.28 C) gene expression as measured by PCR. We detected a significant increase in gene expression of *Il1b*, a critical cytokine in Th17 cell differentiation (Figure 3.28 D; P<0.001), and *Il17a* gene expression (Figure 3.28 E; P<0.01) in the lungs of mice monoclonised with *K. pneumoniae* compared to that seen in both GF and SPF controls. *Ifng* gene expression was
also significantly increased in *K. pneumoniae* monocolonised mice when compared to SPF but not GF controls (Figure 3.28 F; P<0.01).

We next questioned how this cytokine milieu engendered by chronic exposure to *K. pneumoniae* affected T cell responses in the lungs. *K. pneumoniae* monocolonisation did not result in any significant changes in the frequency of CD4<sup>+</sup> Th cells (Figure 3.29 A), CD4<sup>+</sup>CD25<sup>+</sup> Treg cells (Figure 3.29 B), CD8<sup>+</sup> T cells (Figure 3.30 A) or γδTCR<sup>+</sup> T cells (Figure 3.31 A) amongst total CD3<sup>+</sup> T cells in the lungs of monocolonised mice, compared to GF or SPF control mice. In terms of cytokine production, we detected a small but significant increase in IL-10 production by CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> Treg cells (P<0.05) in the lungs (Fig 3.29C). No significant changes in IL-17A, IFNγ, TNFα, IL-4, IL-5 or IL-13 expression by CD4<sup>+</sup> T cells were observed in *K. pneumoniae* monocolonised lungs, compared to lungs from GF or SPF control mice (Figures 3.29 D & F).

When we looked at chemokine expression, we detected a significant decrease in CCR6 expression by CD4<sup>+</sup> T cells (P<0.05) in the lungs of monocolonised mice, compared to GF or SPF mice, but no changes in CXCR3 expression (Figure 3.29 E).

CD3<sup>+</sup>CD8<sup>+</sup> T cells from the lungs of monocolonised mice produced significantly more IFNγ than SPF controls (P<0.05), but no significant differences in IL-17A or TNFα production were detected (Figures 3.30 B- D). Neither did we observe any significant changes in CCR6 or CXCR3 single expression by CD8<sup>+</sup> T cells, however we did see a small, but significant (P<0.05), reduction in the frequency of CCR6<sup>+</sup>CXCR3<sup>+</sup> double-positive CD8<sup>+</sup> T cells compared to GF controls (Figure 3.30 C).

We next looked at γδTCR<sup>+</sup> CD3<sup>+</sup> cells and found a significant increase in the frequency of IL-17A expressing γδ T cells in the lungs of *K. pneumoniae* monocolonised mice, compared to that seen in GF and SPF mice (P<0.01, Figure 3.31 B). We also found a significant increase in TNFα production by γδ T cells in the lungs of *K. pneumoniae* monocolonised mice, compared to GF controls (P<0.01, Figure 3.31 B). We did not see any significant changes in IFNγ<sup>+</sup> or IL-17A<sup>+</sup>IFNg<sup>+</sup> double-positive γδ T cells in the lungs of monocolonised mice compared to controls (Figure 3.31 B). The frequency of CCR6<sup>+</sup> and CCR6<sup>+</sup>CXCR3<sup>+</sup> γδ T cells was also significantly reduced in *K. pneumoniae* monocolonised mice compared to GF mice (P<0.05, Figure 3.31 C). No change in CXCR3 expression by γδ T cells in the lungs was detected (Figure 3.31 C).
We didn’t detect any significant changes in cytokine production or chemokine expression by NKT cells or NK cells (data not shown). Additionally, we carried out the same immunophenotyping on the spleens of GF, *K. pneumoniae* monocolonised and SPF mice. We detected a significant increase in IFNγ production by CD8⁺ T cells in *K. pneumoniae* monocolonised mice compared to SPF mice and a significant increase in CCR6 expression on CD8⁺ T cells in *K. pneumoniae* monocolonised mice compared to GF mice. Apart from these changes in CD8 T cell phenotype, we did not observe any other significant changes in cytokine production by CD4, γδ, NKT cells or NK cells (data not shown). Taken together, this data indicates that long-term exposure to *K. pneumoniae* results in the chronic expression of Th17-associated genes, as well as significantly increased IL-17A expression by γδ T cells, predominantly in the lungs.
Figure 3.27 Stable monocolonisation of the murine airways and gut by *K. pneumoniae*

*K. pneumoniae* was introduced in the drinking water to mice in a GF isolator. Mice were bred and maintained under monocolonised conditions for 7 months and bacterial burden in fecal samples quantified at regular intervals and expressed as log$_{10}$CFU/g fecal sample (A). Lungs, NALT, liver, small intestine and colon were harvested from 2 (C) and 4 (D) wk old pups and 8 wk old adult mice (B) that had been colonised from birth, and bacterial burden assessed and expressed as log$_{10}$CFU/ organ or log$_{10}$CFU/g fecal sample. Results are expressed as mean ± SEM.
Figure 3.28 Monocolonisation with *K. pneumoniae* results in a significant increase in *Il1b, Il17a* and *Ifng* gene expression in the lungs of adult mice

*K. pneumoniae* was introduced in the drinking water to mice in a GF isolator. Mice were bred and maintained under monocolonised conditions. Lungs were harvested from 8 wk old adult mice that had been colonised from birth, RNA extracted and gene expression for (A) *Il23a*, (B) *Il12b*, (C) *Il12a*, (D) *Il1b*, (E) *Il17a* and (F) *Ifng* assessed by RT-PCR. mRNA values are expressed as mean fold change ± SEM, compared to PBS-administered controls, after normalising to a housekeeping control gene (18S rRNA)(n=6-8 per group representative of 3 independent experiments). Statistical analysis performed by Mann–Whitney U Test (A & C) or Students *T* Test (F) *P<0.05, **P<0.01, ***P<0.001 vs controls.
Figure 3.29 Monoclonisation with *K. pneumoniae* promotes increased IL-10 expression by CD4⁺CD25⁺ Treg cells in the lungs

*K. pneumoniae* was introduced in the drinking water to mice in a GF isolator. Mice were bred and maintained under monoclonised conditions. Lungs were harvested from 8 wk old adult mice that had been colonised from birth, and GF and SPF mice at the same age, digested using Collagenase D and DNAse1. Cells were stimulated with PMA and ionomycin with brefeldin A for 4 h and stained for surface CD3, CD4, CD25, CCR6 and CXCR3 and intracellular IL-17A, IFNγ, IL-10, IL-4, IL-13. Results are mean percentages of (A) CD4⁺ cells of total CD3⁺ T cells, (B) CD25⁺ cells of total CD3⁺CD4⁺ cells, (C) IL-10⁺ cells of total CD25⁺CD4⁺ cells, (D) IL-17A⁺, IFNγ⁺, IL-17A⁺IFNγ⁺ DP or TNFα⁺ cells of total CD4⁺ cells, (E) CCR6⁺, CXCR3⁺, or CCR6⁺CXCR3⁺ DP cells of total CD4⁺ cells and (F) IL-4⁺, IL-5⁺ or IL-13⁺ cells of total CD4⁺ cells ± SEM. (n = 11/group from 3 independent experiments. Statistical analysis performed by student’s t test, * P<0.05, ** P<0.01 vs controls.
Figure 3.30 Monocolonisation with *K. pneumoniae* results in a small but significant increase in IFNγ expression by CD8+ T cells in the lungs, compared to that seen in GF and SPF controls. *K. pneumoniae* was introduced in the drinking water to mice in a GF isolator. Mice were bred and maintained under monocolonised conditions. Lungs were harvested from 8 wk old adult mice that had been colonised from birth, and GF and SPF mice at the same age, digested using Collagenase D and DNase1 and cells stained for surface CD3, CD8, CCR6 and CXCR3 and intracellular IL-17A, IFNγ and TNFα. Results are mean percentages of (A) CD8+ cells of total CD3+ T cells, (B) IL-17A+, IFNγ+, IL-17A+IFNγ+ DP or TNFα+ cells of total CD8+ cells and (C) CCR6+, CXCR3+, or CCR6+CXCR3+ DP cells of total CD8+ cells ± SEM (n = 11/group from 3 independent experiments. Statistical analysis performed Mann–Whitney U Test (B) or Students T Test (C), * P<0.05 vs controls. (D) Representative FACS plots for one individual experiment.
Figure 3.31 Monoclonisation with *K. pneumoniae* results in significantly increased IL-17A and TNFα expression by γδT cells in the lungs, compared to that seen in GF and SPF controls.

*K. pneumoniae* was introduced in the drinking water to mice in a GF isolator. Mice were bred and maintained under monoclonised conditions. Lungs were harvested from 8 wk old adult mice that had been colonised from birth, and GF and SPF mice at the same age, digested using Collagenase D and DNAse1 and cells stained for surface CD3, γδTCR, CCR6 and CXCR3 and intracellular IL-17A, IFNγ and TNFα. Results are mean percentages of (A) γδTCR+ cells of total CD3+ T cells, (B) IL-17A+, IFNγ+, IL-17A+IFNγ+ DP or TNFα+ cells of total γδTCR+ cells and (C) CCR6+, CXCR3+, or CCR6+CXCR3+ DP cells of total γδTCR+ cells ± SEM (n = 11/group from 3 independent experiments. Statistical analysis performed Mann–Whitney U Test (B) or Students *T* Test (A & C) * P<0.05 ** P<0.01 vs controls. Representative FACS plots for one individual experiment (D).
3.2.9 Long-term exposure to *K. pneumoniae* primes lung cells for robust memory response

Finally, we next considered how long-term exposure to *K. pneumoniae* might prime a memory response to this bacterium in the lungs and peripherally. We prepared single cell suspensions from the lungs and spleen of monocolonised mice and compared their antigen recall response to heat-killed (HK) *K. pneumoniae* or an unrelated bacterium. Lung cells from mice monocolonised with *K. pneumoniae* displayed enhanced IL-17A recall responses following exposure to HK *K. pneumoniae* at both MOI 10 and MOI 100, compared to cells isolated from the lungs of GF or SPF mice. A small increase in IL-17A concentration was also detected in culture supernatants from lung cells of *K. pneumoniae* monocolonised mice stimulated with HK *S. aureus* (Figure 3.32 A). Restimulation of lung cells from *K. pneumoniae* monocolonised mice with HK *K. pneumoniae* also resulted in increased IFNγ secretion, but not in those stimulated with HK *S. aureus* (Figure 3.32 B). We also found elevated IL-10 concentration in supernatants from lung cells exposed to the higher MOI of HK *K. pneumonia*, but not at the lower MOI or in cells cultured with HK *S. aureus* (Figure 3.32 C). Similarly, robust TNFα secretion was observed in response to HK *K. pneumoniae*, but not HK *S. aureus* (Figure 3.32 D). Splenocytes from GF, *K. pneumoniae* monocolonised and SPF mice readily secreted both IFNγ and TNFα upon restimulation with HK *K. pneumonia*, but not HK *S. aureus*, but failed produce an IL-17A or IL-10 in response to either bacterium (Figures 3.32 E-H). This data indicates that an antigen-specific Th17-type immune response is initiated in the lungs but not in the periphery of *K. pneumoniae* monocolonised mice.
A. Lung

B. IFN-γ pg/ml

C. IL-10 pg/ml

D. TNF-α pg/ml

E. Spleen

F. IFN-γ pg/ml

G. IL-10 pg/ml

H. TNF-α pg/ml

Legend:
- GF
- K. pneumoniae
- SPF

Media
K. pneumoniae MOI 10
K. pneumoniae 100
S. aureus MOI 10
PMA & α-CD3
Figure 3.32 Long-term exposure to *K. pneumoniae* primes lung cells for a robust memory response

*K. pneumoniae* was introduced in the drinking water to mice in a GF isolator. Mice were bred and maintained under monocolonised conditions. Lungs and spleens were harvested from 8 wk old adult mice that had been colonised from birth, and GF and SPF mice at the same age. Lungs were digested using Collagenase D and DNAse1 and a single cell suspension prepared from lungs and spleens. Cells were seeded in triplicate at $2 \times 10^5$ cells/well in a 96 well plate. Cells were stimulated with media alone, heat killed *K. pneumoniae* at MOI 10 and 100, heat killed *S. aureus* at MOI 10 or PMA (1µg/ml) and αCD3 (1µg/ml) and cultured at 37°C/5% CO$_2$. Supernatants were collected at 72 h and the concentration of IL-17 (A & E), IFNγ (B & F), IL-10 (C & G) and TNFα (D & H) quantified by ELISA. Results are expressed as mean ± SEM (n = 11/group representative of 3 independent experiments).
3.3 Discussion

Recent years have seen a surge in correlative information linking changes in the composition of the RT microbiota and chronic inflammatory diseases including COPD, asthma and arthritis [257]. However, there is paucity of information on how specific members of the RT microbiota regulate local and systemic immune responses. There remains a need to elucidate the specific molecular mechanisms by which the microbiota, and its individual members influence host immunity. The goal of this research project was to identify human RT bacteria with immunomodulatory abilities that may influence the pathogenicity of Th17 cells in chronic inflammatory disease. Given the critical role played by IL-23 in driving Th17 cell pathogenicity in chronic inflammatory diseases including MS, we wanted to establish if individual members of the RT microbiota could drive IL-23 expression in innate immune cells and in the lungs.

Our results show that all Proteobacteria species tested induce IL-23 secretion in BMDC (Figure 3.8 & 3.12). In particular, *K. pneumoniae* induced robust IL-23 secretion by BMDC but stimulated only low levels of IL-12p70 production. On the other hand, exposure to *M. catarrhalis* resulted in equally significant production of both IL-23 and IL-12p70. Although with reduced potency, BMDC exposed to multiple Neisseria (*N. meningitidis*, *N. cinerea* and *N. lactamica*) and Haemophilus (*H. influenzae* type B and NTHi) species, as well as *P. aeruginosa*, also secreted IL-23 in a dose-dependent manner and produced comparable levels of IL-12p70 (Figure 3.8). Exposure of BMDC to the Bacteroidetes species *P. melaninogenica* and *P. nigrescens*, also drove IL-23 secretion, equivalent to that seen in Neisseria- or Haemophilus-stimulated BMDC (Figure 3.6 and 3.8). *F. nucleatum* also induced IL-23 secretion by BMDC, although this was relatively low compared to that seen in response to stimulation with the Proteobacteria or Bacteroidetes species (Figure 3.9). We did not detect IL-23 in supernatants from BMDC or BMM that had been exposed to any of the Firmicutes or Actinobacteria species tested in this assay. However, BMDC exposed to *S. pneumoniae*, *S. mitis* and *V. parvula* did secrete low but detectable amounts of IL-12p70 (Figure 3.7 & 3.9), highlighting the selective activation of IL-23 by specific bacterial species (Figure 3.12 & 3.13). Moreover, we found that *M. catarrhalis* could promote IL-23 expression by lung-derived DC ex vivo (Figure 3.14), supporting the theory that DC are the dominant IL-23-secreting cell in the local immune response to these RT bacteria.
Neither *in vitro* BMM or *ex vivo* AM secreted IL-23 or IL-12p70 upon stimulation with RT bacteria from any phyla, despite high TNFα expression and comparable cell viability following exposure to all bacterial strains tested (Figures 3.6 – 3.9; 3.15). AM play an important role in the in the maintenance of lung homeostasis and often display an immunosuppressive phenotype. AM are not considered to have a significant role in antigen presentation and one mechanism by which AM exert their immunosuppressive action is through the regulation of DC recruitment to the airways [258-260]. In addition, bacterial species such as *S. aureus* and *M. tuberculosis* have been shown to manipulate AMs towards an anti-inflammatory phenotype through the induction of IL-10 to promote survival [261-263]. While there are few reports on AM responses to *N. cinerea* or *M. catarrhalis*, *K. pneumoniae* has been shown to promote IL-10 and TNFα expression by AM [264-267]. Cytokine concentration in BAL fluid is frequently used as a surrogate measure of AM cytokine production in both murine and human studies [268, 269]. In the current study, to achieve the maximum cell purity, we FACS-sorted AM populations from the lungs of naïve mice to a high purity (99% +) based on their expression of a stringent set of markers [251]. It might be that, *in vivo*, AM act in concert with other immune cell subsets to promote IL-23 expression, as was reported by Happel and colleagues after i.t. challenge with *K. pneumoniae* [256].

It is striking that all species that induced IL-23 in our *in vitro* assay system are Gram-negative bacteria. However, what is also interesting is that different Gram-negative bacteria have different immunogenic properties. For example, *K. pneumoniae* was a potent stimulator of IL-23 secretion in DC but induced very little IL-12p70 (Figure 3.8). In the same assay conditions, *Prevotella* species, or even alternate Proteobacteria species such as *M. catarrhalis*, *Neisseria*-or *Haemophilus* induced significantly less IL-23 secretion, and equal levels of IL-12p70 (Figures 3.6 & 3.8).

Immune stimulation by bacteria is mediated by a diverse set of cell surface receptors that engage with microbe-associated molecular patterns (MAMPS). Toll-like receptors (TLRs) are a well-characterised family of microbial-sensing proteins [211]. Activation of signalling pathways downstream of microbe-activated TLRs is crucial for the initiation of immune responses in innate immune cells like DC and MΦ. TLRs display agonist specificity, for
example, components of the microbial cell wall such as lipoproteins and peptidoglycan activate TLR2, while TLR9 recognises single-stranded DNA [211].

A recent comprehensive study compared the immunomodulatory properties of human gut commensal bacteria is important to note in this regard. In that study, GF mice were monoclonised with each of 53 individual bacterial species (representative of the five phyla selected in the current study) and host immunologic adaptation to colonization studied. An interesting finding was that membership of a given phylum did not predict immunomodulatory properties, highlighting the importance of characterizing immunogenicity at a species rather than phyla level [270]. A previous study further showed that the cytokine profile induced in DC by intestinal Gram-negative bacteria cannot be attributed to individual MAMPs since purified major cell wall components did not induce an identical cytokine profile to that obtained with whole bacteria [271]. It is important, therefore, to consider the strain-specific immunomodulatory properties of each IL-23-stimulating bacterium.

*N. cinerea, M. catarrhalis* and *K. pneumoniae* and are all Gram-negative members of the phylum Proteobacteria and strongly induced IL-23 expression by BMDC in our assay conditions. IL-23 secretion by DC in response to *K. pneumoniae* is well described [272]. IL-23 is rapidly induced in the lung following *K. pneumoniae* pulmonary challenge and IL-23-deficient mice display increased mortality upon challenge with the bacterium [256]. This *K. pneumoniae* induced IL-23 expression in the lung promotes an IL-17-mediated immune response which is critical for bacterial clearance [83, 273, 274]. TLR4, through its capacity to sense LPS on the outer membrane of *K. pneumoniae*, and TLR2, through its interaction with bacterial lipoproteins including outer membrane protein A expressed by *K. pneumoniae*, contribute to the recognition of *K. pneumoniae* by innate immune cells and the initiation of this immune response [274]. Future studies might further elucidate whether the IL-23-promoting abilities of the selected bacteria is mediated through LPS-TLR4 ligation using primary DC from C3H/HeJ mice that are hypo-responsive to LPS and resistant to toxic shock in the exposure assay system described herein [275]. However, preliminary data from our lab indicates that, while IL-1β responses are largely mediated via TLR4 signalling, IL-23 production by DC following exposure to *K. pneumoniae* and *M. catarrhalis* show greater dependency on
TLR2 signalling (Varadi and Lalor, unpublished). This might support a recent report that demonstrated a role for TLR2 signalling in driving Th17 cell pathogenicity and migratory capacity in CNS autoimmunity [276].

*M. catarrhalis* is a human-restricted, unencapsulated commensal of the URT that relies on its ability to attach to the mucosal surface of the RT for successful colonization and infection [277]. *M. catarrhalis* has an outer membrane that is comprised of phospholipids, lipooligosaccharides (LOS), integral outer membrane proteins (OMPs) and lipoproteins, and the immune response to *M. catarrhalis* appears to be initiated primarily through TLR2 ligation [278]. There are a limited number of studies describing IL-23 production in response to *M. catarrhalis*. Larsen *et al.* found significant induction of IL-23 and IL-12p70 in human DCs stimulated with *M. catarrhalis in vitro*, while one *in vivo* study in mice observed significant levels of IL-17 in BAL fluid following i.n. exposure to the bacterium [63, 279].

*N. cinerea* commonly colonises the oropharynx of healthy adults and children [280, 281]. *N. cinerea* closely resembles other Neisseria species including the commonly encountered commensal *N. lactamica* and the pathogenic species *N. gonorrhea* and *N. meningitides*. It is considered that *N. lactamica* colonises the nasopharynx of children more frequently than *N. cinerea* which dominates in adulthood [282, 283]. Interestingly, *N. lactamica* displays an inverse epidemiological relationship with *N. meningitidis*, a more pathogenic species of this Gram-negative group of bacteria while *N. cinerea* was found to impair *N. meningitidis* colonisation of the nasopharynx [284, 285]. There have been no previous reports of IL-23 induction in response to *N. cinerea*.

*Prevotella* species are strictly anaerobic Gram-negative bacteria that belong to the Bacteroidetes phylum. *Prevotella* are generally considered commensal organisms given their abundance in the human oral, lung and GIT microbial communities, and rare association with infection [286]. Notwithstanding this, certain *Prevotella* species have been linked with inflammatory diseases including periodontitis and RA [287, 288]. Consistent with our findings here, *Prevotella* species have previously been shown to induce IL-23 production in both human and murine DC. *P. nigrescens* promoted Th17-mediated inflammation in a murine model of arthritis, via TLR2 stimulation on APCs [288]. Similarly, *P. melaninogenica* drove an IL-23-dominant response in human monocyte-derived DC, that was also dependent on TLR2
signalling [63]. The penta-acylated lipid A structure of LPS in *Prevotella* may account for the lower immunostimulatory potential of these Bacteroidetes species. Both tetra- and penta-acylated LPS structures are less stimulatory upon TLR4 ligation compared to hepta- and hexa-acylated LPS, expressed by Proteobacteria such as *H. influenzae* and *M. catarrhalis*, respectively [289, 290]. The decreased capacity of *Prevotella* species to stimulate TLR4 may perhaps account for the broadly tolerogenic immune response against this most abundant airway commensal [286].

The Fusobacteria *F. nucleatum* is a fastidious anaerobe that is ubiquitous in the healthy human oral cavity. Nothing is known about its ability to promote IL-23 or related cytokines, although *F. nucleatum* has recently been linked with a number of infectious and inflammatory conditions at peripheral sites [291, 292]. Here, *F. nucleatum* induced IL-23 secretion by BMDC, although this was relatively low compared to that seen in response to stimulation with the Proteobacteria or Bacteroidetes species. Interestingly, *F. nucleatum* did not induce IL-12p70 by BMDC or BMM, at any MOI (Figure 3.9).

Exposure of DC to *S. pneumoniae, V. parvula* or *R. mucilaginosa* did not result in IL-23 secretion in our assay conditions and were selected as controls for future investigations. *S. pneumoniae* is a Gram-positive Firmicutes species which is part of the RT microbiota that commonly colonises the nasopharyngeal niche, but is also an opportunistic pathogen associated with URT infections [293]. Although it has been postulated that IL-23 may play a role in clearance of infection, the role of IL-23 during *S. pneumoniae* colonisation has not been defined [294]. Firmicutes species are generally Gram-positive, however *V. parvula* is a rare Gram-negative member of this phyla and is a dominant component of the RT microbiome [295]. *V. parvula* is a diderm bacteria, expressing both an inner and outer membrane, and shares its LPS biosynthesis pathway with Proteobacteria [296]. Although it is known that *V. parvula* expresses LPS, it’s structure has not yet been fully described [297]. Again, no studies have reported IL-23 secretion by any cell in response to *V. parvula*. However, the LPS from *V. parvula* was found to induce inflammatory cytokine production in both human and murine cells *in vitro*, in a TLR4-dependent manner, but at a potency that is significantly less than LPS from the prototypical Gram-negative bacterium *Escherichia coli* [296].
R. mucilaginosa is an encapsulated Gram-positive bacterium of the Actinobacteria phyla. R. mucilaginosa is part of the core microbiota of the oral cavity URT of healthy individuals, and only causes disease in immunocompromised individuals [298, 299]. Evidence regarding the immunomodulatory competency of R. mucilaginosa is limited, and no studies have reported IL-23 expression in response to R. mucilaginosa stimulation. Moreover, a recent study that analysed the effects of bacterial metabolites on DC activation and T cell priming did not detect IL-12p70 in the supernatants of murine DC following stimulation with metabolites from R. mucilaginosa [300].

Having selected the strongest IL-23-promoting bacteria with which to pursue further studies, and non-IL-23-inducing RT bacteria to use as controls, we next wanted to verify that the cytokine profile observed in vitro was representative of the true response to these bacteria in the upper and lower RT in vivo. Reflecting our in vitro data, exposure to the Bacteroidetes species P. melaninogenica and the Proteobacteria species N. cinerea, M. catarrhalis and K. pneumoniae promoted rapid IL-23p19 expression in the lungs 24 h post-exposure (Figure 3.18). The three Proteobacteria species also promoted IL-23p19 expression in the NALT at 24 h post-administration of bacteria, although only N. cinerea drove a statistically significant response (Figure 3.19). This data demonstrates that individual Proteobacteria species can strongly promote IL-23 expression in the airways, as well as in cultures of BMDC in vitro.

To investigate if this elevated IL-23p19 expression were associated with downstream changes in cytokine production in the LRT, we examined cytokine production by flow cytometry at 3 and 7 d post-bacterial administration. We found that i.n. exposure of mice to K. pneumoniae resulted in increased IL-17A production by CD4 T cells and γδ T cells in the lungs at 3 d post-administration, and a small increase in IFNγ production by both T cell subsets at the same timepoint. No T cell responses were detected by 7 d post-administration, reflecting the clearance of these RT bacteria from the murine airways at that time (Figures 3.21-3.22).

The colonisation of GF mice with human microbes offers a useful tool for the development of our knowledge on how changes in the composition of the microbiota influence host immunity and represent an important recourse as microbiome research moves from correlative studies to those striving to assign specific functions to individual microbes based on their impact on host biology. The rapid clearance of bacteria in SPF mice fails to replicate the lifetime’s
exposure that a human immune system has to various RT bacteria. Additionally, both the murine lung and gut microbiota, as in the human, can vary significantly between subjects [301, 302]. This may be dependent on mouse genotype, vendor, shipping method, housing, and environmental conditions such as bedding, food and temperature in the animal facility [303-305]. Host commensal microbiome variability must therefore be considered as a confounding factor when defining the immunomodulatory capacity of an individual bacterium. To overcome this issue, we exposed GF mice to selected RT bacteria which allowed for the defined assessment of the influence on local and systemic immunity of each bacterium in the absence of a complex host microbiota. To our knowledge, there are no previous reports in the literature describing the monoclonisation of GF mice with *N. cinerea* and *M. catarrhalis* and limited knowledge on the impact of *K. pneumoniae* monoclonisation on local and systemic immune responses.

Using this approach, we found a significant increase in IL-23p19 expression in the lungs of mice 24 h after exposure to *M. catarrhalis* and an almost 10-fold, although not significant, increase in response to *K. pneumoniae* (Figure 3.23 A). The IL-23p19 response to *K. pneumoniae* was maintained for at least 7 d. A similar pattern of expression was observed for IL-17A expression in the lungs of GF mice exposed to *M. catarrhalis* and *K. pneumoniae* at both 24 h and 7 d, while i.n. exposure of GF mice to *N. cinerea* also promoted significantly increased IL-17A expression in the lungs at both timepoints (Figure 3.23 D). We did not see any significant changes in the expression of IL-12p40, IL-12p35 or IL-1β in the lungs in response to any of these bacteria (Figure 3.23 B, C, E). Although small increases in IL-23p19 and IL-17A expression were observed, exposure of GF mice to each bacterium did not lead to any significant changes in cytokine gene expression in the NALT (Figure 3.24) or small intestine (Figure 3.25). These data indicate that i.n. exposure of GF mice to these discrete strains of RT bacteria can preferentially promote an IL-23/IL-17 type response in the lungs of otherwise GF mice.

*K. pneumoniae* commonly colonises both the healthy URT and the gut of both humans and mice [248-250]. Our data demonstrates that acute exposure to *K. pneumoniae in vitro* and *in vivo* in the lungs of SPF and GF mice leads to increased IL-23 and IL-17 expression. However, acute exposure to the bacterium does not replicate the effect of a lifetimes interaction
between the local immune system and the bacterium. To attempt to elucidate the impact of this on local and systemic immune responses, we monocolonised GF mice with *K. pneumoniae* (strain 43816) and bred mice under monocolonised conditions for 7 months. Individual mice were colonized within days (data not shown), and we confirmed stable colonization of the NALT, lungs and GIT throughout the study by culturing fecal pellets and sampling mucosal and peripheral tissues from sentinel mice. Interestingly, bacteria were detected in the liver of both adult mice and pups that had been weaned (Figure 3.27 B). This low bacterial burden in the liver may be accounted for by impaired barrier integrity or “leaky gut” frequently reported in GF mice [306]. These results demonstrate that *K. pneumoniae* monocolonisation of the airways and GIT can be maintained over time and across generations. It is also interesting to note the stable level of bacterial burden in these mice over time, the resistance to infection and the normal breeding of these mice, despite the strong proliferative capacities of *K. pneumoniae* seen in our earlier studies (Figure 3.2).

Immunophenotyping of monocolonised mice was performed on 8-week-old mice that had been monocolonised from birth. This allowed time for colonisation to stabilize and for the immunophenotype to be assessed under steady state conditions. Monocolonised mice displayed significantly upregulated expression of IL-1β, IL-17A and IFNγ in the lungs compared to GF and SPF controls (Figures 3.28 D - F). Elevated, although not significant, IL-23p19 expression was also detected in the lungs of these mice (Figure 3.28 A). Given that a significant increase in the expression of this cytokine was detected within the first 7 d following administration of *K. pneumoniae* to GF mice (Figure 3.23 A), it is likely that this 8 wk timepoint is too late to assess changes in IL-23p19 gene expression.

We did not detect any significant changes in the frequency of any T cell subset in the lung or spleen of 8 wk old *K. pneumoniae* monocolonised mice. However, we did find a significant increase in the frequency of IL-17-expressing and TNFα-expressing γδ T cells in the lungs of *K. pneumoniae* monocolonised mice, compared to GF controls (Figure 3.31 B). We also found that CD4⁺CD25⁺ Treg cells produced significantly more IL-10 in the lungs of monocolonised mice compared to GF or SPF controls (Figure 3.29 C). IFNγ expression by CD8⁺ T cells was also significantly elevated in mice monocolonised with *K. pneumoniae* compared to their SPF counterparts (Figure 3.30 B). Surprisingly, the RORγt-regulated chemokine receptor CCR6 was
significantly downregulated on CD4+, CD8+ and γδ T cells in monocolonised mice compared to GF controls (Figures 3.30 & 3.31 C). Although we did not detect much IL-17A expression by CD4 T cells in mice that had been colonized since birth, ex vivo antigen-recall responses of lung cells from these mice to HK K. pneumoniae promoted strong IL-17A, as well as IFNγ, IL-10 and TNFα, secretion compared to lung cells stimulated with S. aureus or cells derived from the lungs of GF mice (Figures 3.32 A – D). No such IL-17 recall response was seen in splenocytes from K. pneumoniae monocolonised mice. This demonstrates that long-term exposure to K. pneumoniae primes lung-resident T cells against the bacterium, that can promote a robust inflammatory response upon re-exposure.

Our study is the first to address the impact of long-term monocolonisation by K. pneumoniae on T cell dynamics in the lungs. A limited number of previous reports describe the monocolonisation of GF mice with K. pneumoniae, with all studies primarily focused on intestinal inflammation. Lau et al described the nasal and GIT colonisation of GF and SPF mice with K. pneumoniae strain IA565 (KpIA565). However, GIT colonisation had no impact on inflammatory histopathology in either C. rodentium or DSS induced colitis [250]. Another study showed that the composition of the host commensal microbiota determines the ability of a neonate GIT-derived K. pneumoniae strain to influence intestinal inflammation and the development of colonic tumours [307]. Colonisation of GF mice with a strain of K. pneumoniae isolated from the saliva of a Crohn’s disease patient led to significant Th1 cell accumulation in the colon [308]. Finally, Fagundes and colleagues demonstrated that GF mice are more susceptible to K. pneumoniae bacteraemia compared to conventionally-housed mice, and that this resulted from an IL-10-mediated hypo-responsiveness to i.t. K. pneumoniae challenge. Anti-Klebsiella immunity could be rescued through the neutralisation of IL-10 or by the introduction of a conventional microbiota or LPS administration prior to bacterial challenge [309]. Our finding that IL-10-expressing CD25+CD4+ Treg cells were twice as prevalent in the lungs of K. pneumoniae monocolonised mice compared to the lungs of GF or SPF mice, supports the concept that pulmonary colonization with K. pneumoniae can modulate immune responses in the airways that can result in circulation-associated systemic effects.
The use of monocolonised mice in this study allowed, for the first time, the discreet impact of individual bacterial species on host immunity in the airways to be defined. However, it should be noted that although a useful approach in the context of this study, monocolonisation studies do not allow for the potentially critical interactions of the microbe to be studied with other bacterial, fungal, or viral species individually or as part of a complex microbiota which may impact how that microbe influences the host [310].

It is interesting to note that the most immunogenic bacteria, in terms of IL-23 secretion, identified in this study include *K. pneumoniae* and *M. catarrhalis* which, despite their frequency of colonisation are often identified as the causative agents in bacterial pneumoniae and otitis media infections, respectively [278, 311]. This is in sharp contrast to bacteria such as *V. parvula* and *R. mucilaginosa* did not induce any IL-23 secretion and are rarely associated with immunopathology. It is possible that certain Proteobacterial species have developed the capacity to exploit the metabolic changes that are associated with inflammation thus creating an environmental niche in which the cycle of inflammation is perpetuated. Perhaps the production of ROS and RNS during inflammatory responses create an environment in which adaptable strains of bacteria bloom and outcompete resident bacteria that lack this capacity. Future studies should aim to understand early inflammatory events associated with these bacterial species and how these might promote the development of chronic inflammatory disease.

The data presented in this chapter adds to our developing understanding of the immunogenicity of various human RT bacteria. We have demonstrated that members of the phyla Bacteroidetes, Proteobacteria and Fusobacteria are potent inducers of the pathogenic Th17 cell-associated cytokine IL-23 in DC, *in vitro* and *in vivo* and that their ability to induce cytokine secretion is not simply determined by the membership of a specific phylum or expression of LPS. We have shown that individual Proteobacteria species including *N. cinerea*, *M. catarrhalis* and *K. pneumoniae* induce an IL-23/IL-17-type immune response in the airways. The use of a novel gnotobiotic model of *K. pneumoniae* monocolonisation showed that Klebsiella can drive these types of immune responses directly, without the requirement for a complex microbiota. These findings will support future research on the immunogenic potential of human RT bacteria including *N. cinerea*, *M. catarrhalis* and *K. pneumoniae*, and the potential roles some of these microbes may play in the pathophysiology of chronic
inflammatory diseases. Through the use of neutralizing antibodies against components of signalling pathways that lead to IL-23 production, mice and primary immune cells deficient in these same pathways, and mutant strains of bacteria that lack the ability to induce IL-23 in innate immune cells, we may be able to identify critical molecules expressed by RT bacteria that directly or indirectly drive IL-23-mediated licensing of pathogenic Th17 cells in the airways. Ultimately, novel therapeutic agents could be developed that target these factors in patients with chronic inflammatory diseases such as MS.
Chapter 4

The respiratory tract microbiota & Th17 cell pathogenicity in EAE
4.1 Introduction

Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). MS is instigated by the infiltration of autoreactive T cells and other immune cell subsets into the CNS which attack the myelin sheath surrounding nerve axons, resulting in the formation of an inflammatory plaque and reduced signal conductance [95]. The etiology of MS remains elusive, but several studies have suggested a role for the microbiome in disease development [312]. It is widely accepted that both genetic and environmental factors play important roles in disease development [313-315]. The high rate of discordance of MS in genetically identical twins highlights the importance of environmental triggers. Moreover, epidemiological studies have long associated relapses in MS patients with systemic infection. Correale and colleagues demonstrated that bystander activation and increased sensitisation of autoreactive myelin-specific T cells resulted in elevated numbers of circulating IFNγ+ CD4+ T cells and exacerbated disease in RRMS patients shortly following viral or bacterial infection [120]. Interestingly, of the 127 infections reported in that study, 52% occurred in the RT and only 5.5% in the GI tract. The RT harbours a diverse microbial community, and a growing literature indicates this may be an important site in the development of certain chronic inflammatory diseases [200, 202]. Here, we have investigated a role for individual members of the RT microbiota in the modulation of CNS autoimmune inflammation.

Much of our understanding of the pathophysiology of MS has arisen from studies of experimental autoimmune encephalomyelitis (EAE), a preclinical animal model that recapitulates many of the clinical and pathological features of the human disease. EAE can be induced by active immunization with myelin peptides emulsified in complete Freund’s adjuvant (CFA) or by passive transfer of in vitro-generated myelin-specific T cells to naïve recipient mice. Active induction of EAE results in the generation of a polyclonal T cell response whereas the adoptive transfer model allows for the polarization of T cells along specific lineages in vitro and then to specifically assess their effector function upon transfer. Additionally, the passive transfer of donor cells to congenic recipient mice allows these cells to be tracked in vivo after transfer [149, 151].
4.1.1 Th17 cell pathogenicity

Th17 cells play an important role in the maintenance of barrier integrity at mucosal surfaces and in defence against extracellular bacterial and fungal infections. However, myelin-specific Th17 cells have also been identified as the key pathogenic effector cells in MS and in EAE [81, 82]. It was originally thought that Th1 cells were the main orchestrators of disease EAE and MS. However, this theory was dispelled upon observation that mice lacking the p19 chain of IL-23 required for Th17 cell development, but not the p35 chain of IL-12 required for Th1 cell development, were resistant to the induction of EAE [154]. This was a seminal finding in the context of the discovery of Th17 cells and it was later demonstrated that IL-23 induces the expansion of myelin antigen-specific IL-17-producing CD4+ T cells which induce EAE upon transfer to naïve mice [64, 154, 157]. However, it is currently believed that the role of CD4+ T cells in MS and EAE is not fully explained by the action of terminally differentiated Th1 or Th17 cells but instead that Th17 cells exist on a continuum and display a high degree of context-dependent plasticity.

Th17 cells differentiate from naïve precursors cells following recognition of their cognate antigen in the presence of innate cytokines including IL-1, IL-6 and TGFβ [73]. After upregulation of the master transcription factor RORγt, Th17 cells express IL-17A and IL-17F as well as other cytokines including IL-10, IL-21, IL-22 and TNFα which orchestrate the host response at mucosal surfaces. RORγt also promotes the expression of a number of key Th17 cell-associated receptors, including IL-23R and CCR6 [316, 317]. Th17 cells also express the aryl hydrocarbon receptor (AHR), a ligand activated transcription factor that renders Th17 cells susceptible to environmental cues [73, 318].

Self-antigen-specific Th17 cells develop in the same manner as those elicited upon infection but appear to be innocuous immediately following differentiation [73, 152, 168, 172]. For self-reactive Th17 cells to gain pathogenic potential – the ability to migrate to and access tissue sites where self- or autogenous antigens are expressed and, therein, orchestrate the inflammatory response – these cells require exposure to the cytokine IL-23, which regulates their conversion to pathogenic effectors of chronic inflammation. Upregulation of IFNγ, GM-CSF and other inflammatory mediators in IL-23-stimulated Th17 cells appears to be associated with pathogenicity and suggests the role of Th17 cells extends beyond the actions of IL-17.
Hence, these so-called ex-Th17 cells (referred to Th17.1 cells in human studies) are activated and develop differently to those that protect against acute infection with, for example, *Candida albicans* or *Salmonella enterica* that do not require IL-23 signalling or deviation of Th17 cell cytokine expression to mediate their effector functions [152, 173, 174]. In EAE, on the other hand, upregulation of Tbet, IFNγ, GM-CSF and other “Th1-like” inflammatory mediators in IL-23-stimulated Th17 cells appears to be a critical encephalitogenic event. In fact, GM-CSF production by IL-23-stimulated ex-Th17 cells has been shown to be indispensable in the development of T cell pathogenicity in EAE [156, 175].

The critical role of Th17 cell plasticity in autoimmune CNS inflammation was demonstrated by Hirota and colleagues. Using fate mapping reporter mice, they showed that majority of IFNγ, GM-CSF and TNFα producing T cells found in the CNS at the peak of EAE were almost exclusively derived from T cells that had once produced IL-17. It appears that these cells transition through an IL-17/IFNγ double positive phenotype before completely downregulating RORγt and IL-17A expression. Crucially, mice deficient in IL-23p19 lacked the IL-17A+/IFNγ+ double-positive, and IFNγ single-positive Th17 cells observed in their WT counterparts, and IL-23-mediated induction of Tbet, the Th1 associated transcription factor, was critical for the induction of IFNγ expression by “ex-Th17” cells [152].

In MS patients too, CD161+ Th17.1 cells that express IFNγ and GM-CSF were also reported to be the main pathogenic T cell subset associated with disease relapse and progression [188]. And in cerebrospinal fluid (CSF), the dominant CD4+ T cell subset express the Th17-associated chemokine receptor CCR6 but not IL-17; instead expressing IFNγ and GM-CSF, or GM-CSF only [17].

Other factors too have been associated with encephalitogenicity in myelin-reactive Th17 cells. CCR6 is a chemokine receptor that is expressed on various cell types including immature DCs, ILCs, Treg cells and Th17 cells. CCL20 is the only known high-affinity ligand that binds to this receptor and promotes the migration of CCR6+ cells into tissues [319]. It was reported that CCR6 plays an essential role in EAE by allowing for the initial trafficking of autoreactive Th17 cells to the uninflamed CNS to initiate disease [78]. However, CCR6 is downregulated in IFNγ+GM-CSF+ producing “ex-Th17 cells” found at the CNS at the height of clinical disease course in EAE [152]. Moreover, upon persistent antigen exposure, Th17 cells transition to a
CCR2-expressing phenotype [320]. Analysis of cytokine expression by Th17 cells in EAE and in MS patients showed that co-expression of GM-CSF and IFNγ was confined to CCR6+CCR2+ Th17 cells. Mice deficient in IL-23 or IL-23R failed to generate GM-CSF expressing CCR6+CCR2+ Th17 cells demonstrating that this mechanism is primarily driven by IL-23 signalling. This further supports the concept that a transition to an “ex-Th17” GM-CSF and IFNγ-producing phenotype is a critical step in Th17 cell pathogenicity.

CXCR3 is a Th1-associated chemokine receptor which is enriched in T cells that accumulate in MS lesions. The CXCR3 ligands CXCL9, CXCL10 and CXCL11 are expressed by astrocytes and microglia in spatial proximity to perivascular infiltrates [321, 322]. In addition, elevated CXCR3 expression has been reported in IFNγ-producing Th17 cells in MS patients [323]. CXCR3+IFNγ+ T cells assemble in CNS lesions of mice with EAE too, accompanied by upregulated CXCL10 in adjacent astrocytes [324]. However, it has been shown that mice deficient in CXCR3 or CXCL10 are susceptible to EAE and that blockade of CXCR3 or neutralization of its primary ligand CXCL10 had no therapeutic impact on the development of clinical disease [324].

SerpinB1 is a protease inhibitor and neutrophil survival factor that is highly expressed by encephalitogenic CD4+ T cells following induction of EAE and is required for their survival and expansion [185, 325]. EAE was ameliorated in mice lacking Sb1 (Sb1−/−) and this was specifically associated with reduced T cell pathogenicity. SerpinB1-deficient mice had a decreased frequency of T cells that co-express IL-17A, IFNγ and GM-CSF in the brain and spinal cord, which was directly linked to diminished expression of the non-lymphoid tissue homing receptor CXCR6. Interestingly, Sb1-deficient and IL-23R-deficient mice display indistinguishable phenotypes suggesting that Sb1 functions downstream of IL-23 to regulate the encephalitogenic program [185, 186].

Little is known, however, about the pathways that drive development of these self-reactive Th17 cells. Exposure to the cytokine IL-23 appears to be a critical step in their acquisition of pathogenic potential and for conversion to an “ex-Th17” cell phenotype. Naïve CD4+ T cells do not express the IL-23R, however, which is only upregulated following expression of the transcription factor RORγt [168]. When and where IL-23 promotes Th17 cell pathogenicity
remains unknown. Understanding these mechanisms is of critical importance and may identify novel targets for therapeutic intervention in MS.

4.1.2 Reparatory Tract and EAE

In MS and EAE, very little is known about the trafficking of myelin-reactive Th17 cells prior to the onset of clinical disease. Preliminary data from our lab indicates that myelin-reactive Th17 cells traffic to mucosal sites including the gut-draining mesenteric lymph nodes and the lungs in the time between their transfer to naïve mice and the onset of neurological deficits. It was found however, that antibody-mediated blockade of α4β7-MAdCAM-1 interactions or genetic insufficiency in the same pathway preventing transferred Th17 cells from trafficking through the mesenteric lymph nodes, Peyer’s patches and small intestinal lamina propria, had no impact on donor Th17 cell accumulation in the CNS and failed to attenuate disease (Lalor S.J., unpublished data). It remained to be seen if the lungs might be a critical site for the conversion of Th17 cells into pathogenic effector ex-Th17 cells that mediate neuroinflammation and demyelination in EAE.

In support of this theory, a growing number of reports have also implicated the RT in the pathogenesis of CNS autoimmune inflammation [200-203]. Using intra-vital microscopy in a rat model of EAE, Odoardi and colleagues demonstrated that myelin-specific T cells are reprogrammed in the lungs and acquire the capacity to enter the CNS [200]. Phenotypic changes in effector T cells involved downregulation of genes associated with activation and proliferation and upregulation of migration-associated molecules including sphingosine-1-phosphate (S1P) receptors and the adhesion molecules ninjurin 1 and integrin α4. Almost all T cells were found in the lungs following i.v. transfer of GFP-labelled T cell blasts, before they entered the lung draining mediastinal lymph nodes and reappeared in the blood and spleen. Further analysis of T cell trafficking within the lungs, revealed that cells are initially distributed within the peripheral lung parenchyma before they move along bronchial structures and accumulate in bronchus associated lymphoid tissues (BALT). T cells only entered the CNS shortly prior to the onset of clinical disease. Importantly, T_{MBP-GFP} cells accumulated in the lungs after the active induction of EAE, counteracting the argument that the homing of effector T cells to the lungs might be an artefact of i.v. transfer.
Separately, it was found that expansion of a pro-inflammatory population of granulocytic myeloid-derived suppressor cells (MDSC) in the lungs during EAE promotes Th17 cell pathogenicity [202]. MDSCs are a heterogeneous bone marrow-derived immature myeloid cell population that can display both suppressive and immunostimulatory capacities [326]. The authors had previously observed that CD11b$^\text{hi}$Ly6C$^\text{lo}$ MDSCs expand in the lungs after the induction of EAE and demonstrated that the expansion of MDSCs in the lung temporarily coincided with the infiltration of Th17 cells at this site after the induction of EAE [200, 327]. Interestingly, MDSCs isolated from the lungs and co-cultured with CD4$^+$ T cells promoted greater IL-17 production than those isolated from the spleen, again implicating this site in the pathogenic development of Th17 [202].

Two separate studies found that RT infection with influenza A exacerbated clinical signs of disease in EAE [328, 329]. Data presented in one study indicated that myelin-reactive IFN$\gamma^+$ CD4$^+$ T cells accumulated in the lungs prior to entering the CNS and this response was enhanced in mice recovered from influenza A [328]. Conversely, reduced disease severity was observed in mice infected with the respiratory pathogen B. pertussis [201]. Significantly decreased IL-17$^+$ CD4$^+$ T cell accumulation was observed in the brains of mice infected with B. pertussis, suggesting that infection with certain RT bacteria might suppress migration of T cells to the CNS. The authors of that study showed that Treg-derived IL-10 was elevated in infected mice and was associated with reduced expression of the critical adhesion molecules integrin a4 and LFA-1 on myelin-specific Th17 cells in the lungs [201].

Lung inflammation and reduced CNS tropism were also observed by Kanayama et al. during the pathogenesis of EAE [203]. These authors found that mice lacking the Autophagy Related 7 (ATG7) protein displayed delayed onset of disease. ATG7 is required for autophagy and microtubule-associated protein1A/1B-light chain 3 (LC3)-associated phagocytosis (LAP). Mice deficient in ATG7 develop spontaneous subclinical pulmonary inflammation but display normal T cell development [203, 330]. These authors also demonstrated that Th17 cells accumulate in the lungs during the preclinical stages of EAE but that, in ATG7-deficient mice, these cells are stalled in a process involving CCL20 ligation of CCR6, delaying T cell trafficking to the CNS and the onset of clinical disease.
Although these studies indicate the varying effects of microbial exposure or infection on the manifestation of EAE, it is clear that the immune landscape in the lungs can be an important contributor to disease outcomes. These studies, together with our own preliminary data, support the concept that the lungs play an important role in modulating encephalitogenicity in myelin-reactive T cells.

4.1.3 The microbiota and EAE

Numerous studies have implicated the microbiota as an important factor in the pathogenesis of MS and EAE. The importance of the microbiota in EAE development was highlighted by the finding that GF or antibiotic-treated mice are relatively resistant to EAE and that reconstitution of these mice with the commensal bacteria SFB, which is known to promote Th17 cells in the murine gut, can partially restore disease susceptibility [190, 191]. Furthermore, mice expressing a myelin autoantigen-specific T cell receptor and spontaneously develop a relapsing form of EAE, require the presence of commensal bacteria for the development of CNS autoimmune inflammation [89]. Interestingly, the transfer of fecal microbiota from patients with RRMS, but not the microbiota from a paired healthy twin, was sufficient to enhance the incidence of EAE in these mice indicating that the microbiota of MS patients contains factors that regulate CNS autoimmunity [195]. Several studies have demonstrated that changes in the composition of the gut microbiota can alter EAE disease course. However, an etiological connection between specific gut microbes and Th17 cell pathogenicity in MS has not yet been made.

Most studies to date have failed to address the microbiota at other mucosal sites. The RT is also colonised by a vast number of diverse microbes in both health and disease. This community is dominated by bacteria from the phyla Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria and Fusobacteria and, in particular, Streptococci, Veillonella, Prevotella, Rothia, Porphyromonas and Haemophilus on a genus level [8, 9, 17, 207-210]. Knowledge on the immunomodulatory properties of individual members of this community is largely ill-defined. It has been demonstrated that that pathogenic Proteobacteria often enriched in the airways of patients with asthma or COPD, including H. influenzae and M. catarrhalis, induce significantly more IL-23 by host innate immune cells, compared to
*Prevotella* species commonly found in healthy lungs [63]. However, the potential of this microbiome to influence immunopathology in a range of chronic inflammatory disorders is only now being investigated.
4.1.4 Aims and rationale for this study

Th17 cells are the critical pathogenic effector cells in MS and EAE but are innocuous immediately following differentiation [73]. The IL-23-dependent conversion to an IFNγ and GM-CSF-secreting phenotype appears to be a critical step in the development of Th17 cell pathogenicity [152, 156]. The etiology of MS remains elusive but it appears that the microbiota plays a critical role in disease development [89, 190] Studies on the microbiota and MS studies have largely focused on the gut microbiota but the lungs too harbour a diverse bacterial community [15]. However, our knowledge on the immunogenicity of these commensal and pathobiont microbial species in the lung remains in its infancy. Work from our lab has demonstrated that myelin reactive Th17 cells traffic through the lungs in the preclinical stages of EAE, prior to the onset of disease, and mounting evidence suggests that the lungs might be an important staging site during disease pathogenesis [200-203]. In the previous chapter, we identified members of the RT microbiota capable of inducing IL-23/IL-17 type immunity in the airways. We demonstrated that the Proteobacteria species *N. cinerea, M. catarrhalis*, and *K. pneumoniae* strongly induce IL-23 secretion *in vitro* in BMDC and *in vivo* in the lungs of both SPF and GF mice. Hence, we wanted to assess the impact of exposure of the murine airways to these RT symbionts on Th17 cell pathogenicity and the development of EAE. Specifically, we aimed to:

1. Determine the ability of IL-23-stimulating RT bacteria to promote Th17 cell encephalitogenicity and disease development in EAE, a preclinical mouse model of MS.

2. Assess the effect of RT bacteria on donor Th17 cell phenotype in the lungs of recipient mice during the preclinical stages of EAE.

3. Investigate the mechanisms by which RT bacteria might promote conversion of differentiated myelin-specific Th17 cells to a pathogenic ex-Th17 cell phenotype.
4.2 Results

4.2.1 Th17 cell adoptive transfer model of EAE and exposure to RT symbionts

In order to investigate the impact of RT symbionts on the effector function of myelin-specific Th17 cells, we employed an adoptive transfer model of disease that allowed us to polarise cells in vitro, track them following transfer in vivo and for phenotypic changes of in vitro polarized T cell to be studied.

As per figure. 4.2, C57BL/6 mice were immunised by s.c. injection of 100 µg of MOG35-55 emulsified in CFA. 10 - 13 days after immunization, mice were sacrificed, and lymph node cells and splenocytes cultured in Th17 cell polarizing conditions (Section 2.5.2). 72 h later, cells were collected and magnetically enriched for CD4+ T cells. Cells were consistently >95% CD4+ (Figure 4.2 A). Cytokine concentration in cell culture supernatants were quantified by ELISA. IL-17A was consistently the prominent cytokine detected by ELISA in culture supernatants (average 25,024.7 pg/ml) and was detected at higher levels than IFNγ (average 9332.48 pg/ml) and GM-CSF (average 4986.94 pg/ml) following 72 h culture (Figures 4.2 B). An aliquot of cells was restimulated with PMA and ionomycin and cytokine secretion blocked by brefeldin A for 4 h before staining for surface CD4, CCR6, CXCR3, CXCR6, and intracellular IL-17, IFNγ, and GM-CSF and analysed by flow cytometry. Th17-polarised CD4+ T cells predominantly produced IL-17A (avg. 8.29 %) as well as IFNγ (avg. 4.76 %) and GM-CSF (avg. 1.59 %)(Figures 4.2 C & D). An average of 22.05 % of cells expressed the RORγt-regulated chemokine CCR6, whereas CXCR3 was expressed at an average of 12.60 % and approx. 19.16 % of CD4+ T cells expressed CXCR6 expression after 72 h polarization (Figures 4.2 C & E).

1 X 10^6 CD4+ cells were transferred to mice i.p. and animals were monitored daily for signs of clinical disease. Clinical disease typically presented from d 7 post-Th17 cell transfer, beginning with loss of tail tone (stage 1), and progressing through varying degrees of hind limb weakness, leading ultimately to hind limb paralysis (stage 4; Section 2.5 A) (Figure 4.2 F).

Our lab has previously demonstrated that Th17 cells traffic to the lungs and gut associated lymphoid tissue prior to entering the CNS (Lalor S.J., unpublished data). Using polarised Th17 cells from congeneric donor mice, cells were tracked in the periphery daily following transfer. Transferred Th17 cells began to appear in the airways at d 2 post-transfer and donor cell
accumulation peaked in the lungs at d 6 post-transfer. In the current study, we found that *N. cinerea, M. catarrhalis, and K. pneumoniae* are detected in the lungs 3 d after i.n. exposure but cleared by d 7 (Figure 3.20). Additionally, we detected an increase in IL-17A production by CD4\(^+\) T cells and γδ T cells in the lung 3 d after exposure to *K. pneumoniae*, a response which had waned 7 d after i.n. exposure (Figures 3.21 & 3.22). Based on these findings, we chose to expose Th17 recipient mice to selected RT symbionts 2 d after T cell transfer so that the appearance of transferred Th17 cells in the lung should coincide with the immune response induced by bacterial exposure. 6 d post-transfer was selected as an optimal timepoint to assess changes in Th17 cell phenotype in the lungs as this was the time at which the maximum number of donor T cells would be isolated, and prior to their leaving the lungs and trafficking to the CNS (Figure 4.1).

At this time, lungs and spleens were harvested, cells analysed by flow cytometry and gene expression by PCR. At peak disease, mice were perfused with ice-cold PBS and brains and spinal cords harvested and analysed by flow cytometry.
Figure 4.1 Schema for the assessment of the impact of airway exposure to RT bacteria on Th17 cell phenotype and pathogenicity in EAE

Polarized myelin-specific Th17 cells were adoptively transferred to naïve C57BL/6 mice by i.p. injection. 2 d after transfer, groups of mice were exposed to bacteria or PBS via i.n. administration. 6 d after T cell transfer, lungs and spleens were harvested, cells analysed by flow cytometry and gene expression in tissue analysed by PCR. Animals were weighed daily and monitored for signs of clinical disease. At peak disease, mice were perfused intracardially and brains and spinal cords harvested, and mononuclear cells isolated and analysed by flow cytometry.
Figure 4.2 Passive induction of EAE by adoptive transfer of myelin-specific Th17 cells

Mice were immunized with MOG<sub>35-55</sub>/CFA. 10-14 d later, single cell suspensions of auxiliary, brachial, inguinal LN and spleen cells were cultured with MOG<sub>35-55</sub> in the presence of IL-1α, IL-23, anti-IFNγ and anti-IL-4. 72 h later, cells were harvested, enriched for CD4<sup>+</sup> T cells and adoptively transferred to naïve mice (1X10<sup>6</sup> CD4<sup>+</sup> T cells/mouse i.p.) (A). An aliquot of cells was re-stimulated with PMA and ionomycin with brefeldin A for 4 h, stained for surface CD4, CCR6, CXCR3, CXCR6, and intracellular IL-17A, IFNγ and GM-CSF and analysed by flow cytometry. Cytokine concentration in cell culture supernatant was quantified by ELISA. Results are representative dot plots from one experiment (A) which shows % purity of CD4<sup>+</sup> cells of total cells after enrichment and % IL-17A<sup>+</sup>, IFNγ<sup>+</sup>, GM-CSF<sup>+</sup>, CCR6<sup>+</sup>, CXCR3<sup>+</sup> and CXCR6<sup>+</sup> of CD4<sup>+</sup> T cells (C). Average cytokine pg/ml in culture supernatants as measured by ELISA (B) and mean frequency of cytokine (D) and chemokine expression (E) by CD4<sup>+</sup> T cells across all experiments (n=15 independent experiments). Animals were weighed daily and monitored for clinical signs of disease. Disease severity was graded as follows: grade 0 - normal; grade 1 - flaccid tail; grade 2 - wobbly gait; grade 3 - hind limb weakness; grade 4 - hind limb paralysis; grade 5 - tetraparalysis/death (F). Results are mean score +/- SEM (n=15 mice/group from 4 independent experiments).
4.2.2 GF mice are less susceptible to both active EAE and Th17 cell-mediated EAE compared to conventionally-housed SPF mice

GF mice have previously been shown to be develop significantly attenuated MOG-induced EAE compared with conventionally housed mice [190]. Here, we wanted to confirm that this same trend holds true in our facility. We induced in C57BL/6 by s.c. injection of 100 µg of MOG$_{35-55}$ emulsified in CFA, containing 4 mg/ml H37 Ra M. tuberculosis. All mice were injected i.p. with 500 ng pertussis toxin (PT) on days 0 and 2. Animals were monitored daily for weight loss and signs of clinical disease (Section 2.5 A). GF mice displayed significantly decreased disease severity (P<0.001) and significantly reduced weight loss compared to SPF controls (P ≤ P<0.001) (Figure 4.3 A). This confirms previous reports that microbial colonisation is required for autoimmune inflammation to occur in the active model of EAE.

Active induction of EAE results in the generation of polyclonal T cell response. However, we wanted to assess the impact of bacterial exposure on the effector function of Th17 cells. We wondered if the outcome observed in actively immunized GF mice held true upon the passive transfer of in vitro polarized Th17 cells. CD4$^+$ T cells from conventionally house mice were cultured in Th17-polarizing conditions as per Section 2.5. CD4$^+$ T cells were adoptively transferred to naïve conventionally housed SPF mice and GF mice (1X10$^6$ cells/mouse i.p.), and animals monitored daily for weight loss and signs of clinical disease (Section 2.5 A). We found that GF recipients of myelin-specific Th17 cells displayed significantly decreased disease severity (P ≤ 0.0001) and significantly reduced weight loss compared to SPF recipients of the same Th17 cells (P ≤ 0.0001) (Figure 4.3 B). This data indicates that, the commensal microbiota can specifically influence the migration and/or effector function of myelin-specific Th17 cells.
Figure 4.3 GF mice are less susceptible than SPF mice to both active EAE and Th17 cell-mediated EAE

Active EAE was induced in C57BL/6 mice by s.c. injection of 100µg MOG<sub>35-55</sub>/CFA emulsified in CFA. Mice were injected i.p. with 500 ng pertussis toxin (PT) on d 0 and d 2 (A). Th17-polarized MOG-specific T cells were adoptively transferred to naïve SPF recipients via i.p injection (1X10<sup>6</sup> CD4<sup>+</sup> T cells/mouse) (B). Animals were weighed daily and monitored for clinical signs of disease. Disease severity was graded as described in Fig. 4.2. Results are mean score +/- SEM (n=8 mice/group from 2 independent experiments for A, n= 12 mice/group from 3 independent experiments for B). Statistical analysis comparing EAE clinical scores and mouse weight over the course of disease was performed using two-way ANOVA with multiple comparisons. *** P<0.001, **** P ≤ 0.0001 vs controls.
4.2.3 Airway exposure of Th17 cell recipient mice to specific RT symbionts exacerbates clinical signs of EAE

Having demonstrated that microbial exposure is necessary for the full development of Th17 cell mediated EAE, we next wanted to assess how exposure to specific respiratory symbionts might modulate the effector function of Th17 cells during EAE. Based on the immunogenicity of various RT symbionts in the airways, as described in Chapter 3, we selected 4 strains of bacteria to use in this model of i.n. exposure during the development of Th17-mediated EAE. *N. cinerea, M. catarrhalis* and *K. pneumoniae* were selected given their ability to induce IL-23/IL-17 type responses *in vitro* in BMDC and *in vivo* in the lungs (Figures 3.12 and 3.18). *V. parvula* is typically associated with the healthy human airways and did not induce any IL-23 secretion in innate immune cells *in vitro* or *in vivo* in the data presented in Ch.3 (Figures 3.12 & 3.18). Th17 cells were polarized *in vitro* and 1 X 10^6 enriched CD4^+^ T cells transferred to naïve SPF mice. 2 d later, groups of mice were exposed to 1 X 10^6 CFU bacteria or PBS i.n. Animals were monitored daily for weight loss and signs of clinical disease.

We found that exposure to *V. parvula* did not result in any changes in EAE disease course or weight loss in Th17-recipient mice, compared to PBS-administered controls (Figure 4.4). In mice exposed *N. cinerea*, we detected a small but not significant increase in disease severity, compared to PBS-exposed controls (Figure 4.5 A). There was no difference in weight loss between Th17-recipient mice exposed to *N. cinerea* or PBS (Figure 4.5 B). We next examined the impact of airway exposure of Th17-recipient mice to *M. catarrhalis* on EAE disease development. Mice exposed to *M. catarrhalis* developed earlier (d 5) and more severe disease than PBS exposed controls (P<0.05 Figure 4.6 A). *M. catarrhalis*-exposed mice also lost significantly more weight than PBS-administered controls (P<0.05; Figure 4.6 B). Exposure of Th17 cell-recipients to *K. pneumoniae* also significantly exacerbated clinical disease compared to PBS-administered controls (P ≤ 0.0001; Figure 4.7 A), with a similar significant increase in weight loss, compared to PBS-administered controls (P<0.001; Figure 4.7 B). Mice exposed to *V. parvula, N. cinerea, M. catarrhalis or K. pneumoniae* alone did not show any signs of EAE or lose weight over the course of these investigations (Figure 4.4 B - 4.7 B). These findings show that specific members of the airway microbiota, including *M. catarrhalis* and *K. pneumoniae*, can exacerbate Th17 cell-mediated EAE while exposure to other RT symbionts has no effect on clinical disease.
Figure 4.4 Airway exposure to *V. parvula* has no effect on Th17 cell-mediated EAE

Th17-polarized MOG-specific T cells were adoptively transferred to naïve SPF recipients via i.p. injection (1X10^6 CD4^+ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to *V. parvula* via i.n. administration of 20 μl PBS containing 2x10^6 CFU bacteria or PBS alone. Animals were weighed daily and monitored for clinical signs of disease. Disease severity was graded as described in Fig. 4.2. Results are mean score +/- SEM (n = 5 mice/group from 2 independent experiments). Statistical analysis comparing EAE clinical scores and mouse weight over the course of disease was performed using two-way ANOVA with multiple comparisons.
Figure 4.5 Airway exposure to *N. cinerea* does not exacerbate Th17 cell-mediated EAE

Th17-polarized MOG-specific T cells were adoptively transferred to naïve SPF recipients via i.p. injection (1X10^6 CD4+ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to *N. cinerea* via i.n. administration of 20 μl PBS containing 2x10^6 CFU bacteria or PBS alone. Animals were weighed daily and monitored for clinical signs of disease. Disease severity was graded as described in Fig. 4.2. Results are mean score +/- SEM (n = 12 mice/group from 3 independent experiments). Statistical analysis comparing EAE clinical scores and mouse weight over the course of disease was performed using two-way ANOVA with multiple comparisons.
Figure 4.6 Airway exposure to *M. catarrhalis* of recipients of myelin-specific Th17 cell exacerbates EAE

Th17-polarized MOG-specific T cells were adoptively transferred to naïve SPF recipients via i.p. injection (1X10^6 CD4^+ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to *M. catarrhalis* via i.n. administration of 20 μl PBS containing 2x10^6 CFU bacteria or PBS alone. Animals were weighed daily and monitored for clinical signs of disease. Disease severity was graded as described in Fig. 4.2. Results are mean score +/- SEM (n = 18 mice/group from 5 independent experiments). Statistical analysis comparing EAE clinical scores and mouse weight over the course of disease was performed using two-way ANOVA with multiple comparisons. * P<0.05 vs. PBS administered controls.
Figure 4.7 Airway exposure to *K. pneumoniae* of recipients of myelin-specific Th17 cell exacerbates EAE

Th17-polarized MOG-specific T cells were adoptively transferred to naïve SPF recipients via i.p. injection (1X10^6 CD4^+ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to *K. pneumoniae* via i.n. administration of 20 μl PBS containing 1x10^6 CFU bacteria or PBS alone. Animals were weighed daily and monitored for clinical signs of disease. Disease severity was graded as described in Fig. 4.2. Results are mean score +/- SEM (n=18 mice/group from 6 independent experiments). Statistical analysis comparing EAE clinical scores and mouse weight over the course of disease was performed using two-way ANOVA with multiple comparisons. *** P<0.001, **** P ≤ 0.0001 vs PBS administered controls.
4.2.4 *K. pneumoniae* monocolonisation exacerbates Th17 cell-mediated EAE

Mice are not natural hosts for the majority of human RT bacteria. This was demonstrated in Chapter 3 where SPF mice had cleared *N. cinerea*, *M. catarrhalis* and *K. pneumoniae* by d 7 following i.n. exposure (Figure 3.22). Unlike in conventionally-housed SPF mice, GF mice monocolonised with *K. pneumoniae* maintained colonisation in the airways from birth through the entire course of these investigations (Figure 3.27 B). Moreover, SPF mice have a complex microbiota that might influence the immunomodulatory function of individual RT bacteria. Long-term monocolonisation of mice with *K. pneumoniae* provides a unique tool to assess the impact of this bacterium alone on Th17-mediated EAE disease development. Hence, Th17 cells were polarized *in vitro* and 1 X 10^6 enriched CD4^+ T cells transferred to naïve GF mice and mice monocolonised with *K. pneumoniae* from birth. Mice were monitored daily for clinical signs of disease and weight loss. *K. pneumoniae* monocolonisation enhanced disease susceptibility to EAE in otherwise less susceptible GF mice. Only 55 % of GF Th17 cell recipients developed any signs of clinical disease whereas 77 % of *K. pneumoniae* monocolonised mice developed disease (Figure 4.8 A). Disease severity was also increased in *K. pneumoniae* monocolonised mice compared to GF recipients of the same Th17 cells (Figure 4.8 A). Additionally, *K. pneumoniae* monocolonised mice lost more weight than GF mice over the course of EAE (Figure 4.8 B). This data, although not significant, supports the concept that colonisation with individual RT bacteria has potential to enhance the pathogenicity of myelin-specific Th17 cells and exacerbate clinical signs of disease.
Figure 4.8 *K. pneumoniae* monocolonisation exacerbates Th17 cell-mediated EAE compared to that seen in GF hosts

Th17-polarized MOG-specific T cells were adoptively transferred to naïve GF or *K. pneumoniae* monocolonised mice via i.p. injection (1X10^6 CD4+ T cells/mouse). Animals were weighed daily and monitored for clinical signs of disease. Disease severity was graded as described in Fig. 4.2. Results are mean score +/- SEM (n= 9 mice/group from 2 independent experiments). Statistical analysis comparing EAE clinical scores and mouse weight over the course of disease was performed using two-way ANOVA with multiple comparisons.
4.2.5 Altered CD4 T cell phenotype in the CNS of mice exposed to *M. catarrhalis* and *K. pneumoniae* at the peak of Th17 cell-mediated EAE

Having observed a significant increase in disease severity in recipients of myelin-specific Th17 cells exposed to both *M. catarrhalis* and *K. pneumoniae*, we wanted to investigate if exposure to these respiratory symbionts was modulating CD4\(^+\) T cell accumulation in the CNS. At peak disease, we found a significant increase in the frequency and total number of CD4\(^+\) T cells in the spinal cord of mice exposed to *K. pneumoniae* \((P<0.05; \text{Figures 4.9 A and C})\). *M. catarrhalis* exposure resulted in a small but not significant increase in the frequency and total number of CD4\(^+\) T cells in both the spinal cord and brain \((\text{Figures 4.9 A, B & D})\).

Th17 cells have been reported to undergo conversion from an IL-17-dominant cytokine phenotype to an IFN\(\gamma\) and GM-CSF-producing phenotype during the pathogenesis of EAE \([152]\). In fact, GM-CSF production by CD4\(^+\) T cells is indispensable for the development of EAE \([184]\). We questioned if exposure to *M. catarrhalis* and *K. pneumoniae* was promoting conversion to this pathogenic T cell phenotype. We found that airway exposure of mice to *K. pneumoniae* following transfer of myelin-reactive Th17 cells led to a significant increase in the accumulation of IL-17\(^+\) (Figure 4.10 A), IFN\(\gamma\)^\(+\) (Figure 4.10 B) and GM-CSF\(^+\)IFN\(\gamma\)^\(+\) double positive \((\text{DP})\) CD4\(^+\) T \((\text{Figure 4.10 F})\) cells in the spinal cord at peak disease, compared to PBS-administered controls \((P<0.05)\). Exposure to either *K. pneumoniae* or *M. catarrhalis* also resulted in a small, but not significant, increase in accumulation of GM-CSF\(^+\) CD4\(^+\) T cells in the spinal cord \((\text{Figure 4.10 C})\). The number of IL-17A\(^+\)GM-CSF\(^+\) and IL-17A\(^+\)IFN\(\gamma\)^\(+\) CD4\(^+\) T cells was also slightly elevated in mice exposed to either bacterium, compared to PBS-administered controls \((\text{Figure 4.11 D & E})\). *M. catarrhalis*, but not *K. pneumoniae*, exposure following transfer of myelin-specific Th17 cells also led to a moderate increase in accumulation of IL-17\(^+\), IFN\(\gamma\)^\(+\) and GM-CSF\(^+\)IFN\(\gamma\)^\(+\) DP CD4\(^+\) T cells in the brain at the peak of disease \((\text{Figure 4.11})\).

We next looked at the impact of bacterial exposure specifically on donor \((\text{CD3}^+\text{CD4}^+\text{CD45.1}^+)\) and host \((\text{CD3}^+\text{CD4}^+\text{CD45.1}^-)\) CD4\(^+\) T cells in the CNS at the peak of disease. The use of congenic recipient mice allowed us to track donor Th17 cells following transfer and to further delineate between the impact of bacterial exposure in the lung on donor myelin-specific Th17 cells versus stimulation of host CD4\(^+\) T cells that ultimately accumulate in the CNS. In the spinal cord at peak disease, we found a significant increase in the frequency of GM-CSF\(^+\) and
IFNγ-GM-CSF+ DP donor CD4+ T cells in mice exposed to *M. catarrhalis*, compared to PBS-administered controls (P<0.05) (Figure 4.12 A). We found a similar, although non-significant increase in the frequency of GM-CSF+ and IFNγ-GM-CSF+ DP donor T cells in mice exposed to *K. pneumoniae* during the development of EAE (Figure 4.12 A). This heightened donor Th17 cell cytokine response was restricted to the spinal cord because we did not detect increased frequency of GM-CSF+ and IFNγ-GM-CSF+ DP donor T cells in the brains of mice exposed to either *M. catarrhalis* or *K. pneumoniae* (Figure 4.13). No changes in the frequency of IL-17A+ or IFNγ+ CD4+ donor T cells were detected in the spinal cord (Figure 4.12) or brain (Figure 4.13) in response to either bacterium. Production of all cytokines by host CD4+ T cells was markedly lower when compared to donor CD4+ T cells in both the spinal cord and brain of recipient mice and did not appear to be regulated by exposure to human RT bacteria (Figure 4.12 and 4.13). This data suggests that the immune response induced in the lungs upon bacterial exposure to *M. catarrhalis* and *K. pneumoniae* is promoting the conversion of donor myelin-specific Th17 cells to a GM-CSF-producing pathogenic “ex-Th17” cell phenotype.

Myelin-specific T cells have been shown to upregulate their migratory capacity in the lungs as they traffic through this site during the preclinical stages of EAE [200]. We therefore also examined chemokine receptor expression on CD4+ T cells in the brain and spinal cord at peak disease. We found a significant increase in the frequency of CXCR6+ CD4+ T cells in the spinal cord of mice that were exposed to either *M. catarrhalis* or *K. pneumoniae*, compared to PBS-administered controls (P<0.05; Figures 4.14 A). We did not detect any significant changes in CCR6 or CXCR3 expression in CD4+ T cells in the brain or spinal cord (Figures 4.14 A & B), or in the frequency of CXCR6+ CD4+ T cells in the brains of mice exposed to either bacterium (Figure 4.14 B). However, when we looked at the effect of exposure to human RT bacteria specifically on donor and host CD4+ T cells, we saw a significant increase in the frequency of CCR6+ donor CD4+ T cells amongst total CD3+ T cells in the brain of mice exposed to *M. catarrhalis*, compared to PBS administered controls (P<0.05; Figure 4.16 A). We did not detect any effect of *M. catarrhalis* exposure on CCR6 expression by donor Th17 cells that had accumulated in the spinal cord (Fig 4.15 A). Intranasal administration of *M. catarrhalis* or *K. pneumoniae* did not affect the frequency of CXCR3+ or CXCR6+ donor or host CD4+ T cells in the brain or spinal cord, compared to PBS-administered controls (Figures 4.15 & 4.16). While no significant differences were observed in the frequency of CCR6 and CXCR3 expression between donor
and host cells in the CNS, it is striking to note the differential expression of CXCR6 by donor CD4$^+$ T cells in both the brain and spinal cord, compared to that seen on host CD4$^+$ T cells. Almost all donor CD4$^+$ T cells detected in the brain and spinal cord of mice with EAE were CXCR6$^+$ (> 89 %), whereas only a fraction of host CD4$^+$ T cells at both sites expressed this critical chemokine (< 28 %) at the peak of disease (Figures 4.15 & 4.16 A & B).
Figure 4.9 Increased accumulation of CD4+ T cells in the brain and spinal cord of mice exposed to *M. catarrhalis* and *K. pneumoniae* at the peak of Th17 cell-mediated EAE

Th17-polarized MOG-specific T cells were adoptively transferred to naïve SPF recipients via i.p. injection (1X10^6 CD4+ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to bacteria via i.n. administration of 20 μl PBS containing 2x10^8 CFU *M. catarrhalis*, 1x10^6 CFU *K. pneumoniae* or PBS alone. Animals were weighed daily and monitored for clinical signs of disease. At peak disease (d 10-13), mice were perfused intracardially and mononuclear cells isolated from the CNS. Cells were stained for surface CD3 and CD4 and analysed by flow cytometry. Results are mean % CD4+ of CD3+ T cells in the spinal cord (A) and brain (B) and the total number of CD3⁺CD4⁺ T cells in the spinal cord (C) and brain (D) +/- SEM (n= 9 mice/group from 3 independent experiments). Statistical analysis performed by Students T Test *P<0.05 vs. PBS administered controls.
Figure 4.10 Increased frequency of IL-17A, IFNγ and GM-CSF+IFNγ CD4+ T cells in the spinal cord of mice exposed to *M. catarrhalis* and *K. pneumoniae* at the peak of Th17 cell-mediated EAE

Th17-polarized MOG-specific T cells were adoptively transferred to naïve SPF recipients via i.p. injection (1x10^6 CD4+ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to bacteria via i.n. administration of 20 μl PBS containing 2x10^6 CFU *M. catarrhalis*, 1x10^6 CFU *K. pneumoniae* or PBS alone. Animals were weighed daily and monitored for clinical signs of disease. At peak disease, mice were perfused intracardially and mononuclear cells isolated from the spinal cord. Cells were stimulated with PMA and ionomycin with brefeldin A for 4 h, stained for surface CD3, CD4 and intracellular IFNγ, IL-17A and GM-CSF and analysed by flow cytometry. Results are total number of IL-17A+ (A), IFNγ+ (B), GM-CSF+ (C), IL-17A+GM-CSF+ (D) IL-17A+IFNγ+ (E) and IFNγ+GM-CSF+ (F) CD4+ T cells in the spinal cord +/- SEM (n = 9/group from 3 independent experiments). Statistical analysis performed by Mann–Whitney U Test * P<0.05 vs. PBS-administered controls. Representative FACS plots show GM-CSF+ of CD4+CD3+ T cells and IFNγ+ of GN-CSF CD4+CD3+ cells for one control mouse, one *M. catarrhalis*-exposed mouse and one *K. pneumoniae*-exposed mouse (G).
Th17-polarized MOG-specific T cells were adoptively transferred to naïve SPF recipients via i.p. injection (1X10⁶ CD4⁺ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to bacteria via i.n. administration of 20 μl PBS containing 2x10⁶ CFU M. catarrhalis, 1x10⁶ CFU K. pneumoniae or PBS alone. Animals were weighed daily and monitored for clinical signs of disease. At peak disease, mice were perfused intracardially and mononuclear cells isolated from the brain. Cells were stimulated with PMA and ionomycin with brefeldin A for 4 h, stained for surface CD3, CD4 and intracellular IFNγ, IL-17A and GM-CSF and analysed by flow cytometry. Results are total number of IL-17A⁺ (A), IFNγ⁺ (B), GM-CSF⁺ (C), IL-17A⁺GM-CSF⁺ (D) IL-17A⁺IFNγ⁺ (E) and IFNγ⁺GM-CSF⁺ (F) CD4⁺ T cells in the spinal cord +/- SEM (n = 9/group from 3 independent experiments).
Figure 4.12 Increased frequency of donor GM-CSF+ and IFNγ+GM-CSF+ DP CD4 T cells in the spinal cord of mice exposed to *M. catarrhalis* at the peak of Th17 cell-mediated EAE

CD45.1 congenic donor Th17-polarized MOG-specific T cells were adoptively transferred to CD45.2 SPF mice via i.p. injection (1×10⁶ CD4+ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to bacteria via i.n. administration of 20 μl PBS containing 2×10⁶ CFU *M. catarrhalis*, 1×10⁶ CFU *K. pneumoniae* or PBS alone. Animals were weighed daily and monitored for clinical signs of disease. At peak disease, mice were perfused intracardially and mononuclear cells isolated from the spinal cord. Cells were stimulated with PMA and ionomycin with brefeldin A for 4 h, stained for surface CD3, CD4 and CD45.1 and intracellular IFNγ, IL-17A and GM-CSF and analysed by flow cytometry. Results are mean frequency of donor (A) and host (B) IL-17A+, IFNγ+, GM-CSF+ and IFNγ+GM-CSF+ DP CD4+ T cells in the spinal cord +/- SEM (n = 9/group from 3 independent experiments). Statistical analysis performed by Students *T* Test * P<0.05 vs. PBS administered controls. Representative FACS plots for one control mouse, one *M. catarrhalis*-exposed mouse and one *K. pneumoniae*-exposed mouse (C).
Figure 4.13 Frequency of cytokine-expressing donor and host CD4 T cells in the brain of mice exposed to *M. catarrhalis* and *K. pneumoniae* at the peak of Th17 cell-mediated EAE

CD45.1 congenic donor Th17-polarized MOG-specific T cells were adoptively transferred to CD45.2 SPF mice via i.p. injection (1X10^6 CD4^+ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to bacteria via i.n. administration of 20 μl PBS containing 2x10^6 CFU *M. catarrhalis*, 1x10^6 CFU *K. pneumoniae* or PBS alone. Animals were weighed daily and monitored for clinical signs of disease. At peak disease, mice were perfused intracardially and mononuclear cells isolated from the brain. Cells were stimulated with PMA and ionomycin with brefeldin A for 4 h, stained for surface CD3, CD4 and CD45.1 and intracellular IFNγ, IL-17A and GM-CSF and analysed by flow cytometry. Results are mean frequency of donor (A) and host (B) IL-17A^+, IFNγ^+, GM-CSF^+ and IFNγ^+GM-CSF^+ DP CD4^+ T cells in the brain +/- SEM (n = 9/group from 3 independent experiments).
Figure 4.14 Increased frequency of CXCR6+ CD4+ T cells in the spinal cord of mice exposed to *M. catarrhalis* and *K. pneumoniae* at the peak of Th17 cell-mediated EAE

Th17-polarized MOG-specific T cells were adoptively transferred to SPF mice via i.p. injection (1X10^6 CD4+ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to bacteria via i.n. administration of 20 μl PBS containing 2x10^6 CFU *M. catarrhalis*, 1x10^6 CFU *K. pneumoniae* or PBS alone. Animals were weighed daily and monitored for clinical signs of disease. At peak disease, mice were perfused intracardially and mononuclear cells isolated from the spinal cord. Cells were stained for surface CD3, CD4, CCR6, CXCR3 and CXCR6 and analysed by flow cytometry. Results are mean frequency of CCR6+ CXCR3+ and CXCR6+ CD4+ T cells in the spinal cord (A) and brain (B) +/- SEM (n = 9/group from 3 independent experiments). Statistical analysis performed by Students T Test, * P<0.05 vs. PBS administered controls. Representative FACS plots for one control mouse, one *M. catarrhalis*-exposed mouse and one *K. pneumoniae*-exposed mouse (C).
Figure 4.15 Frequency of CCR6, CXCR3 and CXCR6-expressing donor and host CD4 T cells in the spinal cord of mice exposed to *M. catarrhalis* and *K. pneumoniae* at the peak of Th17 cell-mediated EAE

CD45.1 congenic donor Th17-polarized MOG-specific T cells were adoptively transferred to CD45.2 SPF mice via i.p. injection (1X10^6 CD4+ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to bacteria via i.n. administration of 20 μl PBS containing 2x10^6 CFU *M. catarrhalis*, 1x10^6 CFU *K. pneumoniae* or PBS alone. Animals were weighed daily and monitored for clinical signs of disease. At peak disease, mice were perfused intracardially and mononuclear cells isolated from the spinal cord. Cells were stained for surface CD3, CD4, CCR6, CXCR3 and CXCR6 and analysed by flow cytometry. Results are mean frequency of donor (A) and host (B) CCR6+, CXCR3+ and CXCR6+ CD4+ T cells in the spinal cord +/- SEM (n = 9/group from 3 independent experiments).
Figure 4.16 Increased frequency of CCR6+ donor CD4+ T cells in the brain of mice exposed to *M. catarrhalis* at the peak of Th17 cell-mediated EAE

CD45.1 congenic donor Th17-polarized MOG-specific T cells were adoptively transferred to CD45.2 SPF mice via i.p. injection (1X10^6 CD4+ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to bacteria via i.n. administration of 20 μl PBS containing 2X10^6 CFU *M. catarrhalis*, 1X10^6 CFU *K. pneumoniae* or PBS alone. Animals were weighed daily and monitored for clinical signs of disease. At peak disease, mice were perfused intracardially and mononuclear cells isolated from the brain. Cells were stained for surface CD3, CD4, CCR6, CXCR3 and CXCR6 and analysed by flow cytometry. Results are mean frequency of donor (A) and host (B) CCR6+, CXCR3+ and CXCR6+ CD4+ T cells in the brain +/- SEM (n = 9/group from 3 independent experiments). Statistical analysis performed by Students T Test, *P<0.05 vs. PBS administered controls. Representative FACS plots for one control mouse, one *M. catarrhalis*-exposed mouse and one *K. pneumoniae*-exposed mouse (C).
4.2.6 Conversion of donor Th17 cells to an ex-Th17-like phenotype in the lungs of mice exposed to *M. catarrhalis* or *K. pneumoniae* during the preclinical stages of EAE

Having shown that exposure to *M. catarrhalis* and *K. pneumoniae* can exacerbate Th17 cell mediated EAE, and that this was associated with increased GM-CSF+ and GM-CSF+IFNγ+ DP cells in the CNS and increased CXCR6 expression in the spinal cord, we next questioned how exposure to these RT bacteria might modulate the effector function of transferred Th17 cells in the preclinical stages of disease. Previous work in our lab had demonstrated, using CD45.1 congenic donor mice, that myelin-specific Th17 cells traffic through the lungs prior to entering the CNS. This study found that transferred cells began to appear in the lungs by d 2 and peaked at d 6 following transfer. This was also when these donor Th17 cells began to disappear from the lungs and appear in the brain and spinal cord. Hence, we chose d 6 as the optimal time point to assess phenotypic changes in donor CD4+ T cells in the airways and peripheral lymphoid tissues. Confirming this previous data, we found that donor CD4+ T cells trafficked through the lungs in the days before entering the CNS, but there was no difference in frequency of CD4+ T cells in the lungs (Figure 4.17 A & C) or spleen (Figure 4.17 B & C) of mice exposed to *V. parvula*, *M. catarrhalis* or *K. pneumoniae*, compared to PBS-administered controls.

When we looked at the cytokine expression profile of donor (CD3+CD4+CD45.1+) and host (CD3+CD4+CD45.1+) T cells in the lung, we detected a significant increase in the frequency of GM-CSF+ donor CD4+ T cells amongst total CD3+ T cells in the lungs of mice exposed to *K. pneumoniae* compared to PBS-administered controls (P<0.05; Figure 4.18 A & C). Additionally, a significant increase in the frequency of GM-CSF+IFNγ+ DP donor CD4+ T cells amongst total CD3+ T cells was detected in the lungs of mice exposed to *M. catarrhalis* compared to PBS controls (P<0.05 Figure 4.18 A & C). Similarly, we saw increased accumulation of GM-CSF (P<0.05) and GM-CSF+IFNγ+ DP (P<0.05) donor CD4+ T cells in the lungs of mice exposed to *M. catarrhalis*, compared to PBS controls (Figure 4.19 A). Exposure to any bacteria did not lead to any changes in IL-17A or IFNγ production by donor CD4+ T cells (Figures 4.18 and 4.19). However, exposure to *V. parvula* resulted in a 63% reduction in IL-17A production and a 53% reduction in IFNγ production by donor CD4+ T cells compared to PBS controls (Figure 4.18 A). Strikingly, host CD4+ T cells were unresponsive to bacterial stimulation in terms of IL-17A, IFNγ and GM-CSF production, which were markedly less cytokine-responsive than donor CD4+ T
cells in all groups of mice (Figure 4.18 B). This suggests that the bacterial stimulus in the airways primarily activates already primed CD4+ T cells as they traffic through the lungs.

We next questioned if these changes in CD4+ T cell phenotype were a result of factors encountered due to microbial colonisation specifically in the lungs or if the bacterial exposure was inducing a systemic inflammatory response in the mouse. We therefore analysed the cytokine profile of donor and host CD4+ T cells in the spleen as a representative secondary lymphoid organ. Although cells trafficked to the spleen as well as lung, no changes in cytokine production by donor or host CD4+ T cells in response to bacterial exposure was detected in mice exposed to *M. catarrhalis* or *K. pneumoniae* at this site (Figure 4.20). Similar to what we observed in the lung, a small decrease in IL-17A and IFNγ production was detected in donor CD4+ T cells of mice exposed to *V. parvula* (Figure 4.20). Again, cytokine production by host CD4+ T cells was minimal in the spleen compared to cytokine production by donor CD4+ T cells, regardless of bacterial stimulation. Taken together, this data indicates that exposure to *M. catarrhalis* and *K. pneumoniae* promotes phenotypic changes to myelin-specific T cells, including upregulation of IFNγ and GM-CSF expression, specifically in the lungs of recipient mice.

To assess the impact of bacterial exposure in the lungs on the trafficking of CD4+ T cells, we analysed chemokine expression in lung tissue by PCR, 6 d after the adoptive transfer of myelin-specific Th17 cells. We detected an increase in gene expression of the chemokine *Cxcl16* which is trending towards significance in the case of, *K. pneumoniae* (*P* = 0.069) and is increased in lungs of mice exposed to both *V. parvula* and *M. catarrhalis*, compared to PBS-administered controls (Figure 4.21 C). No significant changes were seen in *Ccl20, Ccl2, Cxcl2, Cxcl9* or *Cxcl10* gene expression (Figure 4.21). When we looked at chemokine receptor expression on donor and host CD4+ T cells in the lungs by flow cytometry, we found a significant increase in CCR6 expression on donor CD4+ T cells in the lungs of mice exposed to *K. pneumoniae*, compared to PBS-administered controls (*P*<0.01; Figure 4.22 A & C). No significant changes were detected in the frequency of CXCR3+ or CXCR6+ donor T cells in the lungs of mice exposed to any bacteria, compared to PBS-administered controls (Figure 4.22 A). Similar to our findings regarding cytokine expression by host CD4+ T cells, we did not detect any change in chemokine receptor expression by host CD4+ T cells in the lungs of recipient...
mice, regardless of their exposure to human RT bacteria (Figure 4.22 B). Interestingly, however, almost all donor T cells in the lungs at 6 d post-transfer expressed the chemokine receptor CXCR6 (>88 %), whereas host CD4+ T cells at this site were largely negative for CXCR6 (<5%) (Figure 4.22). Again, changes in donor CD4+ T cell phenotype were limited to the lung at 6 d following exposure to V. parvula, M. catarrhalis or K. pneumoniae. No significant changes in chemokine expression were detected in donor or host CD4+ T cells in the spleens of Th17 cell recipient mice exposed to V. parvula, M. catarrhalis or K. pneumoniae (Figure 4.23). Intriguingly, CXCR6 expression in donor cells in the spleen was high (> 75 %) compared to host cells CXCR6 expression (< 5 %) (Figure 4.23). Taken together this data indicates that bacteria-induced chemokine expression in the lungs, particularly the upregulation of CXCL16, might promote the trafficking of CXCR6+ Th17 cells to this site.
Figure 4.17 Donor CD4+ T cell accumulation in the lungs and spleen of mice exposed to *V. parvula*, *M. catarrhalis* and *K. pneumoniae* in the preclinical stages of Th17 cell-mediated EAE

CD45.1 congenic donor Th17-polarized MOG-specific T cells were adoptively transferred to CD45.2 SPF mice via i.p. injection (1X10^6 CD4+ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to bacteria via i.n. administration of 20 μl PBS containing 2x10^6 CFU *V. parvula*, 2x10^6 CFU *M. catarrhalis*, 1x10^6 CFU *K. pneumoniae* or PBS alone. During the pre-clinical stage of EAE (6 d post-T cell transfer) lungs and spleens were harvested, lungs were digested using Collagenase D and DNAse1 and single cell suspensions prepared from both tissues. Cells were stained for surface CD3, CD4 and CD45.1 and analysed by flow cytometry. Results are mean % CD4+ of CD3+ T cells in the lungs (A) and spleen (B) +/- SEM (n = 6-25 mice/group from 2-5 independent experiments). Representative FACS plots from lung and spleen for one control mouse, one *V. parvula*-exposed mouse, one *M. catarrhalis*-exposed mouse and one *K. pneumoniae*-exposed mouse (C).
Figure 4.18 Increased frequency of GM-CSF$^+$ and IFN$^+$GM-CSF$^+$ DP donor CD4$^+$ T cells in the lungs of mice exposed to *K. pneumoniae* and *M. catarrhalis* in the preclinical stages of Th17 cell-mediated EAE

CD45.1 congenic donor Th17-polarized MOG-specific T cells were adoptively transferred to CD45.2 SPF mice via i.p. injection (1×10$^6$ CD4$^+$ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to bacteria via i.n. administration of 20 μl PBS containing 2×10$^6$ CFU *V. parvula*, 2×10$^6$ CFU *M. catarrhalis*, 1×10$^6$ CFU *K. pneumoniae* or PBS alone. During the pre-clinical stage of EAE (6 d post-transfer) lungs were harvested and digested using Collagenase D and DNAse1. Cells were stimulated with PMA and ionomycin with brefeldin A for 4 h, stained for surface CD3, CD4 and CD45.1 and intracellular IL-17A, IFNγ and GM-CSF and analysed by flow cytometry. Results are mean frequency of donor (A) and host (B) IL-17A$^+$, IFNγ$^+$, GM-CSF$^+$ and IFNγ$^+$GM-CSF$^+$ DP CD4$^+$ T cells in the lungs +/- SEM (n = 6-25 mice/group from 2 - 5 independent experiments). Statistical analysis performed by Mann–Whitney U Test, *P<0.05 vs. PBS administered controls. Representative FACS plots for one control mouse, one *V. parvula*-exposed mouse, *M. catarrhalis*-exposed mouse and one *K. pneumoniae*-exposed mouse (C).
Figure 4.19 Increased accumulation of GM-CSF+ and IFNγ+GM-CSF+ donor CD4 T cells in the lungs of mice exposed to *M. catarrhalis* in the preclinical stages of Th17 cell-mediated EAE

CD45.1 congenic donor Th17-polarized MOG-specific T cells were adoptively transferred to CD45.2 SPF mice via i.p. injection (1X10^6 CD4+ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to bacteria via i.n. administration of 20 μl PBS containing 2x10^6 CFU *V. parvula*, 2x10^6 CFU *M. catarrhalis*, 1x10^6 CFU *K. pneumoniae* or PBS alone. During the pre-clinical stage of EAE (6 d post-transfer) lungs were harvested and digested using Collagenase D and DNAse1. Cells were stimulated with PMA and ionomycin with brefeldin A for 4 h, stained for surface CD3, CD4 and CD45.1 and intracellular IL-17A, IFNγ and GM-CSF and analysed by flow cytometry. Results are total number of donor (A) and host (B) IL-17A+, IFNγ+, GM-CSF+ and IFNγ+GM-CSF+ DP CD4+ T cells in the lungs +/- SEM (n = 6-25 mice/group from 2 - 5 independent experiments). Statistical analysis performed by Mann–Whitney U Test, * P<0.05, ** P<0.01 vs. PBS administered controls.
Figure 4.20 No change in cytokine expression by CD4 T cells in the spleen of mice exposed to bacteria in the preclinical stages of Th17 cell-mediated EAE

CD45.1 congenic donor Th17-polarized MOG-specific T cells were adoptively transferred to CD45.2 SPF mice via i.p. injection (1X10^6 CD4^+ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to bacteria via i.n. administration of 20 μl PBS containing 2x10^6 CFU *V. parvula*, 2x10^6 CFU *M. catarrhalis*, 1x10^6 CFU *K. pneumoniae* or PBS alone. During the pre-clinical stage of EAE (6 d post-transfer) spleens were harvested and single cell suspensions prepared. Cells were stimulated with PMA and ionomycin with brefeldin A for 4 h, stained for surface CD3, CD4 and CD45.1 and intracellular IL-17A, IFNγ and GM-CSF and analysed by flow cytometry. Results are mean frequency of donor (A) and host (B) IL-17A^+, IFNγ^+, GM-CSF^+ and IFNγ^+GM-CSF^+ DP CD4^+ T cells in the spleen +/- SEM (n = 6-19 mice/group from 2 - 5 independent experiments).
Figure 4.21 Elevated CXCL16 expression in the lungs of mice exposed to *K. pneumoniae* and *V. parvula* in the preclinical stages of Th17 cell-mediated EAE

CD45.1 congenic donor Th17-polarized MOG-specific T cells were adoptively transferred into CD45.2 SPF mice via i.p. injection (1X10⁶ CD4⁺ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to bacteria via i.n. administration of 20 μl PBS containing 2x10⁶ CFU *V. parvula*, 2x10⁶ CFU *M. catarrhalis*, 1x10⁶ CFU *K. pneumoniae* or PBS alone. During the pre-clinical stage of EAE (6 d post-T cell transfer), RNA was extracted from the lungs and gene expression for (A) Ccl20, (B) Ccl2, (C) Cxcl16, (D) Cxcl2 (E) Cxcl9 and (D) Cxcl10 assessed by RT-PCR. mRNA values are expressed as mean fold change ± SEM, compared to PBS-administered controls, after normalising to a housekeeping control gene (18S rRNA)(n=5-9/group from 2-3 independent experiments). Statistical analysis performed by Mann–Whitney U Test.
Figure 4.22 Elevated frequency of CCR6\(^+\) donor CD4 T cells in the lungs of mice exposed to *K. pneumoniae* in the preclinical stages of Th17 cell-mediated EAE

CD45.1 congenic donor Th17-polarized MOG-specific T cells were adoptively transferred into CD45.2 SPF mice via i.p. injection (1X10\(^6\) CD4\(^+\) T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to bacteria via i.n. administration of 20 \(\mu\)l PBS containing 2x10\(^5\) CFU *V. parvula*, 2x10\(^6\) CFU *M. catarrhalis*, 1x10\(^6\) CFU *K. pneumoniae* or PBS alone. During the pre-clinical stage of EAE (6 d post-T cell transfer) lungs were harvested and digested using Collagenase D and DNAse1. Cells were stained for surface CD3, CD4, CD45.1, CCR6, CXCR3 and CXCR6 and analysed by flow cytometry. Results are mean frequency of donor (A) and host (B) CCR6\(^+\), CXCR3\(^+\) and CXCR6\(^+\) CD4\(^+\) T cells in the lungs +/- SEM (n = 6-19 mice/group from 5 independent experiments). Statistical analysis performed by Students *T* Test, ** *P*<0.01 vs. PBS administered controls. Representative FACS plots for one control mouse, one *V. parvula*-exposed mouse, one *M. catarrhalis*-exposed mouse and one *K. pneumoniae*-exposed mouse (C).
Figure 4.23 No change in the frequency of CCR6+, CXCR3+, CXCR6+ donor CD4 T cells in the spleens of mice exposed to RT bacteria in the preclinical stages of Th17 cell-mediated EAE

CD45.1 congenic donor Th17-polarized MOG-specific T cells were adoptively transferred to CD45.2 SPF mice via i.p. injection (1X10^6 CD4+ T cells/mouse). 2 d after transfer, groups of T cell recipient mice were exposed to bacteria via i.n. administration of 20 μl PBS containing 2x10^6 CFU V. parvula, 2x10^6 CFU M. catarrhalis, 1x10^6 CFU K. pneumoniae or PBS alone. During the pre-clinical stage of EAE (6 d post-T cell transfer) spleens were harvested and single cell suspensions prepared. Cells were stained for surface CD3, CD4, CD45.1, CCR6, CXCR3 and CXCR6 and analysed by flow cytometry. Results are mean frequency of donor (A) and host (B) CCR6+, CXCR3+ and CXCR6+ CD4+ T cells in the spleen +/- SEM (n = 6-19 mice/group from 5 independent experiments). ** P<0.01 vs. PBS administered controls. Representative FACS plots for one control mouse, one V. parvula-exposed mouse, one M. catarrhalis-exposed mouse and one K. pneumoniae-exposed mouse (C).
4.2.7 Human RT bacteria-stimulated BMDC promote upregulation of pathogenic cytokines in myelin-specific Th17 cells

We have identified bacteria that are capable of promoting expression of the pathogenic cytokines GM-CSF and IFNγ by CD4+ T cells in the lungs and CNS of mice with EAE and exacerbating disease severity. Next, we wanted to investigate whether these specific bacteria mediate this conversion of Th17 cells to a pathogenic phenotype via stimulation of DC, which we have found to respond to RT bacteria by secreting the crucial pathogenic cytokine IL-23 (Figure 3.12). To do this, we cultured myelin-specific Th17 cells and, after enriching for CD4+ T cells, rested them for 72 h (Figure 4.24). After resting, IL-17A remained the most abundant cytokine detected, with lower levels of IFNγ and GM-CSF also present (Figure 4.24 B, C & E). Over half of all CD4+ T cells expressed the RORγt-dependent chemokine receptor CCR6, whereas expression of the T-bet-associated receptor CXCR3 and the IL-23-driven receptor CXCR6 was much lower.

Rested myelin-specific Th17 cells were then cultured with BMDC that had been stimulated with the Proteobacteria species *M. catarrhalis* or *K. pneumoniae* for 1 h before treatment with gentamicin to prevent any bacterial overgrowth (Section 2.4.1) at a ratio of 1 DC: 1 T cell. At 24 h, cytokine expression was measured in cell culture supernatants and intracellular cytokine expression measured by flow cytometry (Section 2.9). We found that co-culture of myelin-specific Th17 cells with BMDC exposed to either *M. catarrhalis* or *K. pneumoniae* resulted in significantly elevated IL-17A, IFNγ and GM-CSF secretion, compared to Th17 cells cultured with unstimulated BMDC or rested Th17 cells alone (Figure 4.25). Using flow cytometry, we also found significantly more IL-17A+ and IFNγ+ CD4 T cells amongst total CD4+ T cells in cultures with bacteria-stimulated BMDC, compared to Th17 cells cultured with unstimulated BMDC or rested Th17 cells alone (Figure 4.26).

We next questioned if factors secreted by BMDC when exposed to selected RT bacteria are sufficient to promote IL-17A, IFNγ and GM-CSF secretion by myelin-specific T cells or if cell-cell contact was required. To assess this, we cultured rested myelin-specific specific Th17 cells with conditioned media collected from BMDC that had been exposed to *M. catarrhalis* or *K. pneumoniae*, in the presence of αCD3 and αCD28 stimulating antibodies. After 24 h culture, we detected an increase in the frequency of IL-17A+ , IFNγ+ , 17A+IFNγ+ DP and GM-CSF+IFNγ+
DP T cells amongst CD4$^+$ T cells cultured in the presence of conditioned media from *M. catarrhalis* and *K. pneumoniae* BMDC by flow cytometry, compared to cytokine expression by Th17 cells cultured with unexposed BMDC (Figure 4.27). This suggests that secreted factors contribute to the conversion of *in vitro* polarised Th17 cells to this “ex-Th17” cell phenotype.

To assess if the observed increases in IL-17A, IFN$\gamma$ and GM-CSF cytokine secretion were a result of stimulation by IL-23 secreted by DCs in response to bacterial exposure, we blocked IL-23 using a monoclonal antibody and compared cytokine secretion to that of cells treated with an isotype control. After 24 h culture with *K. pneumoniae*-stimulated BMDC, Th17 cells treated with $\alpha$IL-23 mAb produced slightly less GM-CSF compared to the same Th17 cells treated with the isotype control (Figure 4.28 C). No changes in IL-17A or IFN$\gamma$ secretion were detected (Figure 4.28). This data supports the concept that RT bacteria can stimulate DC to produce IL-23 that promotes expression of pathogenic molecules in differentiated myelin-specific Th17 cells.
Figure 4.24 In vitro polarized MOG-specific Th17 cells predominantly express IL-17A and CCR6

SPF mice were immunized with MOG<sub>35-55</sub> emulsified in CFA. 10-14 d later, single cell suspensions of auxiliary, brachial, inguinal LN and spleen cells were cultured with MOG<sub>35-55</sub> in the presence of IL-1α, IL-23, anti-IFNγ and anti-IL-4. 72 h later, cells were harvested, enriched for CD4<sup>+</sup> T cells and rested @ 4X10<sup>6</sup> cells/ml. 72 h later, cells were harvested for use in co-culture assays. An aliquot of cells was re stimulated with PMA and ionomycin with brefeldin A for 4 h, stained for surface CD4, CCR6, CXCR3, CXCR6 and intracellular IL-17A, IFNγ and GM-CSF and analysed by flow cytometry. Cytokine concentration in cell culture supernatant was quantified by ELISA. Results are representative FACS plots from one experiment (A) and (C). Average cytokine concentration in culture supernatants (B) and mean frequency of cytokine (D) and chemokine expression (E) by CD4<sup>+</sup> T cells across all experiments (n=9 independent experiments).
Figure 4.25 Enhanced IL-17A, IFNγ and GM-CSF secretion by Th17 cells following co-culture with *M. catarrhalis* or *K. pneumoniae*-stimulated BMDC

BMDC were seeded in triplicate at 2 X 10^5 cells/well in a 96 well plate and rested for 3 h before exposure to *M. catarrhalis* and *K. pneumoniae* at MOI 100. Cells were treated with gentamicin (100 μg/ml) after 1 h and re-cultured with media containing gentamicin as well as 2 X 10^5 rested CD4+ T cells/well. After 24 h, supernatants were collected and the concentration of IL-17A (A), IFNγ (B), and GM-CSF (C) quantified by ELISA. Results are expressed as mean ± SEM (n=8/group from 8 independent experiments). Statistical analysis performed by Mann–Whitney U Test (A & B) or Students T Test (C) * P<0.05, ** P<0.01, *** P<0.001 vs. controls.
Figure 4.26 Increased frequency of IL-17A⁺ and IFNγ⁺ CD4 T cells following co-culture with *M. catarrhalis* or *K. pneumoniae*-stimulated BMDC

BMDC were seeded in triplicate at 2 X 10⁵ cells/well in a 96 well plate and rested for 3 h before exposure to *M. catarrhalis* and *K. pneumoniae* at MOI 100. Cells were treated with gentamicin (100 μg/ml) after 1 h and re-cultured with media containing gentamicin as well as 2 X 10⁵ rested CD4⁺ T cells/well. After 24 h, cells were collected and cytokine secretion blocked with Brefeldin A for 4 h before staining for surface CD3, CD4 and intracellular IL-17A, IFNγ and GM-CSF and analysis by flow cytometry. Results are representative FACS plots from one experiment.
Figure 4.27 Increased frequency of IL-17A\(^+\) and IFN\(\gamma\) CD4 T cells following culture with conditioned media from *M. catarrhalis* or *K. pneumoniae*-stimulated BMDC

BMDC were seeded in triplicate at 2 X 10\(^5\) cells/well in a 96 well plate and rested for 3 h before exposure to *M. catarrhalis* and *K. pneumoniae* at MOI 100. Cells were treated with gentamicin (100 μg/ml) after 1 h and recultured with media containing gentamicin for a further 23 h when supernatant was collected. 2 x 10\(^5\) rested Th17-polarised CD4\(^+\) T cells were cultured for 24 h with conditioned supernatants. Brefeldin A was added for the final 4 h and cells stained for surface CD3 and CD4 and for intracellular IL-17A, IFN\(\gamma\) and GM-CSF and analysed by flow cytometry. Results are expressed as fold change in the frequency of IL-17A\(^+\), IFN\(\gamma\)\(^+\), IL-17A\(^+\)IFN\(\gamma\)\(^+\) DP, GM-CSF\(^+\) and IFN\(\gamma\)\(^+\)GM-CSF\(^+\) DP CD4\(^+\) T cells over controls +/- SEM (A) (n = 2/group from 2 independent experiments). Representative FACS plots from one individual experiment (B).
Figure 4.28 *K. pneumoniae*-induced IL-23 from BMDC may contribute to GM-CSF secretion by CD4+ T cells

BMDC were seeded in triplicate at 2 X 10^5 cells/well in a 96 well plate and rested for 3 h before exposure to *M. catarrhalis* and *K. pneumoniae* at MOI 100. Cells were treated with gentamicin (100 µg/ml) after 1 h and re-cultured with media containing gentamicin as well as 2 X 10^5 rested Th17-polarised CD4+ T cells/well. At the time CD4 T cells were added to each well, some wells were treated with a monoclonal antibody against IL-23 (10 µg/ml) or isotype control (rlgG1; 10 µg/ml). After 24 h, supernatants were collected and the concentration of IL-17A (A), IFNγ (B) and GM-CSF (C) quantified by ELISA. Results are mean +/- SEM (n=4/group from 4 independent experiments).
4.3 Discussion

MS is a chronic inflammatory disease of the CNS which is driven by Th17 cells that attack the myelin sheath surrounding nerve axons leading to reduced signal conductance and axonal loss [331]. The etiology of MS is unclear but high rates of discordance amongst monozygotic twins indicate that environmental factors play a crucial role in promoting disease development [106]. Epidemiological studies have long associated relapses in MS patients with systemic infection and, coupled with studies in GF mice demonstrating an important role for the microbiome in EAE development, have strongly implicated microbial factors as important environmental stimuli in promoting CNS autoimmune inflammation [89, 120, 332]. Th17 cells are the main pathogenic effectors in MS and EAE but are innocuous immediately following differentiation [73]. Exposure of recently differentiated Th17 cells to IL-23 is a critical step in their acquisition of pathogenic potential and drives a Th1-like phenotype characterised by the expression of GM-CSF and IFNγ [154, 184]. However, it is unknown where or when Th17 cells are exposed to this pathogenic signal. Recent studies have implicated the RT, which is home to a diverse microbial community, as an important staging site in the preclinical stages of EAE [15, 200]. Here, we have investigated the potential of selected members of the RT microbiota, with an ability to promote IL-23/Th17-type immunity in vitro and in vivo as identified in the previous chapter, to confer pathogenicity in myelin-specific Th17 cells and exacerbate EAE.

Studies in GF mice have been critical in deciphering a role for the microbiota in EAE development. These studies have largely utilized the active induction and spontaneous models of disease to demonstrate that GF mice are less susceptible to EAE [89, 333]. We confirmed that, in our facility, fewer GF mice develop EAE upon active immunization with MOG and CFA, and those that do develop a less severe form of disease compared to conventionally housed SPF mice (Figure 4.3 A). However, the active induction of EAE results in the generation of polyclonal T cell response. In the current study, we questioned how exposure to individual RT bacteria might influence the effector function of Th17 cells. Utilizing the passive transfer of EAE allowed us to generate MOG-specific Th17 cells in vitro and assess their trafficking and encephalitogenicity following transfer to naïve congenic hosts. It is recognised, however, that commensal microbes are required for the normal development of intestinal Th17 cells [334]. To overcome any defect in Th17 cell development in GF mice,
myelin-specific Th17 cells were generated from developmentally normal MOG-primed SPF mice. In agreement with previous data generated by our lab at another facility (Lalor S.J., unpublished data), we found that GF mice are significantly less susceptible to the passive induction of EAE by transfer of MOG-specific Th17 cells than SPF recipients of the same cells (Figure 4.3 B). These findings confirm that microbial colonisation can influence the migration and/or effector function of already differentiated antigen-specific Th17 cells.

Studies describing a role for the microbiota in EAE development have largely focused on the gut microbiota [89, 333]. However, the lungs are also home to a diverse microbial community and have recently been implicated as an important staging site in the preclinical stages of EAE [15, 200]. In the previous chapter, we identified members of the RT microbiota, including *N. cinerea*, *M. catarrhalis* and *K. pneumoniae*, that were capable of inducing IL-23/Th17-type immunity (Figures 3.12 and 3.18). Here, we examined if airway exposure to these RT symbionts could modulate the pathophysiology of Th17 cell-mediated EAE. We found that i.n. exposure to both *M. catarrhalis* (Figure 4.6 A) and *K. pneumoniae* (Figure 4.7 A) resulted in a significant increase in EAE disease severity upon transfer of myelin-specific Th17 cells. Mice exposed to either *M. catarrhalis* (Figure 4.6 A) or *K. pneumoniae* (Figure 4.7 A) developed disease earlier and exhibited more severe clinical signs of EAE than PBS-administered controls. Disease onset typically occurred 5 or 6 d, respectively, following Th17 cell transfer, compared to PBS exposed recipients of the same Th17 cells which typically began to display signs of clinical disease on d 7. Weight loss in EAE generally precedes the onset of clinical disease and is measured daily as an objective measure of disease severity. Th17 cell recipients exposed to either *M. catarrhalis* or *K. pneumoniae* lost significantly more weight than their PBS-administered counterparts (Figures 4.6 & 4.7 B). However, this was not simply a result of the inflammatory response and sickness behaviour caused by the introduction of a bacterial species into the airways since naïve mice administered either *M. catarrhalis* (Figure 4.6 B) or *K. pneumoniae* (Figure 4.7 B) alone did not experience any weight changes. Moreover, exposure of mice that received myelin-specific Th17 cells to *N. cinerea* or *V. parvula* did not affect EAE severity or weight loss, compared to Th17 cell recipients that were administered PBS i.n. (Figures 4.4 & 4.5). This indicates that the observed changes in the course of clinical disease were not a result of general inflammation caused by the introduction of any RT bacteria to the airways.
Having shown in the previous chapter that monocolonisation of mice with *K. pneumoniae* fosters a Th17-oriented immune response in the airways of adult mice, we investigated if a RT bacterium like this could enhance the pathogenicity of myelin-specific Th17 cells in otherwise GF hosts. We found that monocolonisation of mice with *K. pneumoniae* alone was sufficient to enhance, although not significantly, susceptibility of GF mice to EAE and increased the severity of clinical disease compared to GF recipients of the same Th17 cells (Figure 4.8). This was an exciting finding and the first time that a respiratory symbiont was shown to enhance disease severity in otherwise less susceptible GF mice. Previous studies have demonstrated the restoration of EAE disease severity in GF mice upon transfer of the gut colonizing bacterium SFB which induces Th17 cell development in the small intestine [332]. Conversely, administration of *Bacteroides fragilis* after antibiotic depletion of the gut microbiota attenuated EAE by promoting the generation of IL-10-producing Treg cells in the colon [335]. These findings support the concept exposure to specific microbial species can modulate EAE severity by altering the T cell landscape. We demonstrated in Chapter 3 that *K. pneumoniae* stably colonised the RT of previously GF mice (Figure 3.27) and that this was associated with elevated gene expression of the Th17 associated cytokines IL-1β, IL-23p19, IL-17A (Figure 3.28). It may be that elevated IL-23 or other critical pathogenic signals induced by *K. pneumoniae* colonisation might drive the heightened pathogenicity of myelin-specific Th17 cells following transfer and associated increase in EAE susceptibility and severity. Unfortunately, a breach in our GF facility meant that this avenue of research could not be further explored. Had it been possible, neutralising antibodies would have been utilized in this model to explore the effect of IL-23 and GM-CSF expression on enhanced disease susceptibility.

The pathophysiology of MS and EAE involves the trafficking of myelin-specific CD4 T cells across the BBB and B-CSF-B to orchestrate an inflammatory response against myelin surrounding nerve axons [336]. Consistent with the exacerbated clinical disease observed in mice exposed to *M. catarrhalis* and *K. pneumoniae*, we detected increased accumulation of CD4+ T cells in the brain and spinal cord of *M. catarrhalis*- or *K. pneumoniae*-exposed mice, respectively, compared to PBS-administered controls (Figure 4.9). These results suggest that microbial exposure to *M. catarrhalis* and *K. pneumoniae* enhances CD4+ T cell mobilization and infiltration into the brain and spinal cord. Conversely, Edwards *et al* have demonstrated
that infection with the respiratory pathogen *B. pertussis* delays disease onset and reduces disease severity in EAE in an IL-10-dependent fashion [201]. However, as we reported in Chapter 3, distinct respiratory bacteria promote differential cytokine secretion *in vitro* (Figure 3.12) and *in vivo* in the lungs (Figure 3.18). Together with our finding that neither *N. cinerea* nor *V. parvula* modified disease severity in SPF mice, this data demonstrates that the immune response engendered by individual RT bacteria can differentially modulate Th17 cell effector function during the development of EAE.

The approach taken in this study allowed for the assessment of the influence of individual bacterial strains on Th17 cell pathogenicity in EAE and represents an important step as we begin to move away from correlative microbiome studies towards understanding the role played by individual members of the microbiota in various disease settings. However, complete microbial communities may exert an influence that individual organisms cannot on their own. For example, colonisation of GF mice with a complex microbiota is sufficient to reverse the hyper-IgE syndrome seen in these mice whereas monocolonisation with a variety of microorganisms has been shown to be insufficient to restore this phenotype [337]. Future studies should therefore define the impact of the introduction of the bacterial strains used in this study in concert with each other as well as how they impact the microbes already present in the lungs and address how these members of the RT microbiota might influence EAE pathogenicity. Furthermore, investigations on how the introduction of the lung microbiota from MS patients influences EAE susceptibility when compared to that of a healthy control may provide further insights into the role of the RT microbiota in disease development and could be achieved though the transfer of BAL fluid or sputum from MS patients and healthy controls to mice upon the induction of EAE.

Th17 cells are the critical pathogenic effector cells in MS and EAE. Th17 cells differentiate in the presence of IL-1β, IL-6 and TGFβ following recognition of their cognate antigen. However, Th17 cells are innocuous immediately following differentiation and must be exposed to IL-23 to acquire their pathogenic potential [154, 157]. *In vivo* Th17 cells display a high level of context dependent plasticity, exposure to the cytokine IL-23 drives the conversion of Th17 cells to a pathogenic “ex-Th17” cell phenotype. These Th1-like cells are characterised by the production of IFNγ and GM-CSF and the expression of the Th1 associated phenotype T-bet.
In fact, GM-CSF production by IL-23-stimulated ex-Th17 cells is indispensable in the development of EAE [156, 175]. However, Th17 cells only upregulate the IL-23R following differentiation in peripheral lymphoid tissues and there remains a gap in our knowledge of where and when Th17 cells are exposed to this pathogenic signal. Our data in Chapter 3 indicates that DCs are an important source of IL-23 secretion and that bacteria from the phyla Proteobacteria, including *M. catarrhalis* and *K. pneumoniae*, strongly induce IL-23 secretion *in vitro* in BMDC (Figure 3.12) and *in vivo* in the lungs of mice (Figure 3.18). We therefore questioned if the innate immune response stimulated by exposure to these RT symbionts might be enhancing Th17 cell pathogenicity by promoting conversion to an IFNγ and GM-CSF producing “ex-Th17” cell phenotype.

When we analysed cytokine production by CNS infiltrating CD4 T cells at peak disease we detected a significant increase in the accumulation of IL-17A and IFNγ-producing CD4+ T cells in the spinal cords of mice exposed to *K. pneumoniae* and a significant increase in the total number of CD4 T cells producing both IFNγ and GM-CSF in these mice, compared to PBS-administered controls (Figure 4.10 A, B & F). Elevated numbers of GM-CSF-producing CD4+ T cells were also detected in the spinal cord of mice exposed to both *M. catarrhalis* and *K. pneumoniae* (Figure 4.10 C). We then looked specifically at cytokine production by host and donor CD4+ T cells in the CNS at peak disease, we saw that *M. catarrhalis* and *K. pneumoniae* promoted an increase in the frequency of GM-CSF+ and IFNγ+GM-CSF+ DP donor CD4 T cells in the spinal cord (Figure 4.12 A). This data indicates that i.n. exposure to either *M. catarrhalis* or *K. pneumoniae* can promote conversion to an IFNγ and GM-CSF producing “ex-Th17” cell phenotype which may contribute to the exacerbated EAE disease severity observed in these mice (Figures 4.6 and 4.7). Bacterial exposure only enhanced GM-CSF and IFNγ expression in the donor CD4+ T cell population indicating that already primed CD4+ T cells were vulnerable to restimulation in this manner. Interestingly, the frequency of IL-17A+ donor and host CD4+ T cells was comparable in both the spinal cord and brain, indicating that the host CD4+ T cells recruited to the CNS following transfer of Th17 cells are also predominantly of a Th17 cell lineage (Figures 4.12 & 4.13). Unlike GM-CSF expression or cells producing both IFNγ and GM-CSF, however, IL-17A and IFNγ expression by CNS-infiltrating CD4+ T cells did not appear to be regulated by bacterial exposure (Figures 4.12 & 4.13).
A previous report by Odoardi and colleagues demonstrated that pathogenic T cells traffic through the lungs prior to entering the CNS and that the gene expression profile of activated autoreactive T cells is altered during their migration through the lungs to the CNS [200]. Data from our own lab indicated that upon the adoptive transfer, CD4+ T cells accumulate in the lungs prior to entering the CNS, peaking 6 d post-transfer (Lalor, S.J., unpublished data). At this time-point, we also consistently detected a large population of donor CD4+ T cells in the lungs (Figure 4.17). It is possible, therefore, that airway exposure of Th17 cell-recipient mice to *M. catarrhalis* or *K. pneumoniae* was promoting phenotypic changes in these transferred cells as they trafficked through the lungs. 6 d after adoptive transfer of myelin-specific Th17 cells, we detected increased accumulation of GM-CSF+ and IFNγ+GM-CSF+ DP donor CD4+ T cells in the lungs of mice exposed to *M. catarrhalis* or *K. pneumoniae*, compared to PBS-administered controls (Figure 4.18 A and 4.19 A). Exposure of mice that had received the same myelin-specific Th17 cells to *V. parvula* had no effect on the frequency of GM-CSF+ or IFNγ+GM-CSF+ donor CD4+ T cells in the lungs (Figure 4.18 A). This was an interesting finding since airway exposure of mice to *V. parvula* also had no impact on EAE disease severity (Figure 4.4) compared to the significantly exacerbated clinical disease seen in Th17 cell recipients exposed to *M. catarrhalis* and *K. pneumoniae* (Figures 4.6 and 4.7). This data suggests that RT bacteria-induced conversion to a GM-CSF-expressing ex-Th17 cell phenotype may promote enhanced disease severity in EAE.

In a similar pattern to that seen in the brain and spinal cord, host CD4+ T cells produced very little cytokine in the lungs, and this was unaffected by exposure to any bacteria tested (Figure 4.18 B). This suggests that only activated, antigen-specific T cells are modulated by the local immune response to RT bacteria.

Here, we employed the AT model of EAE as it allowed us to polarize antigen specific Th17 cells *in vitro* and to assess how exposure of Th17 cell recipients to individual bacterial species modulates their effector function. The use of congenic donor CD4+ T cells allowed for the accumulation of donor cells in both the lungs and CNS at the preclinical and peak stages of disease to be studied. This approach does not allow for the trafficking of the same Th17 cells from the lungs to the CNS to be monitored and it therefore cannot be categorically stated that the same Th17 cell traffics to the lung before appearing in the CNS. However, our
previous study showed that after transfer the accumulation of cells in the lungs precedes accumulation in the CNS and the increase of donor Th17 cells in the CNS coincides with the decrease of cells in the lung (Lalor, S.J. Unpublished data). Furthermore, Odoardi and colleagues demonstrated that effector T cells transferred directly into the lung migrated along the airways and maintained their capacity to traffic out of the lung and initiate clinical disease in the CNS [200]. Taken together these studies indicate that cells traffic from the lung to the CNS. Perhaps with the advent of new technologies it will be possible to definitively determine the transit of the same polarised Th17 cells through the course of disease. For example, numerous studies have labelled immune cells in the colon, mesenteric LNs and skin and followed their trafficking [338, 339]. Advances in these technologies may make up for the challenge posed by the anatomical location of the lungs which renders them difficult to access using strategies such as the in vivo photoconversion demonstrated by Hiltensperger and colleagues where CD4+ T cells in the iLN expressing a photoconvertible protein were irradiated directly through in the skin and could be subsequently identified in the CNS during EAE. In the context of this study, the labelling of CD4+ T cells in the lungs of mice exposed or unexposed to bacteria would significantly aid our understanding of the mechanisms modulating pathogenicity at this site and their subsequent trafficking to the CNS.

Several studies have now described how the lung microenvironment can modulate T cell trafficking to the CNS [78, 185, 322]. Subclinical pulmonary inflammation was found to stall CD4+ T cells in the lungs in a CCR6-CCL20 dependent fashion, while airway infection with B. pertussis led to reduced integrin a4 and LFA-1 expression on myelin-specific Th17 cells and delayed migration to the CNS [201, 203]. In the current study, exposure of Th17 cell recipient mice to any RT bacteria had no impact on the accumulation of donor CD4+ T cells in the lungs (Figure 4.17). This indicates that the increased disease severity observed in mice exposed to certain RT bacteria is not due to enhanced recruitment of myelin-specific Th17 to the lungs in the preclinical stages of EAE. Nevertheless, mice administered K. pneumoniae displayed significantly increased CCR6 expression by donor CD4+ T cells in the lungs 6 d following transfer, compared to PBS-administered controls (Figure 4.22). A similar, but non-significant, increase in CCR6 expression by donor Th17 cells in the lungs was also detected in response to M. catarrhalis airway exposure. At peak disease, we also found a significant increase in CCR6 expression by donor CD4+ T cells in the brain of mice exposed to M. catarrhalis, compared to
PBS-administered controls (Figure 4.16). It should be noted, however, that the expression of CCR6 is lower on donor CD4\(^+\) T cells in the CNS at peak disease than on donor cells in the lungs prior to the onset of clinical signs of disease (Figures 4.16 & 4.22). This suggests that CCR6 expression is downregulated on pathogenic T cells would be consistent with an “ex-Th17” cell phenotype [152].

Another homing molecule linked to pathogenicity of Th17 cells is CXCR6 [184]. CD4\(^+\) T cells expressing CXCR6 and producing GM-CSF and IFN\(\gamma\) are enriched in the synovial fluid of patients with arthritis further implicating CXCR6 as a marker of pathogenicity and Hou et al have also demonstrated a requirement for Th17-cell expression of this molecule for the development of EAE [185, 186]. Here, we found that exposure to both \textit{M. catarrhalis} and \textit{K. pneumoniae} led to a significant increase in CXCR6 expression by CD4\(^+\) T cells in the spinal cord compared to PBS-administered controls (Figure 4.14 A). Specifically, donor CD4 T cells that reached the brain and spinal cord were almost entirely CXCR6\(^+\) (Figure 4.16), while only 20% of transferred CD4\(^+\) T cells expressed this chemokine (Figure 4.2). This led us to assess CXCR6 expression in the lungs 6 d following T cell transfer. Again, we found that almost all donor T cells in the lungs at this stage of EAE development expressed CXCR6 (Figure 4.22), coincident with increased expression of the ligand CXCL16 in the lungs of mice exposed to \textit{K. pneumoniae}, \textit{M. catarrhalis} and \textit{V. parvula} (Figure 4.21). CXCL16 is constitutively expressed by bronchial epithelial cells and it is interesting to consider whether CXCR6 was upregulated locally at this site or if it is the case that only donor Th17 cells that express CXCR6 traffic to the lungs in response to CXCL16 expression and subsequently on to the CNS [340]. Future studies investigating the regulation of CXCR6 expression on encephalitogenic Th17 cells in the airways may provide some insight to this question. Nevertheless, our findings are similar to those of Hou and colleagues in that expression of CXCR6 is associated with encephalitogenicity in Th17 cells, and we show that this expression may be regulated in the airways by human RT bacteria [185].

We did not detect any change in expression of the Th1-associated chemokine receptor CXCR3 by donor or host CD4\(^+\) T cells in the lungs or CNS during the course of EAE (Figures 4.14 & 4.22). Neither did we detect any changes in expression of the ligands CXCL9 and CXCL10 in the lungs at the preclinical stage of disease (Figure 4.21). This indicates that RT bacteria-
mediated conversion of myelin-specific Th17 cells to a pathogenic effector phenotype does not involve upregulation of these trafficking molecules and supports previous findings by our lab that CXCR3 expression by encephalitogenic Th17 or Th1 cells is dispensable for the development of EAE [324].

In order to identify other potential mechanisms by which stimulation by *M. catarrhalis* or *K. pneumoniae* might be promoting Th17 cell pathogenicity, we measured a number of molecules that have previously been associated with encephalitogenicity. Downregulation of the interaction protein for cytohesin exchange factors (IPCEF), a scaffolding protein implicated in cell migration, has been described on encephalitogenic Th17 cells upon TLR2 activation [276, 341]. SerpinB1 expression is required for the survival and expansion of encephalitogenic Th17 cells [185]. And Ninjurin 1 has been reported to be involved in cell migration and entry into the CNS, with reduced EAE severity upon neutralization [200, 342]. We questioned if exposure of murine lungs to these human RT bacteria might promote changes in the expression of these Th17 cell pathogenicity-associated genes in the preclinical stages of EAE. However, we didn’t detect any change in IPCEF, SerpinB1 or Ninjurin 1 mRNA expression in the lungs of RT bacteria-exposed recipients of myelin-specific Th17 cells, compared to PBS-administered controls (data not shown). It may have been possible to detect subtle changes in the expression of these genes had it been feasible to FACS-sort donor Th17 cells from the lungs and CNS, or if we had investigated expression of these molecules at earlier time-points. Future investigations may pursue this line of enquiry.

Taken together, our results indicate that exposure to *M. catarrhalis* and *K. pneumoniae*, but not *V. parvula*, might enhance pathogenicity in Th17 cells by promoting conversion to a GM-CSF⁺, IFNγ⁺GM-CSF⁺ DP “ex-Th17” cell phenotype in the lungs during the pre-clinical stages of EAE. To test whether DC stimulation by these RT bugs can directly promote Th17 cell conversion to this pathogenic phenotype, we established a co-culture assay system whereby myelin-specific Th17 cells were generated *in vitro* (Section 2.5) before resting these cells and subsequently re-stimulating them with BMDC that had been exposed to *M. catarrhalis* or *K. pneumoniae*, or BMDC alone (Figure 4.24). We found that co-culture of myelin-specific Th17 cells with bacteria-stimulated BMDC led to a significant increase in IL-17A, IFNγ and GM-CSF secretion into culture supernatants (Figure 4.25). Moreover, when we cultured myelin-
specific Th17 cells in conditioned media alone, recovered from cultures of BMDC exposed to either *M. catarrhalis* or *K. pneumoniae*, we also saw increased frequency of IL-17A- and IFNγ-expressing CD4 T cells, and an increase in the frequency of IFNγ⁺GM-CSF⁺ DP CD4⁺ T cells (Figure 4.27). This suggests that factors secreted by BMDC in response to exposure to *M. catarrhalis* and *K. pneumoniae* might be sufficient to promote the conversion to an “ex-Th17” cell phenotype.

It has previously been demonstrated that IL-23 signalling promotes GM-CSF upregulation in pathogenic Th17 cells [156]. In Chapter 3, we described the potent induction of IL-23 secretion by BMDC in response to stimulation with *M. catarrhalis* and *K. pneumoniae* (Figure 3.12). We therefore questioned if this IL-23 secretion in response to *K. pneumoniae* and *M. catarrhalis* might be a critical factor promoting the enhanced GM-CSF and IFNγ secretion we have observed in vitro and in vivo. We found that neutralisation of IL-23 did not impact the strong IL-17 and IFNγ response by Th17 cells upon re-culture with bacteria-exposed BMDC (Figures 4.28 A & B).

Our preliminary data suggests that blocking IL-23 marginally reduced GM-CSF expression by myelin-specific Th17 cells co-cultured with BMDC that had been stimulated with *K. pneumoniae*. Further experimentation is required to support this finding but given our current understanding that IL-23 signalling is required for the conversion of Th17 cells to an “ex-Th17 cell” phenotype it could be the postulated that *K. pneumoniae*-induced GM-CSF expression by CD4⁺ T cells is partially dependent on IL-23 signalling.

Unfortunately, this experimental setup had a number of limitations. We were unable to determine the efficiency of the blocking antibodies used in this assay. In addition, the high MOI used to stimulate the BMDC in this assay may have been too strong to achieve full IL-23 neutralization in this assay system. To be consistent with other in vitro assays employed throughout this project, BMDC were exposed to each bacterium at MOI 100. To address these limitations, future studies might utilize BMDC from IL-23⁻/⁻ mice or myelin-specific CD4 T cells lacking the IL-23R to investigate the true role of IL-23 in the conversion of pathogenic Th17 cells following co-culture with *M. catarrhalis* or *K. pneumoniae*-stimulated BMDC.
In order to determine the absolute requirement for bacterial induced IL-23 in promoting conversion to an “ex-Th17” cell phenotype in both this *in vitro* culture system as well as in the exacerbation of Th17 mediated EAE *in vivo*, future studies should employ Th17 cell culture conditions that do not require the *ex-vivo* stimulation of cells with IL-23 prior to transfer. This approach may present several challenges given the crucial requirement of IL-23 for the induction of EAE but could be addressed by using donor cells from mice expressing a myelin autoantigen-specific T cell receptor. T cells from these transgenic 2D2 mice induce EAE in wild type SJL/J mice upon transfer and do not require *in vitro* stimulation with IL-23 [89].

Additionally, restoration of Th17 cell plasticity upon the addition of recombinant IL-23 to culture systems in which IL-23 has been depleted could help to confirm the role of this signalling pathway in the conversion of Th17 cells to a pathogenic ex-Th17 state. If ex-Th17 cell conversion could be induced in Th17 cells co-cultured with bacteria exposed DCs, these cells could be transferred to GF mice to determine if this can restore the defect in EAE susceptibility in GF mice in the absence of bacterial colonisation. In order to further investigate the critical role of respiratory tract bacteria-induced IL-23 in the acquisition of encephalitogenic properties by Th17 cells in vivo, mice deficient in IL-23p19 could be rederived into GF isolators, colonized with the specific strains of bacteria that promote conversion of Th17 cells to encephalitogenic ex-Th17 cells, T cell plasticity and clinical signs of EAE in monocolonized offspring monitored.

Taken together, it appears that *M. catarrhalis* and *K. pneumoniae* can promote pathogenicity in myelin-specific Th17 cells by driving their conversion to an “ex-Th17” cell phenotype. Given the increased accumulation of GM-CSF-producing donor T cells in the both the lung and CNS after exposure to *M. catarrhalis*, future studies might investigate the proliferation of CD4 T cells in the lungs of mice exposed to this bacterium. In a recent study, BrdU staining was employed to demonstrate that the homeostatic proliferation of tissue resident memory (Trm) CD4+ T cell in the lungs following immunization with HK *K. pneumoniae* was important for the maintenance of this T cell population, which protected against subsequent i.n challenge with *K. pneumoniae* [343]. Similarly, a study assessing the impact of viral infection on EAE development showed that DCs isolated from the lungs of mice that had recovered from influenza A infection displayed greater capacity to induce CD4 T cell proliferation *ex vivo*; a finding that correlated with increased EAE severity in convalescent mice [328]. Interestingly,
A recent report describing the attenuation of EAE severity using i.t. delivery of nanoparticles loaded with the myelin component proteolipid protein (PLP) demonstrated that therapeutic efficacy may be associated with suppression of autoreactive CD4 T cell proliferation in the lungs [344]. These studies highlight the need for further investigation on the influence of RT bacteria on pathogenic Th17 cell proliferation in the airways.

Additionally, it will be important to assess how bacterial exposure might modulate other immune cell subsets in the lungs and how these could contribute to the acquisition of a pathogenic phenotype by myelin-specific Th17 cells. For example, in Chapter 3 we found that *K. pneumoniae* promoted IL-17A production by γδ T cells in the lungs, an observation that has been well documented in the literature (Figure 3.21)[345]. It has also been demonstrated, however, that IL-17 secretion by γδ T cells amplifies IL-17 production by CD4+ Th17 cells during the development of EAE [346]. In the context of the current study, it could be postulated that IL-17A production by γδ T cells upon *K. pneumoniae* exposure could enhance IL-17 and other cytokine expression by Th17 cells as they transiently reside in the lungs. From another perspective, accumulation of granulocytic MDSCs in the lungs of mice during EAE has been reported to coincide with the trafficking of myelin-reactive Th17 cells through the lung and to enhance their pathogenicity in an IL-6-dependent fashion [202]. Hence, a full assessment of the myeloid and lymphoid cells in the lungs of Th17 cell recipient mice exposed to various RT bacteria might provide novel insights into how pathogenicity is regulated at this site.

Finally, PRR engagement, particularly TLR signalling, has been implicated as a pivotal player in the pathogenesis of numerous chronic inflammatory diseases, including MS and EAE [347]. TLR4 recognises the Gram-negative bacterial cell wall component LPS and is perhaps the most well described TLR, while TLR2 dimerisation with TLR1 or TLR6 can also recognise Gram-negative and Gram-positive bacterial PAMPs. Both *M. catarrhalis* and *K. pneumoniae* are Gram-negative bacteria and express LPS on their surface [274, 348]. TLR2 and TLR4-signalling are important in the host response against these bacteria, which have been shown to upregulate expression of both PRRs on human lung epithelial cells [274, 349, 350]. These same TLRs are also expressed directly on Th17 cells and it has been reported that TLR2 and TLR4 signalling promotes Th17 cell pathogenicity in EAE. Th17-polarised *Tlr4−/−* CD4 T cells produce less IL-17A, IFNγ and GM-CSF and are less pathogenic than WT Th17 cells when
transferred to $\text{Rag1}^{-/-} \text{ hosts}$ [351]. It has also been reported that TLR4-induced activation of DC promotes T cell pathogenicity in EAE [352]. These findings support a role for TLR4 signalling in the acquisition of pathogenic potential by myelin-specific Th17 cells in the lungs of $K. \text{pneumoniae}$-exposed mice in the current study. On the other hand, TLR2-deficiency was reported to substantially impair Th17 cell pathogenicity in EAE [353]. In fact, TLR2 stimulation of MOG-specific Th17 cells was found to be sufficient to induce pathogenicity in Th17 cells and to promote the development of severe EAE [276]. Our findings support the concept that the lungs are an important site in the preclinical stages of EAE and that factors encountered in the airways can promote pathogenicity in Th17 cells. The above reports suggest a role for TLR signalling in EAE pathogenesis and, in the context of the current study, might implicate TLR engagement by $M. \text{catarrhalis}$ and $K. \text{pneumoniae}$ in the airways as an important factor in promoting Th17 cell encephalitogenicity and the exacerbation of CNS autoimmunity. Ongoing studies in our lab should begin to elucidate some of these pathways.
Chapter 5
General Discussion
5. Discussion

It is currently estimated that the human body harbors 1-2 bacteria for every host cell [354]. These microbes colonise all mucosal surfaces and the skin and contain far more genetic material than our own human genome [355]. Advanced sequencing technologies have propelled our understanding of the impact of this diverse microbial community on human health and it is now clear that the microbiota can influence a myriad of complex diseases. Most of these advances have centered around the bacteria that colonise the GIT. Other mucosal sites too are colonized by commensal microorganisms that play critical roles in homeostatic functioning and defence against pathobionts. Once thought a sterile microenvironment, recent advances using culture-independent technologies have identified several bacterial species in healthy human lungs and growing evidence indicates that the upper and lower airways may be an important site for microbial regulation of immune responses. In healthy individuals, the RT microbiota is low in biomass but high in diversity and is dominated by *Prevotella* and *Veillonella* species [7, 15, 21, 23]. Reduced bacterial diversity is a common feature during exacerbation of chronic lung diseases such as asthma, CF, IPF and COPD, and often correlates with increased burden of discrete Proteobacteria species. Furthermore, it appears that inflammation at distinct sites along the respiratory tract can modulate extra-pulmonary diseases including RA [8, 11, 13, 227, 356-359]. However, our knowledge of the mechanisms by which individual bacteria that commonly colonise the human RT can regulate inflammatory responses remains sparse. In the current study, we add to the growing appreciation of the immunogenicity of the RT microbiota and how its individual members modulate chronic inflammatory disease.

The significant role played by the microbiota in the pathogenesis of chronic inflammatory disorders is illustrated by the reduced susceptibility of GF mice in several preclinical models of disease [89-92]. One such disease is EAE, a preclinical model of MS which is a chronic, progressive inflammatory disease of the central nervous system (CNS), the etiology of which is not fully understood [95]. However, with a discordance rate of up to 70% amongst monozygotic twins, it is clear that environmental factors play an important role in the pathophysiology of MS [106]. Epidemiological studies too have long associated relapses in MS patients with systemic infection, supporting the theory that microbial factors may provide some of these pathogenic environmental signals [120]. GF mice are more resistant to actively
induced EAE following immunization with myelin peptides, and to a spontaneous model of disease in which mice express a myelin autoantigen-specific T cell receptor [89].

Studies implicating the microbiome in EAE development have largely focused on colonization of the GIT and some have directly implicated gut bacteria in EAE, SFB, for example is a gut colonizing mouse restricted symbiont which has been shown to promote Th17 cell pathogenicity in EAE. It was demonstrated that SFB exerts its effect by inducing IL-17 producing CD4+ T cells in both the gut and in the CNS, supporting the concept that microbes at mucosal surfaces distant from the CNS can promote CD4+ T cell pathogenicity at this site while also raising questions on the translational relevance of this study given that SFB does not colonise the human GIT [332]. Other studies implicating the microbiota in EAE development have failed to establish whether there are differential contributions from gut and lung microbes. Berer and colleagues for example employed a co-housing strategy to recolonize GF mice with a commensal microbiota thus rendering the mice susceptible to EAE development, in this study the relative effects of gut and lung microbes on neuroinflammation require clarification. [89]. However, although the gut-lung axis received much research attention at the initiation of lung microbiome studies it is now considered that the RT microbiota exists as an entity in itself and is more greatly influenced by bacteria colonizing the oropharynx than the GIT [29]. It must therefore be considered that the influence of the RT microbiota on EAE and MS development can be studied independently to the GIT microbiota.

Although important in the maintenance of homeostasis at mucosal sites, Th17 cells have been identified as the critical pathogenic effector cells in MS and EAE. Th17 cells display high levels of context-dependent plasticity and the IL-23-dependent conversion to a pathogenic “ex-Th17“ cell phenotype appears to be a critical step in their acquisition of pathogenic potential [152, 154, 157, 360]. This conversion is signified by the downregulation of the Th17 cell master transcription factor RORγt, the prototypical cytokine IL-17A, and the Th17-associated chemokine receptor CCR6, concomitant with the upregulation of Th1 cell-associated molecules including Tbet and IFNγ [152]. These converted Th17 cells also upregulate the key pathogenic molecules GM-CSF and CXCR6 in response to IL-23 signalling, and which are critical for encephalitogenicity in these cells [184, 185]. However, myelin-reactive Th17 cells only
upregulate IL-23R following differentiation in peripheral lymphoid tissues and are innocuous following initial differentiation in vitro and in vivo [73, 168]. When and where these cells are exposed to this pathogenic signal is unknown.

In the current study, we have demonstrated that myelin-reactive Th17 cells accumulate in the lungs in the preclinical stages of EAE (Figure 4.17). Furthermore, we found that GF mice develop significantly reduced disease severity compared to SPF recipients of the same Th17 cells (Figure 4.3), indicating that commensal microbes contribute to the effector function of myelin-specific Th17 cells. It has been reported that reconstitution of GF mice with a normal microbiota can restore Th17 cell pathogenicity [89, 332]. Furthermore, a systematic review of relevant human studies failed to find major differences in the gut microbiomes of MS patients and healthy controls thus highlighting the potential importance of microbes at sites distant from the GIT in MS development [198, 199]. In addition, previous work from our own lab found that antibody-mediated blockade of Th17 cells from trafficking to the gut failed to attenuate EAE (Lalor, S.J. Unpublished data). Hence, we were encouraged by recent studies in EAE that suggested a role for the lungs as an important staging site for the development of pathogenic T cells in the early stages of disease [200-203].

In a crucial study, Odoradi and colleagues demonstrated that myelin-specific T cells traffic through the lungs and BALT in the preclinical stages of EAE [200]. They found that myelin-specific T cells undergo functional reprogramming in the lungs where they downregulate their proliferative capacity and upregulate genes associated with migration allowing them to enter the CNS and instigate disease. The findings of this study correlated with previous work in our lab which found that myelin-specific Th17 cells traffic to the lungs within days following transfer i.p. or i.v. and their accumulation at this site begins d 2 post-adoptive transfer and peaks on d 5 or 6, just before their initial appearance in the CNS (Lalor, S.J. Unpublished data). Recently, a number of other reports have also implicated the lungs as an important staging point for Th17 cells in the pathogenesis of EAE. For example, it was found that expansion of a pro-inflammatory population of granulocytic myeloid-derived suppressor cells (MDSC) in the lungs during EAE promotes Th17 cell pathogenicity in an IL-6-dependent fashion [202]. Three separate studies also demonstrated regulation of myelin-specific Th17 cells in inflamed airways during the early stages of disease [201, 203, 361]. However, a connection between human RT bacteria and the exacerbation of CNS autoimmune inflammation has not been
reported. Given the centricity of IL-23 signalling in Th17 cell pathogenicity and the paucity of information on the immunogenicity of the RT microbiota, we questioned whether members of this airway bacterial community were capable of inducing IL-23 secretion by innate immune cells in the RT and if these bacteria could modulate Th17 cell pathogenicity in EAE.

Despite the recent surge in research interest on the microbiota, very little is known about how individual members of RT microbiota modulate local and systemic immune responses. Here, we analysed the immunogenicity of 32 of human RT bacteria which commonly colonise the RT. We have identified a number of bacteria from the phyla Bacteroidetes, Proteobacteria and Fusobacteria that are capable of inducing IL-23 secretion in innate immune cells in vitro (Figures 3.12). Moreover, we demonstrated that the commonly encountered respiratory symbionts *N. cinerea*, *M. catarrhalis* and *K. pneumoniae* promote Th17-type responses in the airways, both in the presence and absence of a complex microbiota (Figures 3.18 & 3.23). And, using a novel model of long-term monocolonisation of mice with *K. pneumoniae*, we demonstrated that this Proteobacteria can, in-and-of itself, drive IL-17-type immunity mediated largely by γδ T cells and memory CD4+ Th17 cells in the lungs (Figure 3.21 B).

This novel model of long term monocolonisation with *K. pneumoniae* represented a unique tool to assess the impact of lifelong bacterial colonisation on host immunity and how this might impact Th17 cell pathogenicity in EAE. Here, we have shown that that long term monoassociation with *K. pneumoniae* was sufficient to promote the development of EAE in otherwise less susceptible GF recipients of the same myelin-specific Th17 cells (Figure 4.8). This was a fascinating finding and, to our knowledge, the first report in the literature describing the impact of monocolonisation of a RT bacterium on EAE.

The ability of RT symbionts to exacerbate EAE disease severity was further demonstrated when we administered bacteria i.n. to conventionally housed SPF mice following transfer of myelin-specific Th17 cells. We found that exposure of the airways to either of the respiratory symbionts *M. catarrhalis* or *K. pneumoniae* significantly exacerbated clinical disease severity (Figures 4.6 & 4.7). Interestingly, we did not observe any exacerbation of disease in mice exposed to the alternate Proteobacteria species *N. cinerea*, or the Firmicutes species *V. parvula*, commonly associated with the healthy human lungs (Figures 4.4 & 4.5). These findings indicate that simply introducing any bacterial species into the airways and the
resultant inflammation is not sufficient to modulate disease course. Moreover, it suggests that *M. catarrhalis* and *K. pneumoniae* but not *N. cinerea* express specific factors that promote the acquisition of pathogenicity by Th17 cells and indicates that the ability of individual bacterial species to exacerbate EAE disease severity cannot simply be attributed to membership of a specific phyla e.g. Proteobacteria. Future studies should elucidate the mechanisms by which these bacterial species modulate the effector function of Th17 cells in this preclinical model of EAE.

The significantly exacerbated course of disease in mice exposed, through the airways, to either *M. catarrhalis* or *K. pneumoniae* was associated with increased frequency of donor CD4 T cells producing IFNγ or GM-CSF, or both cytokines together, in the lungs of recipient mice during the preclinical stages of EAE and in the CNS at the height of disease (Figures 4.10, 4.18 & 4.19). It is known that the IL-23 mediated upregulation of IFNγ and GM-CSF, and the acquisition of Th1-like properties is an indispensable event in the acquisition of pathogenicity by Th17 cells [152, 156, 175], A trait shared by these two bacteria we have identified as drivers of Th17 cell pathogenicity is their ability to induce innate IL-23 secretion both *in vitro* and in the lungs *in vivo*. It is possible that this induction of IL-23 in the lungs by *M. catarrhalis* and *K. pneumoniae* is promoting conversion of Th17 cells to an IFNγ and GM-CSF-secreting “ex-Th17” cell phenotype which, in turn, exacerbates disease in the Th17 cell-mediated model of EAE. This concept is supported by our *in vitro* studies where the co-culture of myelin-specific Th17 cells with DCs that has been exposed to either *M. catarrhalis* or *K. pneumoniae* promoted a significant increase in IFNγ and GM-CSF secretion and that blocking IL-23 secretion in these assays reduced GM-CSF, but not IL-17A or IFNγ, secretion by re-stimulated Th17 cells (Figures 4.25 & 4.27). Future studies might address the absolute requirement for RT bacteria-induced IL-23 in the promotion of encephalitogenicity in Th17 cells *in vivo*. Further studies are required to elucidate the mechanism by which these RT bacteria are exerting their effects. The i.n. administration of neutralizing antibodies against IL-23 in these models might provide some insights into the role of bacteria-induced IL-23 in the lungs as an exacerbating factor in EAE. Alternatively, IL-23R-deficient donor Th17 cells could be transferred to WT recipient mice. Additionally, if bacterial components that specifically drive IL-23 secretion could be identified and mutant strains of bacteria that lack the ability to induce IL-23 in innate immune cells generated, we may be able to elucidate the critical signalling that drive IL-23
expression and license pathogenicity in myelin-reactive Th17 cells. Such a discovery could lead to the targeting of these bacterial components or host signalling pathways with novel therapeutic agents in MS patients with an appropriate respiratory microbiome profile.

Additionally, questions remain on how the colonisation dynamics of each individual bacterial species influence how disease pathogenicity is modulated. Here, given that Th17 cell accumulation peaks in the lungs at d 6 post-transfer (SJL, unpublished data) and our finding that \textit{N. cinerea}, \textit{M. catarrhalis}, and \textit{K. pneumoniae} are detected in the lungs 3 d after i.n. exposure but cleared by d 7 (Figure 3.20), we exposed Th17 recipient mice to selected bacteria 2 d after transfer so that the appearance of transferred Th17 cells in the lung would coincide with the immune response induced by bacterial exposure. It would be interesting to investigate if a higher bacterial burden in the lungs or a bacterial burden that was maintained throughout the course of disease would modulate EAE disease severity. For example, sustained bacterial exposure could be achieved by further monocolonisation studies with different bacterial species including \textit{M. catarrhalis} as described here for \textit{K. pneumoniae}. In SPF mice, the repetitive administration of each bacterial strain into the airways allowed for a sustained bacterial burden to be established in the lungs.

Modification of the migratory capacity of T cells in the lungs during the preclinical stages of EAE has previously been described [200]. CCR6 is the prototypical Th17 cell-associated chemokine receptor, expression of which is driven by the transcription factor RORγt and has been implicated in disease pathogenesis through trafficking of CD4+ T cell to the CNS in the early clinical stages of EAE [78]. In the current study, we found a significant increase in CCR6 expression by donor CD4+ T cells in the brain of \textit{M. catarrhalis}-exposed mice at peak disease, compared to PBS-administered recipients of the same Th17 cells (Figure 4.16). CXCR6 is another chemokine receptor which has recently emerged as an important marker of Th17 cell pathogenicity and it’s ligand, CXCL16, is upregulated in the CSF of MS patients [187]. CXCR6 and is highly expressed by GM-CSF-producing CD4+ T cells in the CNS during EAE and animals treated with an anti-CXCL16 mAb display reduced susceptibility to EAE [184, 185, 362, 363]. Here, we observed a dramatic increase in expression of CXCR6 by donor CD4 T cells in the lungs of recipient mice 6 d following transfer, compared to expression of this receptor on transferred cells (Figures 4.2 E & 4.22 A). Additionally, we found that \textit{M. catarrhalis} and \textit{K. pneumoniae}...
*pneumoniae* promoted a significant increase in the frequency of CXCR6+ CD4+ T cells in the spinal cord at peak disease, compared to Th17 cell recipients administered PBS as a control (Figure 4.16 A). It was previously reported that active induction of EAE generates a population of CXCR6-expressing CD4 T cells in the periphery, which are recruited to the CNS via a CXCL16 gradient [362]. In our current study, we detected an increase in CXCL16 expression in the lungs in response to *K. pneumoniae* i.n. exposure (Figure 4.21 C). It could be, therefore, that certain RT bacteria promote trafficking of myelin-reactive Th17 cells to the lungs where other factors, such as IL-23, license encephalitogenicity. In any case, our data supports further investigation of the CXCR6/CXCL16 axis in the lungs as a potential therapeutic target for MS.

Interestingly, a study just published has described the IL-23-dependent development, upon the immunization with myelin peptides, of a population of pathogenic CXCR6+GM-CSF+IFNγ+ Th17 cells from a natural Th17 cell population in the intestines [362]. These pathogenic Th17 cells migrated specifically to the CNS and mediated inflammatory demyelination. The authors of this study proposed that the GIT act as a reservoir for pathogenic CXCR6+ T cells which might be maintained by the gut microbiota. It is possible that discrete members of the microbiota at other mucosal sites too, including the lungs, could act in a similar manner, and future investigations into this possibility should be pursued.

There is growing appreciation for the role played by tissue resident memory T (Trm) cells in lung immunity. Trm cells persist in previously inflamed, non-lymphoid tissue and provide enhanced local immune memory [364]. Trm cells have been shown mediate enhanced pathogen clearance from the lung and can be established through intranasal vaccination, suggesting the localized development of these cells [365, 366]. Interestingly, non-specific bystander activation of lung Trm cells following local bacterial infection has been demonstrated to promote neutrophil recruitment into the airways, reducing the severity of *S. aureus* bacterial pneumoniae [367]. Conversely, Trm cells generated in response to allergen exposure can contribute to immunopathology and the promotion of reactive airway disease [368, 369]. It is interesting, in this light, that Odoardi and colleagues found memory cells persisting in the lungs 10-14 weeks after i.p. transfer of myelin-specific T cells, which could be reactivated by i.t. administration of MBP antigen and trigger CNS autoimmune inflammation [200]. The CXCR6/CXCL16 axis has been implicated in the maintenance of Trm cells in the lung.
In the current study, the majority of CD4+ T cells in the lungs expressed CXCR6 and we saw a significant increase in the expression of CXCL16 in the lungs of Th17-recipient mice exposed to *K. pneumoniae* and *V. parvula*, compared to PBS-administered recipients of the same cells (Figure 4.21). It is an interesting possibility, therefore, that the lungs could act as a reservoir for myelin-reactive Th17 cells, that could be maintained through CXCR6 ligation to bacteria-induced CXCL16, and which may be primed and ready to be activated by alternate factors to trigger clinical relapse in MS patients.

Current treatment options in MS are efficacious in less than 55% of patients [99]. Additionally, severe adverse side effects including nonspecific immunosuppression leading to infection and cardiac complications are observed in patients receiving many of these treatments. There is an urgent need, therefore, to develop better, more targeted therapies. Several exciting new drugs targeting the IL-17 axis have proven efficacious in the treatment of inflammatory disorders such as psoriasis and inflammatory arthritis [66]. Antibodies targeting IL-17 have also shown some promise in clinical trial for the treatment of RRMS [371]. The crucial roles played by both IL-23 and GM-CSF in the development of pathogenic Th17 cells renders them attractive targets in the quest to find effective treatments for MS, however antibodies targeting either molecule have not come to market for MS patients [156, 372]. A phase II clinical trial investigating the efficacy of a mAb, ustekinumab, that targets the IL-12p40 subunit of IL-12 and IL-23 failed to have any effect on the formation of inflammatory white matter lesions or clinical progression in patients with RRMS [139, 372]. The authors postulated that stage of disease progression of patients enrolled in this clinical trial may have been too late for treatment with anti-IL-23 antibodies. Nevertheless, data presented here, together with the findings of other recent studies on the pathogenicity of Th17 cells in MS and EAE, indicates that the administration of disease modulating treatments targeting IL-23 and GM-CSF directly to the airways warrants further investigation. In fact, dimethyl fumarate (DMF), a first line therapy which is normally taken orally for the treatment of RRMS, has recently been shown to be efficacious in attenuating EAE when delivered as an aerosol [361]. The inhalation of DMF encapsulated in nanoparticles promoted an influx of FoxP3+ cells Treg cells to the lungs and spinal cord, reducing CNS inflammation. Another study used nanoparticles loaded with the myelin component proteolipid protein (PLP) with the aim to induce tolerance in a spontaneous model of EAE [344]. These authors demonstrated that the
direct delivery of particles to the airways via i.t. administration was more effective at reducing EAE disease severity than when delivered i.v. The administration of PLP-specific nanoparticles to the lung resulted in reduced proliferation of autoreactive T cells and altered chemokine responses, impairing trafficking of antigen-specific T cells to the CNS [344]. These studies not only highlight the potential for the use of aerosolization as an effective mode of drug administration in MS/EAE but reinforce the importance of the lungs as a critical site for T cell regulation in CNS autoimmunity.

Whether the lung microbiome can be manipulated therapeutically to change disease progression in several disease settings is an area that requires significant research focus. Data presented here provides an indication that certain perturbations in the RT microbiota can alter disease severity in a preclinical model of MS. It would be interesting to investigate the translational relevance of these findings in the airways of patients with MS during exacerbation. By comparing bacterial composition and burden in the airways of MS patients with healthy controls, as well as the transcriptional profile that these microbial changes might induce, a better insight could be obtained into the mechanism by which discrete bacteria modulate immune responses in the lungs of MS patients and how that might exacerbate disease. Identification of individual strains of bacteria that drive the initial inflammatory events in MS, or those that collectively engender chronic inflammation, may permit prophylactic targeting at the very earliest stages of disease development. If airway microbiome composition could be developed as an early biomarker of disease activity in MS, it might allow for the stratification of at-risk individuals. Altered microbiota structure and increased burden of causal strains may signal for interventional approaches including the use of novel small molecule drugs, aerosol delivery of probiotics or narrow-spectrum antibiotics, the use of vaccines or administration of synthetic bacteriophages that reduce harmful populations of bacteria. These approaches should be more cost effective and may have less off-target consequences than currently available DMTs. Ongoing research in this area should also elucidate critical endogenous signalling pathways and new targets for drug therapies. Importantly, recent research has focused on non-canonical pathways involved in the acquisition of pathogenicity by Th17 cells. These include the RNA helicase DDX5 that controls aspects of RORγt-mediated Th17 cell development and CDL5, a scavenger receptor with PRR activity that is expressed by lung epithelial cells, Th17 cells and macrophages in inflamed...
tissues and acts as a negative regulator of the transition from a non-pathogenic to a pathogenic Th17 phenotype [373-375]. In individuals at risk of developing chronic inflammatory disease, prophylactically targeting molecules that regulate progression to a pathogenic Th17 cell phenotype, while sparing protective Th17 capacity, should maintain a balanced immune response without increased susceptibility to infection and, perhaps, lead to improved patient outcomes compared to those achieved therapeutically.
5.1 Graphical summary

Figure 5.1 Respiratory symbionts modulate Th17 cell pathogenicity and clinical disease in EAE.

The data presented herein indicates that discrete RT bacteria, including *M. catarrhalis* and *K. pneumoniae*, express factors that promote conversion of myelin-specific Th17 cells to a pathogenic CXCR6+ IFNγ and GM-CSF-producing ex-Th17 cell phenotype. Whether by molecular mimicry, shared antigenic targets or through bystander activation and migration of self-reactive T cells, it appears that immune responses in the airways can modulate extra-pulmonary inflammation, including CNS autoimmunity. Together, this data supports the concept that perturbations in the RT microbiota might contribute to the pathophysiology of CNS autoimmune disease.
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6. Bibliography


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# Appendix 1 Bacterial Source and Strain Information

<table>
<thead>
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<th>Phylum</th>
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<th>Strain</th>
<th>Source</th>
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