The role of toll-like receptor 9 (TLR9) in autoimmunity : an examination into the relationship between gut microbiota and TLR9 in Systemic Lupus Erythematosus (SLE)

Yasuga, Hiroko

2019

Yasuga, H. (2019). The role of toll-like receptor 9 (TLR9) in autoimmunity : an examination into the relationship between gut microbiota and TLR9 in Systemic Lupus Erythematosus (SLE). Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/100426>

<https://doi.org/10.32657/10220/48587>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**The Role of Toll-Like Receptor 9 (TLR9) in autoimmunity;
An examination into the relationship between gut microbiota
and TLR9 in Systemic Lupus Erythematosus (SLE)**

Hiroko Yasuga

SCHOOL OF BIOLOGICAL SCIENCES

2019

**The Role of Toll-Like Receptor 9 (TLR9) in autoimmunity;
An examination into the relationship between gut microbiota
and TLR9 in Systemic Lupus Erythematosus (SLE)**

Hiroko Yasuga

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for
the degree of Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

3rd June 2019

.....
Date

.....
Hiroko Yasuga

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it of sufficient grammatical clarity to be examined. To the best of my knowledge, the thesis is free of plagiarism and the research and writing are those of the candidate's except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

3rd June 2019.

.....
Date

Anna-Marie Fairhurst, PhD

Authorship Attribution Statement

This thesis contains materials from 2 papers published in the following peer-reviewed journals where I was a co-author.

Section 1.3 includes a paper published as Celhar T, Yasuga H, Lee HY, Zharkova O, Tripathi S, Thornhill SI, Lu HK, Au B, Lim LHK, Thamboo TP, Akira S, Wakeland EK, Connolly JE, Fairhurst AM, *Toll-Like Receptor 9 Deficiency Breaks Tolerance to RNA-Associated Antigens and Up-Regulates Toll-Like Receptor 7 Protein in Sle1 Mice*. In press, *Arthritis Rheumatol*. 2018 Oct;70(10):1597-1609. DOI: 10.1002/art.40535.

The contributions of the authors are as follows:

- *Celhar T., Lim LHK, Akira S, Wakeland EK., Connolly JE, and Fairhurst AM designed research.*
- *Celhar T, Lee HY, Zharkova O, Tripathi S, Thornhill SI, Lu HK, Au B, Lim LHK, Thamboo TP, and Fairhurst AM performed research.*
- *Celhar T, Au B, Thamboo TP, and Fairhurst AM analyzed data.*
- *Celhar T and Fairhurst AM wrote the paper.*

Section 3.2 includes a paper published as Celhar T, Hopkins R, Thornhill SI, De Magalhaes R, Hwang S, Lee HY, Yasuga H, Jones LA, Casco J, Lee B, Thamboo TP, Zhou XJ, Poidinger M, Connolly JE, Wakeland EK, Fairhurst AM, *RNA sensing by conventional dendritic cells is central to the development of lupus nephritis*. In press, *Proc Natl Acad Sci USA*. 2015 Nov 10;112(45):E6195-204. DOI: 10.1073/pnas.1507052112.

The contributions of the co-authors are as follows.

- Celhar T, Connolly JE, Wakeland EK, and Fairhurst AM designed research.
- Celhar T, Hopkins R, Thornhill SI, De Magalhaes R, Hwang S, Lee HY, Yasuga H, Jones LA, Casco J, and Fairhurst AM performed research.
- Casco J, Poidinger M, Connolly JE, Wakeland EK contributed new reagents/analytic tools.
- Celhar T, Hopkins R, Thornhill SI, Jones LA, Lee B, Thamboo TP, Zhou XJ, Poidinger M, Connolly JE, and Fairhurst AM analyzed data.
- Celhar T, Thornhill SI, and Fairhurst AM wrote the paper.

3rd June 2019

.....
Date

.....
[Hiroko Yasuga]

Acknowledgement

First and foremost, I would like to express my deepest gratitude to my supervisor, Dr. Anna-Marie Fairhurst, for giving me this opportunity to undertake the project. I am extremely grateful for her support, patience and guidance throughout the past 4 years. Thank you for creating a working environment where learning and constant challenges of science was a pleasure and motivation for continual self-improvement. Thank you for being a great teacher, adviser and a role model of a great scientist.

Thanks to the thesis advisor committee members: Dr. Florent Ginhoux, Dr Wu Bin, and Dr. Alex Low for advice, discussions and critical feedback throughout my project.

I also wish to express my most heartfelt gratitude to my colleagues, Dr. Teja Celhar, Lee Hui Yin for their thorough guidance, mentoring and patience every step of the way throughout this project.

I wish to express my most heartfelt gratitude to my colleagues to whom I am indebted for the knowledge and skills I received through their patient training and support: Dr. Hao Kim Lu, Dr. Susannah Thornhill, Dr. Olga Zharkova, and Pang Ann Giat for their thorough guidance and mentoring at every step of the way throughout this project.

In addition, I would like to extend my sincere thanks to students and the former members of the AMF lab of SING for their kind assistance and support during my time there.

I also thank the mouse core and SING flow cytometry facility staffs, it would be almost impossible to finish my project without them. Moreover, I thank our collaborators at IMCB: to Dr. John Connolly and JEC lab members.

Thanks to SINGA for the scholarship and to A*STAR for the financial support of this research.

Lastly, but not least, I would like to sincerely thank my friends, my family, and my partner for their unconditional support throughout this project.

Table of Contents

1	Introduction	1
1.1	Autoimmunity	1
1.2	Systemic Lupus Erythematosus (SLE).....	2
1.2.1	Clinical presentations and diagnosis.....	3
1.2.2	Causes of the disease	4
1.2.2.1	Genetic factors.....	5
1.2.2.2	Environmental factors	5
1.2.3	Mouse models of the disease	6
1.2.3.1	Mouse models and Sle1.....	7
1.3	The role of TLR9 in SLE	7
1.4	Contribution of leukocytes in autoimmune murine models	11
1.4.1	B cells	11
1.4.2	Dendritic cells.....	11
1.5	Other factors in SLE development	13
1.6	Microbiota and Autoimmunity	13
1.6.1	Human microbiome	13
1.6.2	Gut microbiota.....	14
1.6.3	Gut microbiota and immune systems	15
1.6.4	Gut microbiota and Autoimmunity.....	16
1.7	Preliminary work on the microbiome in the Fairhurst lab	17
1.8	Hypothesis and aims of Thesis	21
2	Materials & Methods	22
2.1	Overall flow of the experiments.....	22
2.2	Mice.....	22

2.3	Verification of strain identity by PCR genotyping.....	23
2.3.1	Preparation of DNA from mouse tail lysate	23
2.3.2	PCR genotyping.....	23
2.4	Mouse tissue sample preparation	24
2.4.1	Spleen processing	24
2.4.2	Kidney processing	24
2.4.3	Peyer's patches preparation	25
2.5	Enzyme-Linked Immune Sorbent Assay (ELISA).....	25
2.5.1	ANA productions in the serum	25
2.5.2	Immunoglobulin (Ig) isotypes productions in the serum.....	25
2.6	FACS analysis	26
2.6.1	Cell surface staining	26
2.6.2	Intracellular staining	26
2.6.3	Gating strategies	27
2.6.4	FMO control for detection of true population and MFI in flow cytometry .	28
2.7	Stool sample collection and Antibiotic treatment for Microbiome Studies	28
2.8	Analysis of faecal DNA	28
2.8.1	Transformation	28
2.8.2	Plasmid DNA Extraction	29
2.8.3	Plasmid DNA Concentrations.....	29
2.8.4	Faecal DNA Extraction for real-time Polymerase Chain Reaction (qPCR)	30
2.8.5	Principal of SYBR Green I dye	30
2.8.6	Rational for the choice of SYBR Green	31
2.8.7	Quantitative Real-time PCR amplification of 16S rRNA Sequence (Barman et al. 2008)	31

2.8.8	Specificity of the Primers used in qPCR- Dissociation Curve of the Amplified Products	33
2.8.9	Generation of standard curve.....	34
2.8.10	Slope of standard curve	35
2.8.11	Efficiency of PCR reaction.....	35
2.8.12	Absolute quantification.....	36
2.8.13	Relative quantification.....	36
2.9	Genome sequencing by Genome Institute of Singapore (GIS)	37
2.9.1	Introduction and workflow	37
2.9.2	Bioinformatics and analysis workflow	37
2.9.3	Methods and results - Data visualization and statistical analyses	38
2.10	Statistical Analysis	38
3	Results	40
3.1	Impact of TLR9 on microbiota.....	40
3.2	Impact of gut microbiota on Sle1 and Sle1TLR9 ^{-/-}	52
3.3	16S rRNA sequencing analysis	67
3.3.1	Bacteria compositions is changed by time and strains	68
3.3.2	Microbiome profile altered by Sle1 background.....	69
3.4	Primer design and optimisation for qPCR.....	71
3.4.1	Primer test by PCR	71
3.5	Relative abundance of Oscillospira, Rikenellaceae, and S24.7	72
3.5.1	qPCR validation of the genome sequencing result.....	72
3.5.2	qPCR analysis on new samples	73
3.5.3	Relative bacteria abundance in comparison of B6 and Sle1.....	74
3.5.4	Relative bacteria abundance in comparison of B6 and TLR9 ^{-/-}	80
4	Discussion.....	82

4.1	Antibiotic treatment to autoimmune disease	82
4.1.1	In non-autoimmune prone B6 backgrounds	83
4.1.2	In autoimmune prone backgrounds	84
4.1.2.1	Pre-disease status of SLE mouse model.....	84
4.1.2.2	Impact of Abx treatment on Sle1TLR9 ^{-/-} at pre-disease stage	86
4.2	Gut bacteria profile and disease, environment and mothers	87
5	Conclusion	90
6	References	91
7	Appendix	101
7.1	Appendix I Antibodies for Flow cytometry	101
7.2	Appendix II Primer sequences	104
7.3	Appendix III Pathogens screened in each facility	105
7.4	Appendix IV Reagent recipe	106
7.5	Appendix V Software.....	107
8	Author's publications	108
9	Posters, awards	108

List of Figures

Figure 1.1	Absence of TLR9 on <i>Sle1</i> drives severe disease	9
Figure 1.2	Severe kidney disease in <i>Sle1</i> /TLR9 ^{-/-}	9
Figure 1.3	Elimination of TLR9 breaks self-tolerance	10
Figure 1.4	Anatomy of the intestine, and immune system in the gut	15
Figure 1.5	Comparison of bacteria change in the gut - Pre; 8 weeks old vs. Post; 5-6mos old, in B6 and TLR9 ^{-/-} mice	18
Figure 1.6	Immunological traits in the spleen of B6 and TLR9 ^{-/-} mice and correlation between total bacteria in the gut and immune phenotypes.	18
Figure 1.7	Splenic weight at 5-6mos old mice in absence and presence of antibiotics	19
Figure 1.8	Comparison of immunological traits of T cell subsets in the spleen.....	20
Figure 2.1	Flowchart of experiments	22
Figure 2.2	Gating strategy for live CD45 ⁺ cells.....	27
Figure 2.3	Schematic image of SYBR Green I dye interacting with double-stranded DNA	30
Figure 2.4	Melting temperature of the amplified bacterial 16S rRNA sequences from stool DNA samples.....	34
Figure 2.5	Standard curve plotted using the Ct values from Real time PCR analysis	35
Figure 3.1	Experimental plan for comparison of B6 and TLR9 ^{-/-} mice.....	40
Figure 3.2	Pathological traits in mice and total bacteria copy number measured by 16S rRNA qPCR.....	41
Figure 3.3	Inefficient Abx treatment on <i>Bact</i>	43
Figure 3.4	Immunological traits of T cell subsets in the spleen in B6 and TLR9 ^{-/-} mice	45
Figure 3.5	Immunological traits of B cell subsets in the spleen in B6 and TLR9 ^{-/-} mice	47
Figure 3.6	Immunological traits of myeloid lineage in the spleen in B6 and TLR9 ^{-/-} mice	49
Figure 3.7	Circulating Immunoglobulins in the serum measured by Luminex	50

Figure 3.8	Correlations between total bacteria copy numbers and immunological traits	51
Figure 3.9	Experimental plan for comparison of <i>Sle1</i> and <i>Sle1</i> TLR9 ^{-/-}	52
Figure 3.10	Splenic weight, caecum contents weight, and total bacteria copy number measured by 16S rRNA qPCR	53
Figure 3.11	Immunological traits of T cell subsets in the spleen in <i>Sle1</i> and <i>Sle1</i> TLR9 ^{-/-} mice	55
Figure 3.12	Immunological traits of B cell subsets in the spleen in <i>Sle1</i> and <i>Sle1</i> TLR9 ^{-/-} mice.....	57
Figure 3.13	Immunological traits of myeloid lineage in the spleen in <i>Sle1</i> and <i>Sle1</i> TLR9 ^{-/-} mice.....	58
Figure 3.14	Immunological phenotypes in PPs.....	61
Figure 3.15	Circulating Ig subtypes in the serum measured by Luminex.....	63
Figure 3.16	ANA production in the serum measured by ELISA	64
Figure 3.17	Systemic upregulation of TLR7 on immune cells in the spleen	65
Figure 3.18	Relative TLR7 expressions on splenic immune cells in microbiome cohort	66
Figure 3.19	Bacteria composition in B6, TLR9 ^{-/-} , <i>Sle1</i> , and <i>Sle1</i> TLR9 ^{-/-} mice	68
Figure 3.20	<i>Sle1</i> background alters microbiome profile	69
Figure 3.21	Gel image of PCR products for primer test	71
Figure 3.22	Dissociation curves of the amplified bacterial 16S rRNA sequences.....	72
Figure 3.23	Bacteria relative abundance in the DNA samples from MB cohorts by qPCR	73
Figure 3.24	Comparison of bacteria relative abundance to 16S rRNA total bacteria..	74
Figure 3.25	Comparison of bacteria relative abundance in different facilities	76
Figure 3.26	Comparison of bacteria abundance in different facilities, by strain.	78
Figure 3.27	Comparison of bacteria relative abundance in different facilities	81

List of Tables

Table 1.1	17 criteria for diagnosis of SLE [14, 16]	4
Table 2.1	Primer sequence for identification of specific bacterial strain	31
Table 2.2	qPCR conditions for each bacterial strain	32
Table 2.3	Primer concentrations and master mix preparation for qPCR.....	33
Table 3.1	Summery of immunological changes led by Abx treatment (Spleen).....	44
Table 3.2	Summary of changes in immunological phenotypes in PPs led by Abx treatment	60
Table 3.3	Bacteria strains that were significantly different between B6 and <i>Sle1</i> background	70
Table 3.4	Mouse strains and facilities	74
Table 3.5	List of mouse cages, mothers for comparison of relative abundance of <i>Rikenellaceae</i>	79
Table 3.6	List of mouse cages and mothers for comparison of relative abundance of <i>S24.7</i>	80

Abbreviations

Abx	antibiotics
ANA	anti-nuclear antibodies
APC	antigen presenting cells
BCR	B cells receptor
cDCs	conventional dendritic cells
GC	germinal centre
INF- α	interferon - alpha
Min	minute
mLN	mesenteric lymph node
Mos	month(s)
MS	multiple sclerosis
PCR	polymerase chain reaction
pDCs	plasmacytoid dendritic cells
PMNs	neutrophils -
PPs	Peyer's patches
RA	rheumatoid arthritis
RT	room temperature
Sec	second
SLE	systemic lupus erythematosus
Tfh	follicular helper T cells

TLR	toll-like receptors
Treg	regulatory T cells

Abstract

SLE is a complex autoimmune disease characterized by the production of anti-nuclear autoantibodies in a breakdown of self-tolerance. TLR9 is a receptor which recognises microorganism-associated unmethylated DNA. It is located in the endocytic pathway in B cells, dendritic cells (DCs), plasmacytoid DCs, and murine macrophages. Data from our lab has shown that TLR9 deletion in lupus-prone B6.*Sle1* mice causes severe disease, consistent with other model systems. TLR9 is also expressed on intestinal epithelial cells, where it may play an important role in the recognition of gut microbiota. Several studies have suggested that the development of several autoimmune diseases is influenced by gut microbiota. However, the role of the microbiota in the development of SLE has not yet been clarified.

The aim of this investigation was to assess the impact of the microbiome in the development of autoimmunity in TLR9 deficient *Sle1* mice (*Sle1*TLR9^{-/-}) mice. To assess the impact of TLR9 deficiency in the absence of *Sle1*, we first analysed wild type B6 and B6.TLR9^{-/-} mice. The strategies to evaluate the impact of the microbiome on immunological traits included housing mice in facilities with different levels of cleanliness and using antibiotic treatment to eliminate the gut microbiome. The mice were aged, and we assessed immunological traits in spleen and purified DNA from stool for 16S rRNA sequencing analysis. We then examined the effect of antibiotic treatment on mild and severe autoimmune traits, that are in *Sle1* and *Sle1*TLR9^{-/-} mice. We analysed immunological traits in the spleen, kidneys, Peyer's patches, mesenteric lymph nodes, and performed stool DNA sequencing.

In summary, we determined that gut microbiota has an impact on several immunological phenotypes. However, it does not impact autoimmune traits such as autoantibody production and kidney pathology in *Sle1*TLR9^{-/-}. Furthermore, DNA sequencing analysis revealed that the *Sle1* background influences gut microbiota, but this is dominated by the impact of different animal facilities and their mothers. Taken together, this research highlights the importance of gut microbiota in the disease, and how much environment effects on bacteria abundance.

1 Introduction

1.1 Autoimmunity

The human body is constantly challenged by invading pathogens (such as viruses, bacteria, and protozoa) however is able to protect itself with the help of immune system. The immune system can be divided into innate mechanisms, which provide a non-specific yet rapid response to potential pathogens, and the adaptive immune system, which generates a specific long-term immune response, over a much longer period of time (Janeway C.A. et al., *Immunology*, 2001).

It is essential for the immune system to maintain a balance between strong protection against invaders and tolerance to self [1]. The phenomena of tolerance mean a sophisticated differentiation between self-arising antigens and invading pathogens, which evolve to evade the immune system by trying to resemble body own cells. The mechanisms leading to tolerance can be “central”, occurring in the thymus and bone marrow, or “peripheral”, which take place in the lymph nodes or tissues. Central tolerance facilitates the ability to distinguish self from non-self, whilst peripheral tolerance is a key factor preventing an overreaction to environmental substances (Murphy K., *Immunology*, 2016).

Control of innate immunity occurs at multiple levels, starting from the short life-span of innate immune cells, such as neutrophils (PMNs), and negative feedback loops within innate signalling pathways (Kendrick SFW and Jones DEJ, *Spices*, 2008). The regulation of the adaptive immune system is more advanced, including various stages of B and T cell maturation, multiple checkpoints for B and T cell receptors and the formation and eventual elimination of almost 90% of the cells during tolerance tests (Janeway C.A. et al., *Immunology*, 2001).

However, when the intrinsic balance is disturbed, pathologies arise. A hyper-activated, poorly controlled immune system, which is unable to tolerate self-antigens, becomes a

dangerous enemy to its own body enabling the development of autoimmune disorders. Autoimmunity is estimated to affect approximately 8% of the USA population or approximately 25 million people [2]. This translates to 1 person out of 31 being affected in one way or another by autoimmune pathology. The incidence is becoming higher due to a number of factors, including growing awareness and improved clinical diagnoses [3].

More than 100 autoimmune diseases are currently known, with the number increasing; around 40 other conditions are also suspected to be of autoimmune origin [4]. Autoimmunity in general affects more females than males. Moreover, there is an evidence that it is a leading cause of death in young women within the USA [5]. Typically, autoimmune disorders are classified as either organ specific, such as diabetes mellitus (pancreas), myasthenia gravis (muscles) [6], Hashimoto's thyroiditis (thyroid) etc., or systemic, which affect the whole body. The most prevalent examples of systemic disorders include rheumatoid arthritis (RA), scleroderma, systemic lupus erythematosus (SLE), and multiple sclerosis (MS) and Sjogren's syndrome.

While a person of any racial, ethnic or social economic group at any age may be affected by autoimmunity, some races are more prone [7]. For example, SLE incidence is greater amongst African-American and Hispanic populations than amongst Caucasians [8].

Over the past decade, with the development of new scientific methods and animal models of disease, research has given many insights into the mechanisms of disease. Diagnosis at earlier stages and development of new medicines allow maintenance of a better and longer life for patients affected with autoimmunity. An advancement in kidney dialysis improves life quality for SLE patients with glomerulonephritis, however major questions regarding the causes of the disease pathologies remain to be answered.

1.2 Systemic Lupus Erythematosus (SLE)

SLE is a chronic autoimmune disease characterised by the production of autoantibodies which bind self-antigen in the form of immune complexes (ICs). These ICs accumulated in target organs resulting in inflammation and tissue destruction. These situations reflect a loss of tolerance to self and subsequent dysregulation of the immune

system resulting from both genetic and environmental factors. SLE has a strong sexual dimorphism with 10 times more females developing disease than males. The overall prevalence is considered to be about 1 out of 1000 but this varies on ethnicity. The first presentation of SLE symptoms is typically during the child-bearing age. The heritability of SLE is approximately 66% - the rates of concordance are 24% to 56% in monozygotic twins and 2% to 4% in dizygotic twins suggesting that genetic factors are essential [9].

1.2.1 Clinical presentations and diagnosis

The clinical features of SLE are diverse. Criteria of SLE were established in 1982, with revisions in 1997 by Hochberg [10] [11]. Common symptoms include fatigue, weight loss, unsatisfying sleep and fever and although these are not life threatening, they still have a significant impact on the quality of life. Organ damage results from the inflammation driven by the ICs and this can occur in kidneys, lung and heart. Renal disease affects approximately 30% of SLE patients, usually in the first few years of their disease and is one of the life-threatening complications in SLE [12].

The diagnosis of rheumatic diseases, of which SLE is, is always complicated due to heterogeneity of symptoms. Diagnosis of lupus can take years due to the complicated nature of the disease. Clinicians all over the world try to develop specific and precise guidelines to make diagnosis more standard, which will furthermore help to recruit patients for clinical trials and criteria for diagnosis of SLE is shown in Table 1.1 [13].

Currently diagnosis is based on the clinical manifestations and laboratory tests, including screening for auto-antibodies, functional tests, and imaging data. Usually doctors use the reviewed American College of Rheumatology (ACR) classification criteria for SLE [10, 11, 14]. However, these criteria were originally developed for classification of disease severity and not diagnosis, therefore several significant symptoms, including skin inflammation or neuropsychiatric manifestations are missing [15]. More recently, the NIH has recommended a SLE diagnosis with the presence of at least 4 of 17 criteria (table 1.1) [14].

Clinical criteria	Immunological criteria
1. Acute cutaneous lupus erythematosus, including malar rash	1. ANA level above laboratory reference range
2. Chronic cutaneous lupus (e.g., localized or generalized discoid lupus erythematosus)	2. Anti-dsDNA antibodies above laboratory reference range
3. Oral ulcers	3. Anti-Sm antibodies
4. Non-scarring alopecia	4. Antiphospholipid antibodies; anticardiolipin and anti- β 2-glycoprotein I antibodies, false-positive Venereal Disease Research Laboratory
5. Synovitis or tenderness on palpation involving ≥ 2 joints	5. Low complement; C3, C4, or CH50
6. Serositis	6. Direct Coombs test in the absence of hemolytic anemia
7. Renal involvement (urine protein/creatinine ratio or 24-hour urine protein, >0.5 g)	
8. Neurological involvement (e.g., seizures, psychosis, myelitis)	
9. Hemolytic anemia	
10. Leukopenia or lymphopenia	
11. Thrombocytopenia	

Table 1.1 17 criteria for diagnosis of SLE [14, 16]

SLE is diagnosed when at least four criteria are fulfilled. At least one criterion from clinical and one from immunological needed to be presence. Alternatively, nephritis has to be diagnosed histologically with the Immunological criteria 1 and 2.

1.2.2 Causes of the disease

The specific mechanisms leading to autoimmunity and specifically, the onset of SLE are largely unclear, due to the multiple factors involved and their complex interactions. They include genetic predisposition and the influence of environmental

factors with the addition of the influence of gender. SLE is characterized by the development of ANAs to nuclear containing material [13] however almost 5% of the population has ANAs but will never develop full disease as discussed in [17].

1.2.2.1 Genetic factors

Genetic predisposition is the strongest risk factor associated with the development of SLE. There is approximately 25-50% disease concordance in identical twins and close relatives of SLE patients are at a higher risk of developing the disease [13, 18, 19].

With the advances in genetic research and the possibility of genome-wide association study studies, lupus research has shed light on a number of genes either directly involved or linked to the development of pathology. Polymorphisms in more than 100 genes have been shown to increase the risk of autoimmunity [13]. To understand complex genetic interactions more clearly, it is convenient to divide the pool of available genes into pathways, subsequently analysing genes involved in each particular pathway [13].

Genes involved in pathology could be grouped in several pathways with particular functions: apoptotic debris clearance, phagocyte function, complement system and immune complex deposition, antigen presentation, signalling and TLR function, T and B cell activation, energy metabolism and epigenetic modification for example [20]. The alterations in one or several genes within either single or multiple pathways may ultimately lead to the development of pathology.

A single genetic polymorphism alone is not usually sufficient to cause SLE. Moreover, epistatic interactions between several polymorphisms usually coalesce to generate full blown pathology.

1.2.2.2 Environmental factors

Environmental factors like viral infections, UV light and also certain types of drugs are well described triggers for SLE development. Over 100 currently used drugs

can induce lupus, including blood pressure drugs like hydralazine and methyldopa, which affect DNA methylation [21]. Likewise, anti-acne drugs, anti-TNF α drugs and medicine used against metal poisoning may trigger lupus, however the usage of these types of drugs are decreasing [22]

Chemicals widely used in food and cosmetic production, like pristine, are known to provoke lupus development in non-autoimmune mice [23]. There are references demonstrating that individuals exposed to industrial substances such as silica dust or mineral oils, solvents, pesticides and hair dye are more likely to develop SLE [24, 25].

Tobacco smokers and cocaine users are also known to be more predisposed to autoimmunity symptoms [26]. There is an association between the number of cigarettes smoked per year and the risk of SLE development due to smoking causing excessive necrosis [27, 28].

Viral infections have been associated with the development of SLE. In particular, Epstein-Barr Virus (EBV) can activate innate immune cells, followed by B cell secretion of auto-antibodies directed against amino-acid sequences similar between host and virus [29].

Exposure to UV light brings about damage to DNA, such as hypomethylation [30] resulting in mutations provoking auto-antibody production and disease complications including rashes [31].

1.2.3 Mouse models of the disease

So far, animal models have proved the best way to probe the mechanisms of disease in general, because of their phylogenetic relatedness and physiological similarity to humans, the ease of maintaining and breeding them in the laboratory. The availability of many inbred strains, house mice have long served as models of human biology and disease [32] (Games G. Fox, *et al.*, The Mouse in Biomedical Research, 2007). Genomic studies have highlighted the striking genetic homologies between the two species (Games G. Fox, *et al.*, The Mouse in Biomedical Research, 2007). These studies, together with the development of methods for the creation of transgenic, knock-out, and knock-in mice,

have provided added impetus and powerful tools for mouse research, and have led to a dramatic increase in the use of mice as model organisms. Studies on mice have contributed immeasurably to our understanding of human biology species (Games G. Fox, *et al.*, The Mouse in Biomedical Research, 2007).

The precise pathological mechanisms of SLE are still not fully understood. Given the complexity of the human disease, mouse models and their congenic derivatives have proven to be crucial in dissecting out pathways to severe disease. Traditionally there are 3 main murine lupus models, the BXSB, MRL.Fas^{lpr} (MRLlpr) and the F1 progeny of the New Zealand Black (NZB) and New Zealand White (NZW) mice (NZB/W) [17]. These models, together with human immunological assessments have identified that dysregulation of T cells, B cells, and antigen presenting cells (APCs) lead to the development and progression of SLE.

1.2.3.1 Mouse models and Sle1

Sle1 is a genetic susceptibility locus originally derived from the NZM2410 lupus prone mouse strain. This NZM2410 strain was originally generated through a backcrossing strategy of the NZW with the NZB/W [33]. On a B6 background *Sle1* (B6.*Sle1*), confers a loss of tolerance to chromatin, which results in the production of high levels of auto-antibodies directed against nuclear antigens [34]. Candidate genes within this region on chromosome 1 conferring these mild autoimmune traits are Ly108 and CD84 [35-37].

1.3 The role of TLR9 in SLE

Toll like receptors (TLRs) recognize microorganism-associated molecules that are not found in healthy vertebrate cells. There are 10 and 12 TLRs in humans and mice respectively. TLR9 is located on the endosome and in a resting state, is expressed on B cells, dendritic cells (DCs) and murine macrophages. TLR9 is activated by unmethylated dsDNA signals through the MyD88 signalling pathway [38]. It is thought that TLR9 has

a primary role in the development and disease progression of SLE *in vivo*, however, the precise mechanisms of its role are still unclear.

Initial studies suggested that TLR9 was a major candidate for SLE in activating pDCs, B cells and peripheral blood mononuclear cells (PBMCs) by the interaction self-DNA immune complex or SLE sera [39, 40]. Therefore, it was expected that deletion of TLR9 would ameliorate disease in murine models. However, TLR9 deficiency led to an exacerbation of lupus [41-45]. Schlomchik and colleagues found that elimination of TLR9 in the MRL/lpr lupus models prevented the development of anti-DNA and anti-chromatin autoantibodies. However, the main pathological features of the disease, including glomerulonephritis were augmented [38, 41]. Moreover, they determined an increase in circulating IFN α levels in these mice [46]. Further examination demonstrated that Ifnar1, a receptor for IFN α , contributed to renal disease [47].

Similar findings were observed by Izui and colleagues using the B6.Nba2 lupus-prone strain. Acceleration of disease characteristics were observed with the elimination of TLR9, with decreases in the production of anti-nucleosome and anti-chromatin autoantibodies [48].

We recently published a paper which has also shown that TLR9 deficiency in lupus-prone B6.*Sle* results in severe autoimmunity [49].

Figure 1.1 Absence of TLR9 on *Sle1* drives severe disease

Female B6, TLR9^{-/-}, *Sle1*, and *Sle1*TLR9^{-/-} mice were aged to 4-6.5 months. Splenic weight and immunological traits in the spleen were assessed.

A) Splenic weight (n=30-65), B) Frequency of GC B cells, C) Frequencies of CD4⁺ T cell subsets – naïve and effector memory cells (published in *Celhar et al., 2018* [49]).

We found that deletion of TLR9 on *Sle1* led splenomegaly (Figure 1.1 A) and expansions of germinal centre (GC) B cell and CD4⁺ memory T cell population in the spleen (Figure 1.1 B and C) [49].

Figure 1.2 Severe kidney disease in *Sle1*TLR9^{-/-}

Aged female B6, TLR9^{-/-}, *Sle1*, and *Sle1*TLR9^{-/-} mice were analysed for lupus disease parameters. (A) Representative images of glomerular nephritis in *Sle1* and *Sle1*TLR9^{-/-},

(B) CD45⁺ leukocyte infiltration in kidneys. Statistical significance was assessed by 1-way ANOVA (with Bonferroni's multiple comparisons test) or Student's t test and non-parametric data by Kruskal-Wallis (with Dunn's multiple comparisons test) or Mann-Whitney test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (published in *Celhar et al., 2018* [49]).

We also found that the aged female *Sle1*TLR9^{-/-} mice have severe kidney disease (Figure 1.2 A) and elevated CD45⁺ leukocyte infiltration into kidney (Figure 1.2 B). Furthermore, the levels of anti-nucleosome autoantibodies were significantly decreased in *Sle1*TLR9^{-/-} mice compared to *Sle1* controls (Figure 1.3 A). However, we detected increased anti-dsDNA, snit-snRNP, and anti-RNA antibodies using by ELISA (Figure 1.3 A). Analyses of Ig levels in serum showed that the absence of TLR9 conferred increases in serum levels of IgG2a/c, IgG2b, and IgM compared to *Sle1* controls, while IgA titres were decreased (Figure 1.3 B) [49].

Figure 1.3 Elimination of TLR9 breaks self-tolerance

Production of ANAs and Immunoglobulin (Ig) isotypes in the aged *Sle1* and *Sle1*TLR9^{-/-} mice were analysed. (A) Auto-antibody production in sera, and (B) Level of IgG subtypes in serum. All data is from 6 independent cohorts, each dot represent an individual mouse. Statistical significance was assessed by 1-way ANOVA (with Bonferroni's multiple comparisons test) or Student's t test and non-parametric data by Kruskal-Wallis (with Dunn's multiple comparisons test) or Mann-Whitney test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (published in *Celhar et al., 2018*[49])

1.4 Contribution of leukocytes in autoimmune murine models

There are several studies that implicate a role of different cell types in TLR9-deficient autoimmunity. For instance, Shlomchik and colleagues demonstrated that increased pDC maturation was seen in MRL^{lpr}TLR9^{-/-} mice [46]. They also showed that the absence of TLR9 in B cells prevented the spontaneous production of anti-DNA auto-Abs in MRL^{lpr} mice [50]. However, the exact cellular events of TLR9-deficient autoimmunity are unclear.

1.4.1 B cells

B cells have been implicated as key players in TLR-mediated systemic autoimmune responses, including SLE. Stimulation of murine B cells with TLR7 and TLR9 agonists enhances the expression of B cell co-stimulatory molecules and induces cell proliferation, production of cytokines, differentiation into APCs and switching of Ig sub classes [51, 52]. Germinal centres (GC) are where B cells generate high-affinity pathogenic autoantibodies by interacting with follicular DCs and follicular T helper cells. Spontaneous GC (Spt-GC) B cells play an important role in murine models of lupus and human SLE [35]. Previous works from others and our lab (unpublished) have shown that TLR9^{-/-} deficient lupus models have an increase in GC formation. Therefore, it may be possible that *Sle*/TLR9^{-/-} B cells could be hyperactive and therefore driving disease.

1.4.2 Dendritic cells

Dendritic cells (DCs) are antigen presenting cells (APCs) that play important roles in both innate and adaptive immune system, secretion of several inflammatory cytokines and chemokines and presentation of antigens to T and B cells. There are two main functional groups of DCs – conventional DCs (cDCs) and plasmacytoid DCs (pDCs) [53, 54]. In mice, cDCs are further classified into CD8 α ⁺ and CD103⁺-lymphoid DC as cDC1, and CD11b⁺ myeloid DCs as cDC2 [54]. TLR9 is expressed on both CD11b⁺ DCs and CD8 α ⁺ DCs but not on CD8 α ⁻-lymphoid DCs [55, 56].

Functionally cDCs are phagocytic and specialised for antigen-presentation [53]. Immature cDCs express low levels of MHC and B7 (CD80/CD86) molecules. After activation by potential pathogens, MHC-I, II and B7 receptors are upregulated on the cell surface enabling their interaction and stimulation of naive T cells. Upon activation, cytokines are also secreted [57].

Our published study which has shown a contribution of cDCs in a severe kidney disease in autoimmune mouse model – *Sle1Tg7* [58]. A mouse model *Sle1Tg7* has overexpression of TLR7 (2-fold compared to B6 mouse) and develops severe disease like *Sle1TLR9^{-/-}* mice including splenomegaly, remarkably higher score for glomerular nephritis with leucocyte infiltration in kidney, and increased activation of B cells and T cells in the spleen [59]. In this study, we found that a mouse model crossed *Sle1Tg7* with CD11c^{Cre} reporter strain (CD11c^{Cre}*Sle1Tg7*), which has reduction of splenic CD11b⁺ DC [60], had improved disease phenotypes that were observed in *Sle1Tg7* [58]. This suggests that cDCs also play a role in driving disease.

pDCs are characterised by the surface expression of B220, BST2, CD11c^{lo} and Siglec-H in mice and BDCA2 in human [53, 54]. TLR7 and 9 are highly expressed in both mouse and human [17, 54]. Development of pDCs is dependent on the transcript factor E2-2 and growth factor fms-like tyrosine kinase 3 (Flt-3) [61, 62]. Activation of the cells also results in increased upregulation of co-stimulatory and MHC class II molecules and survival [54]. In addition to this, vast amounts of IFN α are secreted following stimulation [63]. Several reports have described detectable levels of IFN α in the sera of patients with SLE [64, 65], but more consistently an IFN α -gene signature has been described in the peripheral blood monocytes (PBMCs) of patients with SLE [66]. More recently Rodriguez-Pla and colleagues demonstrated that in addition to type I IFN, SLE blood monocytes require signals by TLR agonists such as LPS to upregulate CCR7 transcript factor in blood monocytes and gain the migratory DCs property [67].

1.5 Other factors in SLE development

There are many of other factors that are associated with SLE; among these, hormones are known to be important in dictating the severity of disease. Svenson et al. demonstrated that oestrogen receptor alpha (ER α) knockout lupus prone female mice had remarkable less pathogenesis of the disease and prolonged survival [68]. Furthermore, Cunningham and colleague have demonstrated that the inflammatory response to TLR9 activation was diminished in ER α^{ko} mice and the population of pDC was reduced in NZM2410 ER α^{ko} mice compared to NZM2410 ER α^+ group [69]. Therefore, they suggest that the receptor plays an important role in TLR-mediated inflammation and generation of pDCs [69].

1.6 Microbiota and Autoimmunity

1.6.1 Human microbiome

The human microbiome comprises a rich ecosystem of microbes that are essential to human health and physiology.

The initial development and maturation of the neonatal microbiome is largely determined by maternal–offspring exchanges of microbiota. Regardless of intrauterine exposure, massive bacterial colonization of the newborn occurs at birth upon exposure to vaginal, fecal, and skin microbiota [70-72]. Chu and colleagues investigated the microbiota structural and functional changes from birth to age 4–6 weeks in several body sites, and they showed that microbial-community structure and function substantially expand and diversify in all body sites from birth to age 4–6 weeks. It then resembles microbiota from its corresponding maternal body site, independently of the infant's mode of delivery or other prenatal factors [73]. In the adult, the microbiota inhabiting each body site is characterized by a distinct microbial community structure and function [74-77]

Enormous number of microorganisms reside on the surface of the body - the skin, oral, gastrointestinal, respiratory and urogenital tracts. The microorganisms play important roles in maintaining each area's and systemic health, and also are relate to

diseases. For instance, microorganisms from the oral cavity have been shown to cause a number of oral infectious diseases, including caries (tooth decay), periodontitis (gum disease), endodontic (root canal) infections, alveolar osteitis (dry socket), and tonsillitis. Evidence is accumulating which links oral bacteria to a number of systemic diseases [78, 79], including cardiovascular disease [79, 80], stroke [81], preterm birth [82], diabetes [83] and pneumonia [84].

The skin also carries millions of bacteria, fungi and viruses that compose the skin microbiota. skin microorganisms have essential roles in the protection against invading pathogens, the education of our immune system and the breakdown of natural products [85-87]

1.6.2 Gut microbiota

The gut microbiota is a large complex community of bacteria in gastrointestinal tract (GIT), and the number of bacteria is thought to be as many as 10^{14} in the gut [88]. The estimated number of bacterial species in the human gut varies across studies, but a recent investigation suggested that the collective human gut microbiota consists of over 35,000 bacterial species [89]. Furthermore, the composition of the gut microbiome is different from individual to individual and it also varies between different sites within the gut. Frank *et al* reported that *Actinobacteria* the *Bacilli* class of the Firmicutes were enriched in the small intestine. On the other hand, the *Lachnospiraceae* family of the Firmicutes and *Bacteroidetes* were more abundant in colon [89].

Colonisation of microbes in the gut starts at birth. Infants are exposed to bacteria through birth canal. The intestinal microbiota is established during the first year of life, and the bacteria composition in the gut is relatively simple at that point. However, after 1-year time, the gut microbiota in children start to have similar microbiota as young adult and it stabilises [90, 91].

Additionally, it was reported that the gut microbiota of monozygotic and dizygotic twins was similar to gut microbiota in their siblings. This suggested that the colonization by the microbiota from a shared mother was more critical in determining their adult microbiota than their genetic background [92].

On top of the microbiota passed from mothers, there are many factors suggested to affect building up the microbiome in mammalian. Numerous studies have shown that genetic background has an impact on the bacteria composition in the gut. Sex difference is one of genetic factor influence gut microbiota [93]. Benson et al showed host genetic control in shaping individual microbiome diversity in murine models [94]. Moreover, mice carrying a mutation in high density lipoprotein had different microbiota. [95].

1.6.3 Gut microbiota and immune systems

Under healthy conditions, the gut microbiota is regularly maintained by the mucosal immunity. Besides the physical barrier between the bacteria in the lumen and the epithelial cells, other types of barrier such as a thick layer of mucus, lamina-propria resident macrophages and DCs, secretory IgAs, and anti-mycobacterial peptides guard the mucosal surface for pathogenic agents [96] (Figure 1.4, [97]). Intestinal macrophages kill pathogens directly by phagocytosis. DCs prime B cells and T cells by presenting antigens to them to add another line of defence against pathogens in the gut [96].

Figure 1.4 Anatomy of the intestine, and immune system in the gut

A single layer of intestinal epithelial cells (IECs) provides a physical barrier that separates the trillions of commensal bacteria in the intestinal lumen from the underlying lamina propria. The IECs lining the lumen are bathed in nutrients, commensal bacteria, IgA and goblet cell-produced mucus. Epithelial stem cells proliferate and give rise to daughter cells with the potential to proliferate. These IECs then differentiate into villous or colonic enterocytes, which absorb nutrients (small intestine) and water (colon). In addition to differentiated enterocytes and goblet cells, progenitor IECs differentiate into both enteroendocrine cells, which secrete enteric hormones, and Paneth cells at the base of the small intestinal crypts. Beneath the IECs, the lamina propria is made up of stromal cells (myofibroblasts), B cells (especially IgA-producing plasma cells), T cells, macrophages and dendritic cells. Certain subsets of T cells and dendritic cells localize between the IECs. The small intestine has regions of specialized epithelium termed follicle-associated epithelium and microfold (M) cells that overlie the Peyer's patches and sample the intestinal lumen. IEL, intraepithelial lymphocyte.

Figure obtained from Aberu MT, 2010

<https://www.nature.com/reprints/permission-requests.html>

The use of animal models has largely contributed to understand how the gut microbiota impacts the development or maintenance of immune system. Germ-free (GF) mice, that are born and raised in a sterile environment lack commensal gut microbiota. They have smaller isolated lymphoid follicles [98] and Peyer's patches compared to mice in specific-pathogen free (SPF) [99], as well as other defects in number and function of both myeloid and lymphoid cells [99].

1.6.4 Gut microbiota and Autoimmunity

Recently, there is increasing evidence suggesting that there is a relationship between intestinal microbiota and autoimmunity in patients and murine models.

The K/BxN strain is a spontaneous mouse model of arthritis. Recent studies show that commensal bacteria influence the autoimmune phenotypes of this strain. K/BxN mice did not exhibit arthritis when the mice were housed in a germ-free environment. This was associated with a decrease in the production of autoantibodies, decreased GC formation, and reduced Th17 cells compared to conventionally housed K/BxN mice [100]. It was shown that mono-colonisation of segmented filamentous bacterium (SFB) to the small intestine of K/BxN mice was sufficient to induce CD4⁺ T helper cells that produce IL-17

and IL-22 (Th17 cells) in the lamina propria [100, 101]. Furthermore, Th17 cells induced by SFB in lamina propria were able to re-circulate to the brain, and it resulted in inflammation in experimental autoimmune encephalitis (EAE), a mouse model of multiple sclerosis [102].

More recently, there is emerging evidence that there is dysbiosis and gut-pathology across many lupus models. MRL/lpr mice had decreased levels of in *Lactobacillaceae* together with an increase the family *Lachnospiraceae* [103] compared to MRL controls. Interestingly, unlike other autoimmune disorders that have a decrease in diversity in the gut microbiota associated with disease, there was an increase in diversity in MRL/lpr mice compared to non-disease mice [103]. In the study by Zhang H. *et al.*, there were also sex-related changes in the gut microbiota in lupus- prone mice compared to healthy controls. Female mice were more likely to have an increase in *Lachnospiraceae* and this was associated with the development of severe disease [103].

1.7 Preliminary work on the microbiome in the Fairhurst lab

In preliminary studies, the Fairhurst lab sought to determine the impact of TLR9 deficiency on the microbiota. Data from these investigations demonstrated that there was a significant increase of total bacteria in TLR9^{-/-} mice after the mice were transferred from BRC3 (SPF+) to BRC2 (SPF) compared to B6 mice (Figure 1.5 A). Despite the increase of total bacteria in TLR9^{-/-} mice the composition of the major bacterial species measured were similar in both comparisons of pre and post and B6 and TLR9^{-/-} (Figure 1.5 B). This total increase correlated with disease phenotypes which included splenic weight and the increased frequency and activation of splenic CD4⁺ T cells in TLR9^{-/-} mice (Figure 1.6, B). Further strain-dependent analysis did not reach statistical significance (data not shown). Whether this plays an important role in the development of disease in TLR9-deficient lupus strains remains to be determined.

Figure 1.5 Comparison of bacteria change in the gut - Pre; 8 weeks old vs. Post; 5-6mos old, in B6 and TLR9^{-/-} mice

Stool samples were collected at 8 wks old as Pre and 5-6 mos old as Post. Purified faecal DNA was analysed by 16S rRNA qPCR for (A) total bacteria copy number, (B) composition of bacteria sub-strains; *Erec*, *Bact*, *Lact*, and *SFB* out of total bacteria. N=10 per group.

Figure 1.6 Immunological traits in the spleen of B6 and TLR9^{-/-} mice and correlation between total bacteria in the gut and immune phenotypes.

Splenic weight and immunological traits in the spleen in B6 and TLR9^{-/-} mice were assessed at 5-6 mos old. (A) splenic weight (left), and frequency of CD4⁺ memory T cells. These data were correlated with bacteria copy number in faecal DNA samples. (B) correlation between total bacteria copy number and splenic weight (left), and with memory T cells (right).

To ascertain whether the bacterial number affected the splenic immunological phenotype, mice were treated with antibiotics (Penicillin and Streptomycin) for 12 weeks to disrupt the microflora. These studies showed that TLR9^{-/-} mice had higher splenic weights compared to B6 controls in both the absence and presence of antibiotics (Figure 1.7). However, this difference was not significant following antibiotic treatment and may be due to increased variability observed in the B6 mice post treatment (Figure 1.7). Furthermore, a major confounding element of this experiment was that the untreated group and the treated group were assessed on different days.

Figure 1.7 Splenic weight at 5-6mos old mice in absence and presence of antibiotics
One group of B6 and TLR9^{-/-} mice were treated with a mixture of antibiotics; Penicillin and Streptomycin, for 3mos and another group of each strain were given normal water as controls. Mice were euthanised at the age of 5-6mos. N=7-10 mice per group.

Analyses of immunological phenotypes indicated that TLR9^{-/-} mice had a lower frequency of CD62^{hi}CD44^{lo} CD4⁺ naive T cells and a corresponding increase in memory-like CD62^{hi}CD44^{lo} T cells compared to B6 controls. (Figure 1.8 A and B). Antibiotic treatment had no impact on these changes. Furthermore, while CD4⁺CD25⁺foxp3⁺ regulatory T cells (T_{reg}S) were more frequent in untreated TLR9^{-/-} mice compared to B6 controls, antibiotic treatment decreased this change so that there was no difference in T_{reg} frequencies (Figure 1.8 C). CD4⁺PD1⁺CXCR5⁺ T follicular helper cells (T_{fh}S) were more frequent in TLR9^{ko} in absence or presence of antibiotics compared to B6 mice (Figure 1.8 C).

Figure 1.8 Comparison of immunological traits of T cell subsets in the spleen
Mice were euthanised at 5-6 mos age and immunological traits in the spleen were analysed by Flow cytometry for (A) CD4⁺ naïve T cells, (B) CD4⁺ memory T cells, (C) T_{reg}; CD25⁺Foxp3⁺, and (D) T_{fh} cells.

1.8 Hypothesis and aims of Thesis

Taken together, the evidence in the literature together with the preliminary data generated in the Fairhurst lab suggests that gut microbiota influences on disease phenotypes in autoimmune prone mice. Moreover, gut microbiota is altered by diseases. However clear evidence for relationships between SLE and microbiome is not determined yet. We therefore hypothesised that the SLE is also related to microbiota, possibly to specific bacteria. Also, disruption of gut microbiota may cause change in the disease phenotypes and severity.

The overall goal was to evaluate whether the microbiota influences inflammation and disease development and determine whether the deficiency of TLR9 in severe disease has an impact on gut microbiome. The aims to achieve this, the first aim is to investigate immunological traits together with gut microbiota and compare wild type (B6) control and TLR9^{-/-} on B6 background to investigate a role of TLR9 on non-disease background. We also investigated facility differences that are the mice aged in a less clean facility (BRC2) and in a clean facility (BRC3).

Secondly, the aim was to investigate an impact of TLR9 in the microbiome in severe disease of SLE and to investigate whether there was the same trend as the comparison between B6 and TLR9^{-/-}. Lastly, the aim was to analyse relationships and trends between mouse strains and each bacteria species by 16S rRNA analysis. Facility difference was also investigated.

The third aim was to analyse 16S rRNA genome sequencing data to correlate mouse strains in order to determine which bacteria is related to an absence of TLR9 and disease. In addition to the facility differences, differences in mothers of the mice were also investigated as environmental changes and mothers largely affects microbiome too.

2 Materials & Methods

2.1 Overall flow of the experiments

Figure 2.1 Flowchart of experiments

Figure 2.1 shows overall flow of the experiments. This chapter explains about materials and methods used in this study. As it mentioned in the figure, 16S rRNA genome sequencing analysis was conducted by a collaborator at Genome Institute of Singapore (GIS).

2.2 Mice

Female C57BL/6 mice (B6) were originally obtained from the Jackson laboratory (BarHarbor, ME). Female TLR9-deficient (TLR9^{-/-}) mice were originally made by Akira and colleagues and backcrossed onto the B6 background more than 8 times [104]. *Sle1*

breeding pairs were generously donated by Dr. Ward Wakeland. The *sle1* region is flanked by the microsatellite markers, D1Mit17 and D1Mit202 [105].

All animals were maintained in pathogen-free conditions at the Biomedical Resource Centre, A-*STAR, Singapore. Mice were housed in either BRC2 which was certified specific pathogen free (SPF) and BRC3, which was certified SPF+ (a cleaner level of facility). Table 2.1 shows differences in numbers of pathogen screened in each facility. All procedures conformed to IACUC guidelines.

2.3 Verification of strain identity by PCR genotyping

2.3.1 Preparation of DNA from mouse tail lysate

To confirm the genotype of the mice before experiments were started, tails (0.5 cm or less) were processed for DNA extraction and genotyping by polymerase chain reaction (PCR). Tail snips were transferred to a 1.5 ml centrifuge tubes and 75 μ L of alkaline lysis buffer (recipe in Appendix IV) per tail was added. The centrifuge tubes containing the tail and lysis buffer were heated at 95° C for 1 h. Following rapid cooling at 4° C for 5 min, 75 μ L of neutralizing buffer was subsequently added. Lysed tails were vortexed and centrifuged 10 secs at 10000 g, room temperature (RT). Supernatant containing DNA was stored at 4° C.

2.3.2 PCR genotyping

PCR reactions in a total volume of 25 μ l contained 10-100 ng of template DNA, 200 μ M of each of dNTP, 25 mM MgCl₂, reverse and forward primers at 660 nM in 10X reaction buffer (Taq polymerase buffer) diluted in distilled water to a 1 X final concentration. Cycle conditions were as follow: 35 cycles of 95°C for 30 seconds, 55-58°C for 30 seconds, 72°C for 1 minute.

2.4 Mouse tissue sample preparation

2.4.1 Spleen processing

Spleens were harvested, smashed and filtered immediately into chilled plain Roswell Park Memorial Institute-1640 (RPMI) media. Splenocytes were spun down at 1500 rpm for 5 minutes at 4°C. The pellet was resuspended in 1 mL of plain RPMI for cell counting and viability was assessed by Trypan blue exclusion. Splenocytes were then diluted to a concentration of 2×10^6 cells/mL using RPMI.

2.4.2 Kidney processing

Following perfusion with warm PBS to remove excessive blood from the tissue both kidneys were extracted and put in cRPMI. Connective tissue was removed using two curved forceps and one kidney was chopped by razor blade in a 4 cm petri dish. After homogenisation, the tissue was carefully transferred into 1.7 mL tube and plate was washed with 500 μ L of ice-cold PBS. Samples were spun down at 13000 rpm, 7 min, 4 C. The supernatant was carefully aliquoted and stored at -80 °C until cytokine analysis. Tissue was transferred to 50 mL falcon tube and incubated 30 min at 37 °C, 1500rpm with 10 mL collagenase A (Roche). After incubation, cells were syringed with 23G twice and 21G once and spun down at 4 C, 1500 rpm, 5 min. After washing, syringing was repeated, and cells were transferred through 70 μ m (micron) nylon mesh and topped up with fresh cRPMI to 20 ml. The cell suspension was layered over 40% percoll and spun down at 2000g (no break) for 20 min, in order to pellet the immune cells.

Following centrifugation, the supernatant was carefully aspirated, and cells were washed 2 times with PBS. The cells were resuspended in 1000 μ L of SB and 200 μ L per well was aliquoted for flow cytometry staining (see below).

2.4.3 Peyer's patches preparation

The small intestine and mesenteric lymph nodes were removed. Peyer's patches were separated from the small intestine using a microscope. The mesenteric lymph nodes and Peyer's patches were placed into 12-well plate containing 1mL of 10% FCS RPMI per well. 1mL of 0.2mg/mL of collagenase/ 0.01mg/mL of DNase was added per well. Each lymph node was torn apart with 25G needles, and then the samples were incubated for 30mins at 37°C. The samples were homogenised with an 18/19G syringe and filtered through 70um nylon mesh directly into FACS tubes. The plate was washed with 1mL of staining buffer. The tubes were centrifuged for 4 minutes at 1350rpm and 4°C and the supernatant discarded. The cell pellet was then resuspended in 1mL of staining buffer and the number of cells counted using trypan blue with a microscope.

2.5 Enzyme-Linked Immune Sorbent Assay (ELISA)

2.5.1 ANA productions in the serum

Serum autoantibodies were measured using ELISAs detecting anti-nucleosomes (histones and dsDNA), anti-dsDNA, anti-snRNP or anti-RNA. Bound IgG was detected with alkaline phosphatase-conjugated anti-mouse IgG (Jackson Immuno Research Laboratories) using para-nitrophenyl phosphate as a substrate (Sigma). Absorbance was measured at 405/410nm. Results are shown as arbitrary units (AU) that were calculated as absorbance at 405 nm (sample minus blank). For anti-RNA, serial dilutions of standards which is serum pooled from disease mice were used to generate a standard curve.

2.5.2 Immunoglobulin (Ig) isotypes productions in the serum

Ig subtypes (IgA, IgG1, IgG2a/c, IgG2b, IgG3 and IgM) were measured using the mouse immunoglobulin isotyping bead panel (EMD Millipore), following manufacturer's

instructions. Luminex plates were read on the Flexmap 3D System (Luminex Corp) with the Bio-plex Manager version 6.0.

2.6 *FACS analysis*

2.6.1 *Cell surface staining*

The cell suspension and the antibody cocktail were prepared in staining buffer (S.B.) consisting of 1 x PBS (Gibco) with 2% FBS (Hyclone) and 15mM HEPES (Gibco). An fc-block solution (anti-Fc γ RII/III) was added to the cells in order to block unspecific binding and incubated for 15 minutes. The Fc-block solution was prepared from the supernatant of the 2.4G2 hybridoma which produces anti-Fc γ RII/III monoclonal antibodies. Supernatants were titrated for optimal dilutions for inhibiting non-specific binding to the Fc receptors. This blocking step was followed by incubation with the antibody cocktails as indicated. After 30min of incubation, the compensation controls and samples were washed three times with S.B. The samples and the compensation controls were resuspended in S.B.

2.6.2 *Intracellular staining*

Cells stained with surface markers were washed 3 times in 150ul of staining buffer and spun down at 1500 rpm, 4 °C, 5 min. Subsequently cells were washed in 100ul (1X) permeabilization buffer and stained for 30 min on ice with antibodies of interest diluted in 1X Fix/Perm buffer. After staining, cells were washed 3 times in 1X Fix/Perm buffer spun down at 1500 rpm, 4 °C, 5 min, resuspended in 200ul of staining buffer and run in flow cytometer.

Both compensation controls and samples were analysed by FACS on a BD LSR Fortessa flow cytometer, BD Symphony, BD LSRII 5L or BD FACS CantoII. Data was analysed with Flowjo software version 7.6.1 (Tree Star, Ashland, USA).

All the antibodies were purchased from Ebioscience, Biolegend, Invitrogen or BD pharmingen. Further antibody details are included in Appendix I .

2.6.3 Gating strategies

Compensation was checked on every sample, batched experimental recompensation was completed if necessary using FlowJo and checked by an independent user. A specific gating strategy was used depending on cell tissue or cell type according to the figures below, following an initial gating strategy to eliminate duplicates and dead cells (figure 2.1): First, leukocytes were gated using forward and side scatter (FSC and SSC respectively) parameters. Doublets were excluded using FSC and SSC height and area parameters. Live cells were further gated using CD45 and Aquazombie⁻ (L/D). Where possible the live CD45⁺ population was further gated on EpCAM⁻, which is expressed on non-immune cells (Figure 2.2).

Figure 2.2 Gating strategy for live CD45⁺ cells
These images are from spleen samples as an example.

2.6.4 FMO control for detection of true population and MFI in flow cytometry

Median Fluorescence Intensity (MFI) is a representation of the amount of antibodies bound to the cells. The gating and subsequent calculation was performed based on fluorescence minus one (FMO) control samples, where all the fluorochromes were added except the fluorochrome of interest. Gating was set on positive values above the FMO value.

2.7 Stool sample collection and Antibiotic treatment for Microbiome Studies

The stool samples were collected by placing each mouse in a small container separately and faeces were collected from the container. The time points for sample collections were pre1; 8 weeks old in BRC3 (all), pre2; 10 weeks old in both BRC3 (no-transfer) and BRC1 (transferred mice) and post; 5-6 months old. The samples were kept in individual Eppendorf tubes at -80°C until DNA extraction.

Antibiotic water was given to groups of B6, TLR9^{-/-}, *Sle1*, *Sle1*/TLR9^{-/-} mice in BRC3. 1000mg of Streptomycin (Sigma Aldrich) and 500mg of PenicillinG (Sigma Aldrich) were dissolved in 500mL of ultrapure water, according to protocols by our collaborator, Pavelka [106]. The antibiotic water was changed every 3-4 days.

2.8 Analysis of faecal DNA

2.8.1 Transformation

Plasmid transformation was performed using 4 different bacteria plasmids which include sequences coding for 16 rRNA specific for *Ruminococcus productus* (RUM), *Lactobacillus acidophilus* (LACT), *Bacteroides fragilis* (BACT) and *Segmented Filamentous bacteria* (SFB plasmid CT5-6). All plasmids contain the Ampicillin

resistance cassette. The plasmids were a kind gift from Nita Salzman lab (Children's Research institute, Medical College of Wisconsin, USA).

100µl of thawed XL10-Gold Ultracompetent cells (Agilent Technologies) were transferred into a new 1.5ml microcentrifuge tube. Subsequently, 4µl of β-ME came with the cell was added to each aliquot of the cell. The cells were incubated on ice for 10 minutes with swirling the cell gently every 2 minutes. For each strain of bacteria, add 50ng of plasmid and swirl the tubes gently. After 30 minutes of incubation at 4°C, heat-shock was performed by placing the tubes 42°C for 30s, followed by incubation on ice for another 2 minutes. 900µl of *Luria* Bertani (LB) broth (Biopolis supply facility, A*STAR) was added into individual tubes of bacteria and the cells were incubated at 37°C for 1 hour with shaking at 250rpm. 5µl of the bacteria culture was added onto LB agar plate supplemented with 100ng/ml Ampicillin (Sigma). The plates were carefully streak and incubated the plates overnight at 37°C.

2.8.2 *Plasmid DNA Extraction*

Plasmid DNA was extracted from the transformed cells containing the plasmids using the QIAprep Spin Miniprep Kit (Qiagen). DNA concentration was measured using the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific) as described (Rajilić-Stojanović, 2007). The extracted plasmid DNA samples were stored at -80°C.

2.8.3 *Plasmid DNA Concentrations*

After plasmid transformation, DNA was extracted from the colonies of transformed cells and the concentration was measured using NanoDrop (Thermo Fisher Scientific). The DNA concentrations obtained for each type of plasmids are shown in Table 5 which includes the DNA concentrations as well as the purity of the DNA expressed as the ratio of A260/280 and A260/230. The ratio of A260/280 measures the purity of nucleic acid and the accepted range is from 1.8 to 2.0 (Thermo Fisher Scientific).

2.8.4 Faecal DNA Extraction for real-time Polymerase Chain Reaction (qPCR)

Bacterial genomic DNA was extracted from frozen faecal samples using the QIAamp DNA Stool Mini Kit (Qiagen) as per manufacturer's protocol [107]. DNA was extracted from the columns with 100µl for samples that have less than 100mg of stool and with 200µl for samples more than 100mg. DNA concentration was measured using the NanoDrop 2000 UV-Vis Spectrophotometer. The extracted DNA was stored at -20°C.

2.8.5 Principal of SYBR Green I dye

SYBR Green-I can bind to the minor groove of double-stranded DNA, which greatly enhances its fluorescence. It does not bind to single-stranded DNA. During the consecutive PCR cycles, the amount of double-stranded PCR product will exponentially increase, and therefore more SYBR Green I dye can bind and emit its fluorescence (at 520 nm). The fluorescence signal will gradually increase during the extension phase and will be maximal at the end of each extension phase. The signal will be low or absent during the denaturation phase (Van der Velden VH *et al.*, 2003). The increase in fluorescence intensity is directly proportional to the amount of amplicon produced.

Figure 2.3 Schematic image of SYBR Green I dye interacting with double-stranded DNA

2.8.6 Rational for the choice of SYBR Green

This method allows detection of the amplification of any double-stranded DNA sequence. No probe is required therefore use of this dye can reduce assay setup and running costs.

2.8.7 Quantitative Real-time PCR amplification of 16S rRNA Sequence (Barman et al. 2008)

Genomic DNA extracted from the stool samples was subjected to real time PCR analysis for Eubacteria (all bacteria), *Eubacterium rectale*, *Lactobacillus*, *Bacteroides* and *Segmented Filamentous Bacteria* (SFB) using specific groups of 16S rRNA gene primers (listed in table 2) that target the 16S rRNA bacterial gene sequences. Bacterial copy numbers were determined using standard curves constructed using known copy number of 16S rRNA incorporated into plasmid standards specific for each bacterial group analysed. Raw data from real-time PCR does not provide the actual number of bacteria or colony-forming unit (CFU) directly, but we can calculate the bacteria copy numbers by Ct values of known 16S rRNA gene copy from standards [107].

Target Organisms	Primer	Sequence (5' to 3')	Tm (°C)
Total bacteria (<i>Eubacteria</i>)	Forward	ACT CCT ACG GGA GGC AGC AGT	66.5
	Reverse	ATT ACC GCG GCT GCT GGC	64.5
<i>Eubacterium rectale</i>	Forward	ACT CCT ACG GGA GGC AGC	64.5
	Reverse	GCT TCT TAG TCA GGT ACC GTC AT	62.8
<i>Lactobacillus acidophilus</i>	Forward	AGC AGT AGG GAA TCT TCC	58.0
	Reverse	CAC CGC TAC ACA TGG AG	59.6
<i>Bacteroides fragilis</i>	Forward	GGT TCT GAG AGG AGG TCC C	64.5
	Reverse	GCT GCC TCC CGT AGG AGT	64.5
<i>Segmented Filamentous Bacteria</i>	Forward	GAC GCT GAG GCA TGA GAG CAT	64.5
	Reverse	GAC GGC ACG GAT TGT TAT TCA	60.2

Table 2.1 Primer sequence for identification of specific bacterial strain

Write legend here

The plasmids' copy numbers are calculated from their respective concentrations (g/μl) based on the formula below:

$$\text{Copy number in molecules per } \mu\text{l} = \frac{\text{Concentration in grams per } \mu\text{l}}{\frac{\text{Molecular weight}}{\text{Avogadro's number}}}$$

For each plasmid the DNA concentration was measured using NanoDrop and the molecular weight was calculated by multiplying the number of molecule of template per gram (660g/mole per primer pair) with the length of the plasmid (5400bp), giving an estimated molecular weight of 356,400. The Avogadro's number is 6.022×10^{23} . The copy number dilution required differs between different strains of bacteria (determined previously in the Fairhurst lab) and the dilution was done according to Table 2.2 (below).

Bacteria genera	Primer stock concentration (μM)	Primer concentration for qPCR (nM)	Copy number dilution for standard curve	Annealing temp. (°C)
Total (RUM plasmid)	10	100	$10^8, 10^7, 10^6, 10^5, 10^4$	63
Erec (RUM plasmid)	10	100	$10^7, 10^6, 10^5, 10^4$	63
Lact (LACT)	10	100	$10^7, 10^6, 10^5, 10^4$	60
Bact (BACT)	10	100	$10^8, 10^7, 10^6, 10^5, 10^4$	63
SFB (SFB)	10	50	$10^6, 10^5, 10^4, 10^3, 10^2, 10^1, 10^0$	60

Table 2.2 qPCR conditions for each bacterial strain

Including primers' concentrations, plasmid copy number dilutions for the generation of standard curves and annealing temperature.

Real-time PCR was completed using Power SYBR® Green PCR Master mix (Applied Biosystems). For each real-time PCR reaction, the final volume for each sample including the plasmid DNA was 10μl. For different strains of bacteria, different final primer concentrations were required (determined previously in the Dr Fairhurst lab, listed in Table 2.3). The master mixes were prepared according to Table X3. The final amount used for each stool DNA sample was 20ng for the DNA from untreated mice and 50ng for the DNA sample from antibiotic treated mice, and hence the DNA samples were

diluted with ultrapure sterile water to a concentration of 10ng/μl or 25ng/μl and 2μl of template was used for each reaction (Table 2.3, below).

	For reaction with 100nM final primer concentration	For reaction with 50nM final primer concentration	
Syber Green (2x stock)	5	5	
Forward primer (10μM)	0.1	0.05	
Reverse primer (10μM)	0.1	0.05	
Nuclease-free water	2.8	2	
template	2	2.9	
Total volume per well	10	10	(μL)

Table 2.3 Primer concentrations and master mix preparation for qPCR

Sample preparation was completed on ice and the real-time PCR analysis was done using 384-well plates. The master mixes were pipetted into each well, followed by the 2μl of the selected DNA sample. All pipetting steps were completed on ice.

After pipetting, the plates were sealed with plastic film (Applied Biosystems) and centrifuged for 2 minutes at 1500rpm. Real time PCR was performed using 7900HT Fast Real-Time PCR System and SD2.4.2. software for Windows.

The enzyme activation step was completed at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 sec and annealing steps at 63°C for 60 sec. (RUM and BACT primers) or or 60°C for 60 sec (LACT and SFB primers) (refer to table 1 for primer sequences). This was followed by a dissociation stage at 95°C for 15 seconds, 60°C for 15 seconds and 95°C for 15 seconds.

2.8.8 Specificity of the Primers used in qPCR- Dissociation Curve of the Amplified Products

The dissociation curve step after qPCR was performed to obtain the melting temperature of the amplified products. The melting temperature shows the temperature

of the double stranded DNA separating into single stranded DNA. When the primers used in the qPCR reaction are specific, only one specific sequence is amplified resulting in one unique peak in the dissociation curve. As shown in the Figure 2.4, the dissociation curves of Total bacteria, *Erec*, *Lact*, *Bact* and *SFB* amplicons gave one specific peak.

Figure 2.4 Melting temperature of the amplified bacterial 16S rRNA sequences from stool DNA samples

The dissociation curves of the products amplified with primers for (A) Total Bacteria, (B) *Eubacterium rectale* (*Erec*), (C) *Lactobacillus* (*Lact*), (D) *Bacteroides* (*Bact*) and (E) *Segmented filamentous bacteria* (*SFB*).

2.8.9 Generation of standard curve

A series of dilution of known bacteria plasmid template concentrations can be used to establish a standard curve for determining the initial starting amount of the target template in experimental samples or for assessing the reaction efficiency. The log of each known concentration in the dilution series (x-axis) is plotted against the Ct value for that concentration (y-axis). From this standard curve, information about the performance of the reaction as well as various reaction parameters (including slope, y-intercept, and correlation coefficient) can be derived. The concentrations chosen for the standard curve

should encompass the expected concentration range of the target in the experimental samples.

2.8.10 Slope of standard curve

The slope of the log-linear phase of the amplification reaction is a measure of reaction efficiency. To obtain accurate and reproducible results, reactions should have an efficiency as close to 100% as possible, equivalent to a slope of -3.32 (see Efficiency, below, for more detail).

Figure 2.5 Standard curve plotted using the Ct values from Real time PCR analysis
Graphs were plotted using the log of the diluted plasmid copy numbers (x-axis) against the Ct values (y-axis).

2.8.11 Efficiency of PCR reaction

A PCR efficiency of 100% corresponds to a slope of -3.32, as determined by the following equation:

$$\text{Efficiency} = 10^{(-1/\text{slope})} - 1$$

Ideally, the efficiency (E) of a PCR reaction should be 100%, meaning the template doubles after each thermal cycle during exponential amplification. The actual efficiency can give valuable information about the reaction. Experimental factors such as the length, secondary structure, and GC content of the amplicon can influence efficiency. Other conditions that may influence efficiency are the dynamics of the reaction itself, the use of non-optimal reagent concentrations, and enzyme quality, which can result in efficiencies below 90%. The presence of PCR inhibitors in one or more of the reagents can produce efficiencies of greater than 110%. A good reaction should have an efficiency between 90% and 110%, which corresponds to a slope of between -3.58 and -3.10.

2.8.12 Absolute quantification

Absolute quantification describes a real-time PCR experiment in which samples of known quantity are serially diluted and then amplified to generate a standard curve. Unknown samples are then quantified by comparison with this curve.

2.8.13 Relative quantification

Relative quantification describes a real-time PCR experiment in which the expression of a gene of interest in one sample (i.e., treated) is compared to expression of the same gene in another sample (i.e., untreated). The results are expressed as fold change (increase or decrease) in

expression of the treated in relation to the untreated. A normalizer gene is used as a control for experimental variability in this type of quantification.

2.9 *Genome sequencing by Genome Institute of Singapore (GIS)*

2.9.1 *Introduction and workflow*

The V3-V6 region on prokaryotic 16S rRNA was amplified from total DNA. Amplicons were sheared using the Covaris LE220 sonicator (Covaris, Inc., USA) and built into sequencing libraries using GeneRead DNA Library I Core Kit (Qiagen) according to the manufacturer's protocol. DNA libraries were multiplexed by 96 indices, pooled, and sequenced on the Illumina HiSeq 2500 using paired-end (2x76bp) sequencing. Sequencing reads were demultiplexed (Illumina bcl2fastq 2.17.1.14 software) and filtered (PF=0) before conversion to FASTQ format. Reads were trimmed by removal of trailing bases with quality score ≤ 2 . Read pairs containing reads shorter than 60bp were also removed.

2.9.2 *Bioinformatics and analysis workflow*

Full length 16S rRNA reconstructions were produced from the short sequencing reads using the EMIRGE amplicon (Expectation Maximisation Iterative Reconstruction of Genes from the Environment) algorithm. The analysis is largely based on the workflow described in 2 with updated tools and pipeline (https://github.com/CSB5/GERMS_16S_pipeline). EMIRGE uses 16S rRNA sequences on the SILVA database for template guided assembly of reconstructions, followed by an expectation-maximization algorithm to iterate, align, and assign reads to candidate 16S rRNA sequences. This iterative mapping of paired-end reads also prevents chimeric sequences from mapping. Reconstructed sequences that have at least 99% sequence similarity are collapsed into OTUs, and Graphmap 6 was used to map these OTUs to the Greengenes global rRNA database (dated May, 2013; greengenes/13_5/99_otus.fasta). OTUs were called at various levels of identity (phylum, family, genus, species, etc.) dependent on the sequence similarity to the Greengenes database as defined by Yarza et al., 8. Hits below predefined percent identity (97% at the species level, 94.5% at the genus level, 86.5% at the family level and 75% at the phylum level) were not considered for classification and removed. EMIRGE assigns abundance estimates to reconstructed

sequences. The relative abundance of OTUs was generated for each sample and converted to relative abundances at the phylum, family, genus and species level.

2.9.3 Methods and results - Data visualization and statistical analyses

Differences in bacterial community composition among the four infant groups were assessed using multivariate analysis based on Bray-Curtis distances:

1. The distance-based redundancy analysis (db-RDA) with the capscale function of vegan package in R (version 3.4.2).
2. The principle coordinate analysis (PCoA) with the capscale function of vegan package in R.

Sankey plots are drawn by group and by timepoint and represent the flow of taxonomical information from domain to species, with a custom script (<https://github.com/BetaCollins/Sankey>). To assess the variability in the dataset we plotted individual stacked area plots, which were plotted on R using package ggplot2. Individual bacterial species were visualized by interquartile bar plots, which were plotted on R using package ggplot2. Statistical significance of relative abundance between two groups was assessed by non-parametric Mann-Whitney U-test ($p < 0.05$). The network plots are constructed by Spearman's rank correlation coefficients between bacteria using the Hmisc packages in R, and the Spearman's rank correlation coefficient > 0.5 were visualized in the Cytoscape software (version 3.6.1). Shannon's alpha diversity was obtained for each sample and plotted on R using package ggplot2.

2.10 Statistical Analysis

Comparison of the mean values was completed with GraphPad Prism (Version 7.04) software. Assessment of normal distribution was done by using the Kolmogorov-Smirnov test. Gaussian data was analysed by a Student's t-test for 2 comparisons and ordinary one-way ANOVA with post hoc Bonferroni's multiple comparison test for

comparisons of 3 or more. Non-parametric data was assessed using Mann-Whitney test for 2 comparisons and Kruskal-Wallis test with post hoc Dunn's multiple comparisons test for 3 or more comparisons. using the unpaired two-tailed Student's *t* tests, paired two-tailed Student's *t* tests and one-way ANOVA test. $p < 0.05$ was considered to be statistically significant. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

3 Results

3.1 Impact of TLR9 on microbiota

The overall goal was to assess the impact of the microbiome in *Sle1*TLR9^{-/-} mice. To initiate these series of experiments we began by assessing the relationship between the microbiome and TLR9 in the absence of the *Sle1* susceptibility region.

Immunological traits for T cells, B cells, leukocyte lineage, and DCs were assessed by flow cytometry at the endpoint.

To measure the 16S rRNA absolute bacteria copy number by qPCR, stool samples were collected, and bacteria DNA was purified as described in the materials & methods (section 2.8.4) at two time-points: pre: 8-10 weeks old and post: the end of the experiment (5-6 months old). The DNA samples were also sent to a collaborator at Genome Institute of Singapore (GIS) for genome sequencing and its data analysis.

All the data shown in section 3.1 is cumulative from two separate cohorts, total n = 26 (B6) and n = 24 (TLR9^{-/-}).

Figure 3.1 Experimental plan for comparison of B6 and TLR9^{-/-} mice

Schematic image of experimental plan. B6 and TLR9^{-/-} in BRC3 were rearranged into groups at 8 weeks old. The mice were then transferred to BRC2 and the half of the mice

in each strain were treated with a mixture of antibiotics; Streptomycin and Penicillin. The mice were euthanised at the age of 5-6 month. N=6-10 per abx treated or untreated mice groups.

To determine whether antibiotic (Abx) treatment alters the immunological phenotypes in B6 and TLR9-deficient mice, we examined splenic weight, immunological traits in spleen and immunoglobulin (Ig) production in serum.

Figure 3.2 Pathological traits in mice and total bacteria copy number measured by 16S rRNA qPCR.

Mice were euthanised at the age of 5-6 mos. The faecal DNA was purified from stool samples 1-2days before mice were euthanised. (A-left) splenic weight from aged mice, (A-middle&right) comparisons of cecum contents weight after 3 mos of abx treatment, and a representative image of caecum with and without abx, (B) Absolute total bacteria 16S rRNA copy number in the gut. Statistical values were determined using by 1-way ANOVA (with Bonferroni's multiple comparisons test) for parametric data or non-parametric data by Kruskal-Wallis (with Dunn's multiple comparisons test) for comparisons of 3 or more; *. Unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) for 2 comparisons; #. Error bars represent mean $\pm$ SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. N=7-10 mice, two independent experiments.

Previous data from the Fairhurst lab has shown that TLR9^{-/-} mice develop mild splenomegaly compared to B6 mice [49]. However, in this study we did not observe an increase in splenic weight. Furthermore, antibiotic (Abx) treatment did not affect splenic weight in both strains. The cecum content weight was significantly higher in both B6 and TLR9^{-/-} mice following Abx treatment showing an effective response to Abx [108] (Figure 3.2 A).

Assessment of total bacteria using the 16S rRNA copy number in the purified faecal DNA showed that Abx effectively eliminated the microbiota in TLR9-deficient mice. However approximately half (n=5) of B6 mice showed antibiotic resistance, with detectable 16S rRNA levels that were around 50% of original levels (Figure 3.2 B). Therefore, we assessed sub-strains of bacteria in the DNA samples by qPCR, and analysis revealed that *Bacteroides fragilis* (*Bact*) remained, and other 3 sub-strains; *Eubacterium rectale* (*Erec*), *Lactobacillus*, *Bacteroides* (*Lact*) and *Segmented Filamentous Bacteria* (*SFB*) were eliminated (Figure 3.3).

Figure 3.3 Inefficient Abx treatment on *Bact*

16S rRNA total bacteria copy number was analysed at the end-point of the experiment, the red square indicates the samples that was inefficient Abx treatment. Statistical values were determined using unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) for 2 comparisons; #. Error bars represent mean ± SEM. # $P \leq 0.05$, ## $P \leq 0.01$. N=3-5 mice per group, from one experiment.

We next analysed immunological traits in the spleen to assess systemic immune responses. Changes in systemic immunological traits are summarised in Table 3.1. Previous data has shown an expansion in the T follicular helper population (T_{fh}) across multiple lupus-prone strains [49, 109]. Furthermore, germinal centre (GC) B cells are increased and myelopoiesis is present in NZW and BXSB derivative strains [35, 49, 110].

Cell types	Subsets	B6	TLR9 ^{-/-}
T cells	CD4 ⁺ T cells	-	-
	CD4 ⁺ memory T cells	-	-
	CD4 ⁺ naïve T cells	-	-
	T _{fh}	-	↓ [#]
	CD25 ⁺ Foxp3 ⁺ T _{reg}	-	-
	CD8 ⁺ T cells	-	-
	CD8 ⁺ memory T cells	↓ [#]	-
	CD8 ⁺ naïve T cells	↑ [#]	-
B cells	B cells (B220 ⁺ CD138 ⁻)	-	-
	Plasma cells	-	-
	Plasmablast	-	-
	GC B cells	-	↓ (<i>p</i> =0.0635)
	Marginal zone B cells	-	↓ [*]
Myeloid cells	CD11b ⁺ Gr1 ⁺ mono	↓ (<i>p</i> =0.0609)	↓ [#]
	PMNs	↓ ^{####}	↓ [#]
	CD11b ⁺ cDCs	-	-

Table 3.1 Summary of immunological changes led by Abx treatment (Spleen)

Immunological traits in the spleen were analysed by Flow cytometry Images of gating strategy and comparison of frequencies for CD4⁺ T cells, CD4⁺ T cell subsets, CD8⁺ T cells, CD8⁺ T cell subsets, (B) Naïve and memory T cells within CD4⁺ or CD8⁺ compartment, (C) T_{fh} cells, and (D) T_{reg} cells. The B6 mice that were inefficient Abx treatment were highlighted in blue. Statistical values were determined using by 1-way ANOVA (with Bonferroni's multiple comparisons test) for parametric data or non-parametric data by Kruskal-Wallis (with Dunn's multiple comparisons test) for comparisons of 3 or more; *. Unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) for 2 comparisons; #. Error bars represent mean ± SEM. */# P ≤ 0.05, **/### P ≤ 0.01. n=7-10 mice, two independent experiments.

Figure 3.4 Immunological traits of T cell subsets in the spleen in B6 and TLR9^{-/-} mice

Immunological traits in the spleen were analysed by Flow cytometry Images of gating strategy and comparison of frequencies for (A) CD4⁺ and CD8⁺ cells, (B) Naïve and memory T cells within CD4⁺ or CD8⁺ compartment, (C) T_{fh} cells, and (D) T_{reg} cells. The B6 mice that were inefficient Abx treatment were highlighted in blue. Statistical values were determined using by 1-way ANOVA (with Bonferroni's multiple comparisons test) for parametric data or non-parametric data by Kruskal-Wallis (with Dunn's multiple comparisons test) for comparisons of 3 or more; *. Unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) for 2 comparisons; #. Error bars represent mean $\pm$ SEM. */# $P \leq 0.05$, **/## $P \leq 0.01$. n=7-10 mice, two independent experiments.

In these studies, we defined T follicular helper cells (T_{fh}) cells as CD3⁺CD4⁺ and ICOS⁺CXCR5⁺ (Figure 3.4 C). Consistent with our previous findings (Figure 1.8), the frequency of T_{fh} was significantly higher in the spleens from TLR9^{-/-} mice compared to B6 controls. Furthermore, Abx administration resulted in a decrease in the T_{fh} population in TLR9^{-/-} mice but not in B6 counterparts (Figure 3.4 A).

Figure 3.5 Immunological traits of B cell subsets in the spleen in B6 and TLR9^{-/-} mice

Immunological traits in the spleen were analysed by Flow cytometry Images of gating strategy and comparison of frequencies for (A) Plasma cells and Plasmablasts, (B) Total B cells, (C) GC B cells, (D) Marginal zone (MZ) B cells, and Follicular (Fo) B cells. The B6 mice that were inefficient Abx treatment were highlighted in blue. Statistical values were determined using by 1-way ANOVA (with Bonferroni's multiple comparisons test) for parametric data or non-parametric data by Kruskal-Wallis (with Dunn's multiple comparisons test) for comparisons of 3 or more; *. Unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) for 2 comparisons; #. Error bars represent mean $\pm$ SEM. */# $P \leq 0.05$, **/## $P \leq 0.01$. n=7-10 mice, two independent experiments.

Analysis of additional leukocytes within the spleen identified other changes as a result of Abx. Germinal centre (GC) B cells were gated as B220⁺CD138⁻CD19⁺GL7⁺ (Figure 3.5 C). Although not significant, we observed an increase in GC B cells in untreated TLR9^{-/-} mice compared to B6 controls ($p=0.0635$). In a similar manner to our findings on T_H cells, Abx administration decreased the frequency of the GC B cell population significantly in TLR9^{-/-} mice but not in B6 counterparts (Figure 3.5 C).

Taken together this data suggests that the increase observed in T and B cell activation with TLR9-deficiency may be mediated by microbiota.

Figure 3.6 Immunological traits of myeloid lineage in the spleen in B6 and TLR9^{-/-} mice

Immunological traits in the spleen were analysed by Flow cytometry. (A) Images of gating strategy, and comparison of frequencies for (B) Eosinophils, (C) CD11b⁺Gr1⁺ monocytes, (D) PMNs, (E) CD11b⁺cDCs (MZ) B cells, and Follicular (Fo) B cells. The B6 mice that were inefficient Abx treatment were highlighted in blue. Statistical values were determined using unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) for 2 comparisons. Error bars represent mean $\pm$ SEM. # $P \leq 0.05$, ### $P \leq 0.001$. n=7-10 mice, two independent experiments.

Analysis of the myeloid lineage showed that Abx treatment also had an impact on frequencies of Gr1⁺ myeloid cells and polymorphonuclear leukocytes (PMNs) in both B6

and TLR9^{-/-} mice. There was no difference observed following Abx in conventional dendritic cells (cDC) in either strain (Figure 3.6 D).

Figure 3.7 Circulating Immunoglobulins in the serum measured by Luminex

Production of Ig in the serum was measured by Luminex. IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM were assessed. The B6 mice with inefficient Abx treatment were highlighted in blue. Statistical values were determined using by 1-way ANOVA (with Bonferroni's multiple comparisons test) for parametric data or non-parametric data by Kruskal-Wallis (with Dunn's multiple comparisons test) for comparisons of 3 or more; *. Unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) for 2 comparisons; #. Error bars represent mean ± SEM. */# P ≤ 0.05. N=6-10 mice, two independent experiments.

GC B cells and T_h cells play an important role in the generation of antibodies. Therefore, we went on to examine the serum immunoglobulin titres by Luminex. We did not observe any strain dependent differences in untreated mice, except the level of IgG3. It was significantly high in TLR9^{-/-} compared to B6 controls (Figure 3.7). However, in B6 mice,

Abx reduced IgG1 and IgG2a titres. In contrast, Abx treated TLR9^{-/-} mice had a higher level of IgG2b compared to B6 mice with the treatment (Figure 3.7).

Figure 3.8 Correlations between total bacteria copy numbers and immunological traits

The mice with inefficient Abx treatment were highlighted in blue. Correlation was computed by GraphpadPrism as the value of the Pearson correlation coefficient, r. * P ≤ 0.05 N=6-10 mice per genotype, two independent experiments.

We then assessed the association of total bacteria (using the 16S rRNA copy number) with different splenic immunological phenotype known to be associated with the deficiency of TLR9 [46]. There were strain specific significances in T_{fh} cell frequencies and the GC B cell population. In TLR9^{-/-} mice, there were positive correlation between total bacteria copy number and frequencies of T_{fh} and GC B cells, but no was observed in B6 mice. Therefore, this data suggests that bacteria in the gut may contribute to the immune activation observed in the absence of TLR9 on the B6 background.

3.2 Impact of gut microbiota on *Sle1* and *Sle1TLR9*^{-/-}

Previous data has shown that the administration of antibiotics improves symptoms of rheumatoid arthritis [100, 111]. Moreover, a recent study showed that antibiotics ameliorated lupus-like symptoms in MRL/lpr mice, including splenomegaly [112]. Therefore, we went on to evaluate whether antibiotics affected the severe disease traits observed in *Sle1TLR9*^{-/-} mice compared to *Sle1* controls. Furthermore, in these studies we set out to determine if immunological phenotypes are related to the gut microbiota composition.

Due to the limitations of institutional housing, all experiments were completed in BRC2. All the data shown in 3.2 is total n = 18 (*Sle1*) and n = 17 (*Sle1TLR9*^{-/-}).

Figure 3.9 Experimental plan for comparison of *Sle1* and *Sle1TLR9*^{-/-}

Schematic image of experimental plan. *Sle1* and *Sle1TLR9*^{-/-} in BRC2 were rearranged into groups at 8 weeks old. The half of each strain of mice were treated with a mixture of antibiotics; Streptomycin and Penicillin. The mice were euthanised at the age of 4-4.5 months. N=7-9 per groups

We followed the same experimental setup described for B6-strains shown in in Figure 3.1. However, the period of Abx administration was shortened by 2 months to avoid complications in the experiment from the mortality, observed at 6mos *Sle1TLR9*^{-/-} mice. Mice were euthanised at approximately 4-4.5-month age. and immunological traits in the

spleen, kidney, mesenteric lymph node (mLN), and Peyer's patches (PPs) were. Stool samples were collected according to the same times as the previous cohort, and bacterial DNA was extracted for qPCR analysis and genome sequencing by GIS. In addition, serum titters of immunoglobulin subtypes and ANAs were measured by Luminex and ELISAs respectively. Kidney function was assessed by measuring blood urea nitrogen (BUN) and Creatinine levels.

Figure 3.10 Splenic weight, caecum contents weight, and total bacteria copy number measured by 16S rRNA qPCR

The mice with inefficient Abx treatment were highlighted in blue. (A) left; splenic weight from aged mice, middle & right; graph of cecum contents weight at 5-6 mos age, and representative image of cecum with and without Abx at the end-point, (B) Indication

of kidney disease by ratio of albumin/creatinine (left) and Protein urea (right), (C) Absolute total bacteria 16S rRNA copy number in the gut. The faecal DNA was purified from mice 1-2days before the date the endpoint; 4-5mos age. Statistical values were determined using by 1-way ANOVA (with Bonferroni's multiple comparisons test) for parametric data or non-parametric data by Kruskal-Wallis (with Dunn's multiple comparisons test) for comparisons of 3 or more; *. Unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) for 2 comparisons; #. Error bars represent mean $\pm$ SEM. */# $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. N=7-9 mice per group, one experiment.

Consistent with our previous findings [49], *Sle1*/TLR9^{-/-} developed an increase in splenomegaly compared to *Sle1* controls. However, Abx administration did not reduce splenomegaly, in contrast to the findings by Mu Q and colleague in MRL-lpr mice [112] (Figure 3.2 A).

Analysis of caecum content weight showed increases in both *Sle1* and *Sle1*/TLR9^{-/-} mice, as a result of Abx treatment, with no difference between the strains (Figure 3.10). However, despite the enlargement of ceca which is consistent with effective Abx administration, our PCR on 16S rRNA showed that the total gut bacteria were not efficiently depleted in both strains (Figure 3.10). This may be caused by antibiotic-resistant bacteria to the type of antibiotics we used to this. In these studies, we utilized a combination of Streptomycin and Penicillin (Pen/Strep), according to our collaborators protocol. Recent studies have shown similar resistance following Pen and Strep administration [113], and that it can be overcome by using a different mixture of antibiotics [112, 114, 115].

Figure 3.11 Immunological traits of T cell subsets in the spleen in *Sle1* and *Sle1TLR9*^{-/-} mice

Immunological traits in the spleen were analysed by Flow cytometry. Comparisons of frequency for (A) CD4⁺ and CD8⁺ T cells, (B) Naïve and memory T cells in CD4⁺ and CD8⁺ compartments, and (C) T_h cells, and (D) T_{reg} cells. The mice that were inefficient Abx treatment were highlighted in blue. Statistical values were determined using by 1-way ANOVA (with Bonferroni's multiple comparisons test) for parametric data or non-parametric data by Kruskal-Wallis (with Dunn's multiple comparisons test) for comparisons of 3 or more; *. Unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) for 2 comparisons; #. Error bars represent mean $\pm$ SEM. */# P $\leq$ 0.05, ** P $\leq$ 0.01, *** P $\leq$ 0.001, **** P $\leq$ 0.0001. N=7-9 mice, one experiment.

Evaluations of immunological traits in the spleen were completed in a similar way to B6/TLR9^{-/-} comparisons. Analysis of T cell subsets in the spleen showed that, the frequency of T_h cells was significantly higher in *Sle1*/TLR9^{-/-} mice compared to *Sle1* controls consistent with previous findings on a 6mos cohort [49] (Figure 3.11 A). In contrast to the findings in the B6/TLR9^{-/-} cohort, there was no reduction following Abx treatment. Moreover, the frequencies appeared to be slightly higher in both strains following Abx. The frequency of CD4⁺ T cell was higher while the frequency of CD8⁺ T cell was lower in *Sle1*/TLR9^{-/-} mice compared to *Sle1* controls (Figure 3.11 A). Expansion of CD4⁺ memory T cell compartment and reduction in CD4⁺ naïve T cell population in *Sle1*/TLR9^{-/-} mice were observed. These were consistent with our previous finding [49]. However, these populations were not altered by Abx treatment (Figure 3.11 B).

Figure 3.12 Immunological traits of B cell subsets in the spleen in *Sle1* and *Sle1TLR9*^{-/-} mice

Immunological traits in the spleen were analysed by Flow cytometry. Comparison of frequencies for (A) Plasma cells and Plasmablasts, (B) Total B cells, (C) GC B cells, (D) MZ B cells, and (E) Fo B cells. The mice that were inefficient Abx treatment were highlighted in blue. Statistical values were determined using by 1-way ANOVA (with Bonferroni's multiple comparisons test) for parametric data or non-parametric data by Kruskal-Wallis (with Dunn's multiple comparisons test) for comparisons of 3 or more; *. Unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) for 2 comparisons; #. Error bars represent mean $\pm$ SEM. */# $P \leq 0.05$. $n=7-10$ mice, two independent experiments.

GC B cells were gated on Fas⁺GL7⁺ after gating on the total CD138⁺B220⁺CD19⁺ B cell population. The frequency of GCs B cells was also significantly higher in 4.5mos old *Sle1*TLR9^{-/-} mice compared to their *Sle1* counterparts (Figure 3.12 C). Abx treatment had no significant impact on GC B cell frequency in both *Sle1* and *Sle1*TLR9^{-/-} mice.

Figure 3.13 Immunological traits of myeloid lineage in the spleen in *Sle1* and *Sle1*TLR9^{-/-} mice

Immunological traits in the spleen were analysed by Flow cytometry. Comparisons of frequencies for (A) Eosinophils, (B) CD11b⁺Gr1⁺ monocytes, (C) PMNs, (D) CD11b⁺cDCs. The mice that were inefficient Abx treatment were highlighted in blue. Statistical

values were determined using by 1-way ANOVA (with Bonferroni's multiple comparisons test) for parametric data or non-parametric data by Kruskal-Wallis (with Dunn's multiple comparisons test) for comparisons of 3 or more; *. Unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) for 2 comparisons; #. Error bars represent mean $\pm$ SEM. * $P \leq 0.05$, ** $P \leq 0.01$, ### $P \leq 0.001$. n=7-10 mice, two independent experiments.

A wider analysis of leukocyte lineages in the spleen revealed that CD11b⁺ conventional dendritic cells (cDCs) were increased in *Sle1TLR9*^{-/-} compared to *Sle1* controls, consistent with our previous data [49]. Abx treatment had no effect on this population in either strain. We did not observe an increase in the frequencies of PMNs and Gr1⁺ populations in *Sle1TLR9*^{-/-} mice at this age, in contrast to our data from an older 6mos cohort [49]. However, interestingly, Abx treatment reduced the frequency of PMNs regardless of the strain (Figure 3.13 C). These data suggest that first line innate immune cells, such as neutrophils and monocytes may be affected by antibiotic treatment. However, this is not sufficient to affect the characteristic and severe autoimmune phenotypes exhibited by *Sle1TLR9*^{-/-} mice.

Peyer's patches (PPs) are the major organized lymphoid organs of the small intestine. Their location enables immune cells to interact with bacteria and their metabolites, or their components through M cells in the gut. Furthermore, PPs have important relationships with innate and adaptive immune responses [116]. Secretory IgA is the most abundant antibody secreted by intestinal plasma cells. It plays important roles in maintaining homeostasis by interacting with the microbiome. IgA also plays a role in protection from infections in the intestine [117, 118]. It is produced by intestinal plasma cells following B cells interaction and activation by subepithelial DCs in PPs [119].

Therefore, we decided to assess if there was any difference in immune phenotypes in B cells in PPs. We also checked T cell populations. Table 3.2 is a summarised list of changes in immunological phenotypes in PPs.

Cell types	Subsets	<i>Sle1</i>	<i>Sle1</i> TLR9 ^{-/-}
T cells	CD4 ⁺ T cells	-	↓ ****
	CD4 ⁺ memory T cells	-	-
	CD4 ⁺ Naïve T cells	-	-
	T _{reg}	↓ **	↑ ****
	T _{fh}	-	-
	CD8 ⁺ T cells	-	↓ *
	CD8 ⁺ memory T cells	-	-
	CD8 ⁺ Naïve T cells	-	-
	CD8 ⁺ Naïve T cells	-	-
B cells	B cells (B220 ⁺ CD138 ⁻)	-	-
	GC B cells (GL7 ⁺)	-	-
	Plasmablasts	-	-
	IgA ⁺ GC B cells	↓ #	↓ #
	IgA ⁺ non-GC B cells	-	-
	IgG ⁺ GC B cells	-	-
	IgG ⁺ non-GC B cells	-	-

Table 3.2 Summary of changes in immunological phenotypes in PPs led by Abx treatment

Immunological traits in PPs were analysed by Flow cytometry. Comparison of frequencies for T cell subsets, total B cells, B cell subsets, and IgA⁺ B cells within GC (GL7⁺). These data are shown in Figure 3.14. Statistical values were determined using by 1-way ANOVA (with Bonferroni's multiple comparisons test) for parametric data or non-parametric data by Kruskal-Wallis (with Dunn's multiple comparisons test) for comparisons of 3 or more; *. Unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) for 2 comparisons; #. Error bars represent mean ± SEM. */# P ≤ 0.05, ** P ≤ 0.01, ***/#### P ≤ 0.001, ****P ≤ 0.0001. N=7-9 per group.

Figure 3.14 Immunological phenotypes in PPs

Immunological traits in PPs were analysed by Flow cytometry. Comparison of frequencies for (A) T cell subsets, (B) Total B cells, and (C) IgA⁺ B cells within GC (GL7⁺). Statistical values were determined using by 1-way ANOVA (with Bonferroni's multiple comparisons test) for parametric data or non-parametric data by Kruskal-Wallis (with Dunn's multiple comparisons test) for comparisons of 3 or more; *. Unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) for 2 comparisons; #. Error bars represent mean $\pm$ SEM. */# $P \leq 0.05$, ** $P \leq 0.01$, ***/### $P \leq 0.001$, **** $P \leq 0.0001$. N=7-9 per group.

Cellular composition of PPs in wild type (WT) mice was evaluated by Barreau *et al.* [120]. It was shown that there were mostly B cells (B220⁺) at approximately 60%, around 25% of T cells (CD3⁺), 10% of DCs (CD11c⁺) and less than 5% of macrophages (F4/80⁺) or PMNs (Ly-6G⁺) [116, 120]. Within T cell compartment, approximately 45% was CD4⁺, 35% was CD8⁺, and 20% of the population was double negative (CD4⁻CD8⁻) T cells. Moreover, about 85% of CD4⁺ T cells were memory T cells (CD25⁻CD45RB^{lo}), 10% were Naïve (CD25⁻CD45RB^{hi}), and 5% were T_{reg}s (CD25⁺CD45RB^{lo}) [116, 120].

In this study we assessed T cells and B cells in the PPs. Consistent with the study by Barreau *et al.*, majority of CD45⁺ cells were B cells (B220⁺CD138⁻) in both *Sle1* and *Sle1TLR9*^{-/-} mice. Frequency of B cells was significantly low in *Sle1TLR9*^{-/-} mice

compared to *Sle1* controls (Figure 3.14 B). The second largest population was T cells. Unlike the composition of CD4⁺ T cells in WT mice [120], there was mostly CD4⁺ T cells and CD8⁺ T cell compartment was little (Figure 3.14 A). *Sle1*TLR9^{-/-} had a significantly high frequency of CD4⁺ T cells compared to *Sle1* mice, while there was no difference in a frequency of CD8⁺ T cells between the two strains (Figure 3.14 A). In addition, *Sle1*TLR9^{-/-} mice had a remarkably low frequency of T_{reg}s; CD4⁺CD25⁺Foxp3⁺ compared to *Sle1* controls (Figure 3.14 A right). These results suggested that immune cells in PPs from the mice lack TLR9 in *Sle1* were more sensitive to bacteria change in the gut.

Investigation on the immunological traits in PPs revealed that frequencies of CD8⁺ and CD4⁺ cells were reduced with Abx in *Sle1*TLR9^{-/-} mice but not in *Sle1* controls (Figure 3.14 A). Treated *Sle1* mice had reduced ICOS expression on CD4⁺ T cells, which is a marker for activation (Figure 3.14 A). The T_{reg} compartment in treated *Sle1*TLR9^{-/-} was restored to the same level as untreated *Sle1* controls. In contrast to this, treated *Sle1* mice had a lower frequency of T_{reg}s compared to untreated mice (Figure 3.14 A right). Moreover, the populations of total B cells in both strains were reduced. Similarly, the frequencies IgA⁺ B cells were also reduced in the GC following Abx, regardless of strain (Figure 3.14 C).

Figure 3.15 Circulating Ig subtypes in the serum measured by Luminex
Production of Ig in the serum was measured by Luminex. IgG1, IgG1, IgG2a, IgG2b, IgG3 IgA, and IgM were assessed. Statistical values were determined using by unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) for 2 comparisons. Error bars represent mean $\pm$ SEM. # $P \leq 0.05$, ## $P \leq 0.01$. N=7-9 per group.

In our previous work by our lab, we showed that deficiency of TLR9 in *Sle1* mice resulted in significantly higher concentrations of serum immunoglobulin (Ig) G2a, IgG2b and IgM antibodies, and lower level of IgA antibodies compared to *Sle1* controls [49].

Data from these 4-4.5 mos old mice presented here showed no increase in Ig G2a, IgG2b and IgM antibodies or decrease in IgM in the serum in *Sle1*TLR9^{-/-} compared to *Sle1* mice (Figure 3.15). However, this evaluation of serum Ig subtype levels reflected the decrease of IgA⁺ B cell population observed in PPs. The IgA levels were significantly lower in Abx treated *Sle1* and *Sle1*TLR9^{-/-} mice (Figure 3.15 left). On the contrary, IgM levels were higher following Abx administration, possibly due to less switching to IgA⁺ by B cells in PP GCs (Figure 3.15). Furthermore, Abx treatment had no effect on the serum levels of any Ig subtype.

Figure 3.16 ANA production in the serum measured by ELISA

Serum was diluted at 1:100 for anti-RNA and 1:200 for the rest. AU (arbitrary units) represent absorbance at 405nm (sample - blank). Statistical values were determined using by unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) for 2 comparisons. Error bars represent mean ± SEM. # $P \leq 0.05$, ## $P \leq 0.01$. N=7-9 per group.

Production of auto-antibodies such as anti-nucleosome, anti-dsDNA, anti-snRNP, and/or anti-RNA antibodies is a characteristic feature of SLE. Our previous findings indicated that *Sle1TLR9^{-/-}* mice had remarkably high levels of anti-dsDNA, anti-snRNP, and anti-RNA antibodies in the serum compared to *Sle1* controls at 6mos of age [49].

Consistent with the finding, analysis of ANA productions by ELISA showed that levels of anti-dsDNA antibodies and anti-RNA antibodies were significantly higher in 4mos old *Sle1TLR9^{-/-}* mice compared to *Sle1* controls (Figure 3.16). There was no difference observed in the levels of anti-snRNP or anti-nucleosome autoantibodies. Abx administration did not affect auto-antibody titers in either strain.

In the previous study on severe disease in the mouse model, we also found that expression of TLR7 was upregulated systemically in absence of TLR9 in *Sle1* [49].

Figure 3.17 Systemic upregulation of TLR7 on immune cells in the spleen
Expression levels were verified using B6.TLR7^{-/-} mice and fluorescent minus one (FMO) control. Statistical values were determined using by Student's t test and by Mann-Whitney test for non-parametric data. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. (published in *Celhar et al., 2018* [49])

TLR7 expressions were significantly higher on splenic pDC, F4/80⁺ macrophages, CD11b⁺ DCs, CD11c⁺MHCII⁺ cells, and B cells (as B220⁺) (Figure 3.17). In addition, the majority of cells infiltration in kidney in *Sle1*/TLR9^{-/-} were CD11b⁺Gr1^{neg-lo} DCs. The infiltrated cDCs in kidney in the mice also overexpressed TLR7. Furthermore, we performed DC functional assays and determined that the severe disease model was driven by the infiltrated cDC compartment [49].

Therefore, we went on to assess TLR7 expression on cells in this microbiome cohort.

Figure 3.18 Relative TLR7 expressions on splenic immune cells in microbiome cohort

Expression levels were verified using B6.TLR7^{-/-} mice and fluorescent minus one (FMO) control. Statistical values were determined using by Student's t test for parametric data and by Mann-Whitney test for non-parametric data. *P<0.05, **P<0.01, ***P<0.001, **** P<0.0001.

At pre-disease, some of changes were consistent with our finding from aged mice. TLR7 expressions on pDCs and B cells were higher compared to *Sle1* mice (Figure 3.18). The expression on CD11b⁺ DC in *Sle1*TLR9^{-/-} mice was the same level as *Sle1* mice (Figure 3.18), which was not consistent to the published result. In contrast to the published study, CD11c⁺MHC⁻ cells in *Sle1*TLR9^{-/-} expressed lower level of TLR7 compared to the cells in *Sle1* (Figure 3.18). It was shown in our previous work that CD11c⁺MHC⁻ cells were associated to the disease [58]. Mild autoimmunity at this pre-disease stage may be associated with these lower TLR7 expressions on CD11b⁺ DC and CD11c⁺MHC⁻ cells compared to the expressions in post-disease. The kidney disease in *Sle1*TLR9^{-/-} was not severe yet due to young age but the infiltrated CD11b⁺ DC in kidney in the mice expressed

higher TLR7 than the DCs in *Sle1* mice. As we expected, there was no TLR7 expression on CD4⁺ and CD8⁺ T cells. Taken together, our SLE mouse model at 4-4.5 mos did not have severe disease as it's still in pre-disease, however, the mice already started to develop some autoimmune phenotypes in terms some of immune cell activations, serological profile and expressions of TLR7 on pDC and B cells.

3.3 *16S rRNA sequencing analysis*

Addition to our faecal DNA analysis by qPCR, we sent DNA samples for 16S rRNA sequencing to a collaborator at Genome Institute of Singapore (GIS) in order to go into more detailed analysis of gut microbiota. All the processing and analyses for genome sequencing was completed as described in the methods section, 2.9.

3.3.1 Bacteria compositions is changed by time and strains

Figure 3.19 Bacteria composition in B6, TLR9^{-/-}, Sle1, and Sle1TLR9^{-/-} mice

Bacteria composition in faecal DNA was analysed and compared. Stool samples for each cohort; cohort1, cohort 2, and a cohort for Sle1/Sle1TLR9^{-/-} mice, were collected at different timing. The graph was generated by data programming software, R. N=8-10 per group for B6/TLR9^{-/-}, and n=7-9 per group for Sle1/Sle1TLR9^{-/-}.

We analysed bacteria compositions in the 4 mouse strains by cohort. A comparison of B6 and TLR9^{-/-} microbiota implicated that the composition was different between the two cohorts. Cohort1 was conducted approximately 6mos earlier than cohort 2.

We observed that the bacteria composition of *Clostridiales* was lower in B6 mice in the second cohort compared to the first. In contrast, this bacteria strain was expanded in TLR9^{-/-} mice in cohort 2 compared to cohort 1 (Figure 3.19, left). Interestingly, there were

This analysis revealed that there was clear separation between mice with a B6 background and those with the *Sle1* locus (Figure 3.20). This suggests that the *Sle1* background has a significant impact on the gut microbiota. However, it was uncertain that whether this separation was due to the different facilities where the mice were born. All B6 and TLR9^{-/-} mice were born in BRC3 and transferred from BRC3 to BRC2, while *Sle1* and *Sle1*TLR9^{-/-} mice were born in BRC2 where they stayed until the experiment was terminated.

Therefore, we decided to take more stool samples from other mice in all the facility/rooms that we house our mice and assessed gut microbiota to ascertain whether this separation was truly an effect of the *Sle1* background.

We began by validating the results of the Genome sequencing using PCR. We chose bacteria strains that were significantly high or low in *Sle1* mice compared with B6 controls.

Name of bacteria strain	B6 background	<i>Sle1</i> background
<i>Prevotellaceae</i>	Low	High
<i>Bacteroides</i>	Low	High
<i>Oscillospira</i>	Low	High
S24.7	High	Low
<i>Rikenellaceae</i>	High	Low

Table 3.3 Bacteria strains that were significantly different between B6 and *Sle1* background

5 bacteria strains were chosen from a list of analysis of bacteria abundance by GIS. We picked the bacteria strains that are significantly different between B6 background and *Sle1* background (Table 3.3). *Prevotellaceae*, *Bacteroides*, and *Oscillospira* were the top 3 bacteria strains higher in *Sle1* background mice. S24.7 and *Rikenellaceae* were the top two bacteria strains lower in mice with the *Sle1* background compared to the B6/TLR9^{-/-} mice.

3.4 Primer design and optimisation for qPCR

We designed primers for those 5 bacteria strains by using Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Partial or full genome sequences of the chosen bacteria were searched on NCBI website and the sequences were used for designing primers.

3.4.1 Primer test by PCR

To check specificity of each primer, we ran PCR by following the same method as genotyping (section 2.3). Two types of DNA samples were used from the samples in our microbiome cohorts for this test; “high” as a sample contains the target bacteria higher, and “low” as a sample contains the target bacteria lower than others. Those were picked by the analysis of bacteria abundance by GIS. We also ran water as a control.

Figure 3.21 Gel image of PCR products for primer test
(a) Water, (b) DNA sample contains the target bacteria high, (c) DNA sample contains the target bacteria low. This applies to all the 5 primers of bacteria strains

The results of the PCR showed that there are specific bands in expected product size of the primers for *Oscillospira* (408-418bp), *Rikenellaceae* (220bp), and S24.7 (340bp) (Figure 3.21). However, the PCR products of the primers for *Prevotellaceae* and *Bacteroides* had a few bands (Figure 3.21). This means that the primers for these two

were not specific, therefore, we decided to exclude these two primers from validation of bacteria relative abundance.

Next, we ran the three sets of primers for *Oscillospira* (*Osc*), *Rikenellaceae* (*Rik*), and *S24.7* for qPCR analysis using SybrGreen. Optimization of this protocol would enable fast and semi-quantitative assessment of a large number of samples. We began by using the same DNA samples as the ones used for the primer tests by gel PCR (Figure 3.21). We ran the qPCR under the same conditions as the qPCR for total bacteria (section 2.8.4), but the setting for the temperature was changed to 60°C.

Figure 3.22 Dissociation curves of the amplified bacterial 16S rRNA sequences

The dissociation curves of the qPCR products amplified with the designed primers.

Figure 3.22 shows dissociation curves of the three primers. All of them showed clear one peak, therefore, there was no unspecific amplification by the primers.

3.5 Relative abundance of *Oscillospira*, *Rikenellaceae*, and *S24.7*

3.5.1 qPCR validation of the genome sequencing result

We then moved to validation of the result from the genome sequencing to see whether the result was reproducible by qPCR. All the faecal DNA samples in the microbiome cohorts, except the sample from the Abx treated mice, were used for this validation. The samples were run at the same condition as the optimisation of primers (section 3.4.1).

Oscillospira

Rikenellaceae

S24.7

Figure 3.23 Bacteria relative abundance in the DNA samples from MB cohorts by qPCR

Statistical values were determined using by Unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) for 2 comparisons. Error bars represent mean $\pm$ SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. N=14-30 mice per strain.

The validation by qPCR showed the same result as the analysis of genome sequence; *Osc* was higher in *Sle1* background while *Rik* and *S24.7* were lower in *Sle1* background compared to B6 background.

3.5.2 qPCR analysis on new samples

New stool samples were collected from B6 and *Sle1* from the facilities BRC3 and BRC2 and faecal DNA was extracted for analysis of relative abundance for *Osc*, *Rik*, and *S24.7*. Our mice were housed in two rooms in BRC3; SAH2 and SAH5. B6 mice were housed in BRC3-SAH2 and BRC3-SAH5, TLR9^{-/-} mice were housed in BRC3-SAH5 and BRC2, and *Sle1* mice were housed in BRC3-SAH2 and BRC2. In BRC2, we keep mice only in one room (Table 3.4).

	BRC3, SAH2	BRC3, SAH5	BRC2
B6	x	x	
TLR9 ^{-/-}		x	x
<i>Sle1</i>	x		x

Table 3.4 Mouse strains and facilities
X indicates where the mice were housed.

3.5.3 Relative bacteria abundance in comparison of B6 and *Sle1*

To address the question whether the separation in microbiome profile was led by purely the genetic background of the mice, we analysed relative abundance of the three bacteria strains in faecal DNA samples from B6 and *Sle1*.

Figure 3.24 Comparison of bacteria relative abundance to 16S rRNA total bacteria

Relative abundance of bacteria strains to control bacteria; 16S rRNA total bacteria, in faecal DNA samples was analysed by qPCR. Comparisons for (A) *Oscillospira*, (B) *Rikenellaceae*, and (C) *S24.7*. Statistical values were determined using by unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) for 2 comparisons. Error bars represent mean $\pm$ SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. N=23-29 mice per strain.

We compared all B6 samples with all *Sle1* samples regardless of facility. The result showed that the abundance of *Osc* was significantly high in *Sle1* mice and this is consistent with the finding in the genome sequencing analysis. However, abundance of *Rik* and *S24.7* contradicted with the previous findings being higher in *Sle1* mice compared to B6 controls (Figure 3.24). Although we saw significance in the relative abundance in *Osc* and *S24.7*, there are some clusters within a data set of each strain (Figure 3.24), therefore we investigated the data to check what caused the clusters in a group in each strain.

For process of qPCR analysis, the samples were loaded into 4 separate plates for, and analysis was done two plates a day. We investigated whether there is plate difference or day difference, but we found there was no batch difference or day difference.

Figure 3.25 Comparison of bacteria relative abundance in different facilities

Comparisons of bacteria abundance for (A) *Oscillospira*, (B) *Rikenellaceae*, and (C) *S24.7*. Statistical values were determined using by Unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) for 2 comparisons. Error bars represent mean $\pm$ SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. N=23-29 mice per strain.

We then further analysed the data to assess a facility difference of bacteria abundances in B6 and *Sle1* mice. Comparisons of different facilities revealed that there was no facility difference in relative abundance of *Osc* in *Sle1* mice, and no difference between B6 and *Sle1* mice in the same facility (Figure 3.25 A).

Analysis of the relative abundance of *Rik*, in contrast to previous genome sequencing analysis, revealed that *Sle1* mice had higher abundance than B6 mice within BRC3-SAH2. Facility difference affected the *Rik* abundance in *Sle1* mice, and it was significantly lower in the *Sle1* mice housed in BRC2 compared to ones in BRC3 (Figure 3.25 B).

For *S24.7* bacteria, the abundance was higher in *Sle1* in BRC3, which was also contradict to the finding in the sequencing analysis, however, there was no facility difference in between the *Sle1* mice in BRC3 and BRC3 (Figure 3.25 C). Relative abundance of *S24.7* in *Sle1* mice in BRC3, the data was clearly split several clusters in both B6 mice and *Sle1* (Figure 3.24 C, Figure 3.25 C). There was also a separation in the result of *Rik* relative abundance in B6, so we further investigated what made the difference in those data groups (Figure 3.26).

Figure 3.26 Comparison of bacteria abundance in different facilities, by strain.

(A) B6; BRC3-SAH2 vs. BRC3-SAH5, and (B) *Sle1*; BRC3(SAH2) vs. BRC2. Statistical values were determined using by unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) for 2 comparisons. Error bars represent mean $\pm$ SEM. * $P \leq 0.05$, **** $P \leq 0.0001$. N=23-29 mice per strain.

The investigation of the data separation in bacteria abundance revealed that facility, time, and mothers/cages had impacts largely on its abundance.

In B6 mice in BRC3-SAH5, the abundance of *Rik* was lower in the mice came from Biomedical Resource Centre (BRC) which is a department maintains and breeds mice (in a different room but within the same facility) compared to the mice that were bred and kept by our lab (Figure 3.26 A). The mice were transferred to our room at least 3 weeks after they were born. The mothers of the B6 mice in BRC3-SAH2 were all from Mouse

core, and abundance of *Rik* was low. The relative abundance of *S24.7* in B6 mice in BRC-SAH5, it was higher in the mice that have mothers born in late 2017 and early 2018, while it was lower in the mice that were born from mothers born in mid-2017. The B6 mice in BRC3-SAH2 were born from mothers born in late 2017, and abundance of *S24.7* was low. List of mice, their mothers, and other information for relative abundance for *Rik* are in Table 3.5.

Strain	Number of mice	Facility - room number	DOB	Age at collection (month)	DOB of mothers	Origin of mothers	Abundance of <i>Rikenellaceae</i>
B6	4	BRC3-SAH2	16-Mar-18	2.32	4-Dec-17	BRC	Low
	4	BRC3-SAH2	16-Mar-18	3.46	4-Dec-17	BRC	Low
	4	BRC3-SAH5	14-Apr-18	1.32	27 Dec '17 / 3 Jan '18	Our lab	High
	3	BRC3-SAH5	14-Apr-18	1.32	27 Dec '17 / 3 Jan '18	Our lab	High
	4	BRC3-SAH5	21-Aug-17	9.75	3-Aug-17	Our lab	High
	4	BRC3-SAH5	18-Apr-18	2.36	21-Sep-17	BRC	Low

Table 3.5 List of mouse cages, mothers for comparison of relative abundance of *Rikenellaceae*

Is it a strain difference / diet?

We observed that for *Sle1* mice in BRC3-SAH2, mice with high levels of *S24.7* (Figure 3.26 B) were born in 2018 while the cluster of mice with low abundance of *S24.7* were born in 2017. List of mice, their mothers, and other information for relative abundance of *S24.7* are in Table 3.6.

Strain	Number of mice	Facility - room number	DOB	Age at collection (month)	DOB of mothers	Origin of mothers	Abundance of S24.7
B6	4	BRC3-SAH2	16-Mar-18	2.32	4-Dec-17	BRC	Low
	4	BRC3-SAH2	16-Mar-18	3.46	4-Dec-17	BRC	Low
	4	BRC3-SAH5	14-Apr-18	1.32	27 Dec 17 / 3 Jan 18	Our lab	Low
	3	BRC3-SAH5	14-Apr-18	1.32	27 Dec '17 / 3 Jan '18	Our lab	Low
	4	BRC3-SAH5	21-Aug-17	9.75	3-Aug-17	Our lab	High
	4	BRC3-SAH5	18-Apr-18	2.36	21-Sep-17	BRC	High
Strain	Number of mice	Facility - room number	DOB	Age at collection (month)	DOB of mothers	Origin of mothers	Abundance of S24.7
Sle1	4	BRC3	22-Nov-17	6.39	11-Mar-17	Our lab	Low
	5	BRC3	8-Apr-18	1.50	2-Nov-17	Our lab	Low
	5	BRC3	3-May-18	1.75	26-Jan-18	Our lab	High
	5	BRC3	5-May-18	1.68	26-Jan-18	Our lab	High
	2	BRC2	18-Jan-18	3.25	23-Aug-17	Our lab	Low
	3	BRC2	26-Mar-18	2.07	5 / 10 Oct 17	Our lab	Low
	2	BRC2	1-Jun-17	12.75	15-Feb-17	Our lab	Low
	3	BRC2 (breeders)	15-Aug-17	10.07	11-Jan-17	Our lab	Low

Table 3.6 List of mouse cages and mothers for comparison of relative abundance of S24.7

3.5.4 Relative bacteria abundance in comparison of B6 and TLR9^{-/-}

We also compared B6 and TLR9^{-/-} to see an impact of TLR9 in bacteria abundance. In comparison of B6 and TLR9^{-/-} mice, relative abundance of these 3 bacteria within the same facility was not significantly different between the strains (Figure 3.27 A, B, C). For relative abundance of *Osc* and *Rik*, TLR9^{-/-} mice had no facility difference however, there was a facility difference in abundance of S24.7 bacteria in TLR9^{-/-} (Figure 3.27 A, B, C).

Figure 3.27 Comparison of bacteria relative abundance in different facilities

Comparisons of bacteria abundance between B6 and TLR9^{-/-} mice for (A) *Oscillospira*, (B) *Rikenellaceae*, and (C) *S24.7*. The relative abundance of these 3 bacteria was also compared by facility in TLR9^{-/-} mice (D). Statistical values were determined using by Unpaired t test (parametric data) or Mann-Whitney test (non-parametric data) for 2 comparisons. Error bars represent mean $\pm$ SEM. ** $P \leq 0.01$. N=23-25 mice per strain.

These results suggested that *S24.7* was highly sensitive to time or environment change regardless of strains. On the other hand, an abundance of *Rik* seemed to be affected by mothers only in non-disease B6 mice. *Osc* seemed to be not influenced by those environment or mothers.

4 Discussion

There are many studies indicating relationships between microbiota and autoimmunity. Recently, there are increasing evidences that gut microbiota is also related with SLE. Therefore, we assessed gut microbiome by extracting bacterial DNA from mice stool samples and analysing immunological traits in spleen, kidneys, PPs, and mLNs. In this study, we compared different facilities and strains with absence and presence of antibiotics to see relationship between the microbiome and TLR9.

Overall, we determined that long-term antibiotic treatment increases the size of the caecum and reduces populations of splenic Gr1⁺ CD11b⁺ cells and PMNs that play roles in innate immunity, regardless of strain. Our data also showed that mice lack TLR9 became more sensitive to gut bacteria change. In addition, bacteria abundance seemed to be affected by *Sle1* background in a comparison with B6 background, however, it was largely affected by environment and mothers.

4.1 Antibiotic treatment to autoimmune disease

The administration of broad-spectrum antibiotics (Abx) is one of the most commonly used methods to cause intestinal dysbiosis or to create germ free like environment in the gut in experimental animal models. There are several studies have suggested that the use of antibiotics improves autoimmune disease. For instance, administration of Tetracyclines led a clinically significant improvement in disease activity in Rheumatoid arthritis (RA) with no remarkable side effect [121]. In RA animal models, the elimination of commensal gut bacteria by either housing under GF condition or antibiotic treatment attenuated the severity of RA-like symptoms [100, 114]. While there are numbers of studies reporting the effect of antibiotics to RA, there are only little studies investigating the use of antibiotics to SLE. In lupus-prone SNF1 mice, although this was not antibiotic treatment, the mice given acidified water had delayed onset of

nephritis and reduced level of circulating anti-nuclear antibodies with less bacterial diversity in the gut compare to SNF1 mice treated with neutral pH drinking water [122]. More recently, Mu Q *et al* showed that antibiotic treatment on post disease in MRL/lpr mice improved lupus-like symptoms by reducing IL-17 producing CD4⁺ cells and increasing IL-10 in the serum compared to untreated MRL/lpr controls.

These studies showed that deletion of gut bacteria ameliorates autoimmune disease therefore we hypothesised that an antibiotic treatment also attenuates severe disease in our lupus models with changes in systemic immune responses in the absence of TLR9. Unlike those studies, we did not see improvement of disease phenotypes in both TLR9^{-/-} mouse which has mild autoimmunity and severe disease mouse model of SLE; Sle/TLR9^{-/-}. However, there were several changes found in Abx treated mice in this microbiome study.

4.1.1 *In non-autoimmune prone B6 backgrounds*

Many studies reported that TLR9 is expressed on the surface of epithelial cells and in Paneth cells in the gut in both human and mouse [123-127], however, TLR9 was not expressed in the gut in GF mice [123]. Ewaschuk *et al* found that the expression of TLR9 was upregulated on HT-29 cells, that resemble real colon tissue *in vitro*, upon a stimulation by pathogenic bacterial DNA, but not by probiotic bacteria DNA. Secretion of IL-8, a mediator in the innate immune response, was increased by a pathogenic bacterial DNA compared to a stimulation by DNA from probiotic bacteria [123]. Thus, this study showed that TLR9 senses pathogens selectively in the gut, and initiates responses following stimulations by bacteria.

In our data, Abx treatment reduced frequencies of splenic Gr1⁺ CD11b⁺ cells and neutrophils, regardless of strain (Figure 3.4 C). In addition, TLR9^{-/-} mice appeared to be more susceptible to gut microbiota changes. Antibiotic treatment to TLR9-deficient mice prevented the increase of T_H and the formation of GC (Figure 3.4 C, Figure 3.5 C). Furthermore, the number of total bacteria in the gut and T_H and GC B cell populations were positively correlated only in TLR9^{-/-} mice (Figure 3.8). B6 mice showed no

association between bacterial numbers in the gut and splenic immune phenotypes. This may implicate that TLR9 is necessary for regulation of immune activations initiated by bacteria in the gut. Ewaschuk *et al.* has shown that TLR9 selectively recognises pathogenic bacteria [123].

Some B6 mice showed antibiotic resistance, with moderate levels of bacteria post treatment. We assessed sub-strains of bacteria in the DNA samples by qPCR, and analysis revealed that *Bacteroides fragilis* (*Bact*) was remained, and other 3 sub-strains; *Eubacterium rectale* (*Erec*), *Lactobacillus*, *Bacteroides* (*Lact*) and *Segmented Filamentous Bacteria* (*SFB*) were eliminated (Figure 3.3).

To ascertain what bacteria remained post-treatment, we sent the faecal DNA samples from Abx treated mice for genome sequencing. The same amount of DNA from an individual mouse in the same group/strain were pooled into one sample. Analysis showed that there was mostly *Parabacteroides distasonis* remaining in B6 mice when the treatment was not efficient. It was not *Bact* as we saw in our qPCR analysis but this bacteria belongs to the same order as *Bact*. Consistent with this data, Nakano *et al.* showed that *Bacteroides* species, including *Bact* and *Parabacteroides distasonis*, had high resistance to Penicillin [128].

We need to repeat this with different antibiotics that are efficient enough to create germ-free environment in the gut in order to assess immunological changes in complete absence of gut microbiota. This may give a clearer picture of relationships between gut microbiota and systemic immune activation.

4.1.2 In autoimmune prone backgrounds

4.1.2.1 Pre-disease status of SLE mouse model

In this study, the mice were euthanised and analysed at 4-4.5 mos which was pre-disease state for our mouse models, therefore we did not see significant acceleration so much in disease phenotypes that were observed our previous study with aged disease model.

At this stage, *Sle1*TLR9^{-/-} mice already started to have some autoimmune phenotypes although indications of kidney disease as level of protein in urine and ratio of Albumin/creatinine were not significantly high compared to *Sle1* controls. Leukocyte infiltration in kidney was increased in *Sle1*TLR9^{-/-} in comparison with *Sle1*. In the spleen in *Sle1*TLR9^{-/-}, there were expansions of CD4⁺ memory T cell and T_h cell population as a result of elevated T cell activation. Formation of GC was also increased in the spleen in *Sle1*TLR9^{-/-} compared to *Sle1* counterparts. These phenotypes were consistent with our published previous findings [49] (Figure 1.1, Figure 1.2).

In our aged mouse model, they have severe disease with increased level of anti-RNA antibodies and higher concentration of IgG2a, IgG2b, and IgM. Concentration of IgA in the serum was significantly lower [49] (Figure 1.3). In this microbiome study, we observed a decrease in IgA level and an increase in IgM level in the serum. Moreover, concentrations of IgG2a was already increased in the serum in *Sle1*TLR9^{-/-} mice, however, other Ig subtypes were not significantly changed yet. Evaluations of auto-antibodies at pre-disease showed that although we did not see significant change in anti-ANA and anti-snRNP antibodies in *Sle1*TLR9^{-/-}. Levels of anti-dsDNA antibodies and anti-RNA antibodies were higher in *Sle1*TLR9^{-/-} mice compared to *Sle1* controls. However, our previous study showed that detection of anti-dsDNA antibody ELISA was not accurate [49].

Analysis of TLR7 expressions in splenic immune cells revealed that there were upregulations of the receptor in pDC and B cells, which was consistent to our previous finding [49]. There was no upregulated expression of TLR7 in cDC and F4/80⁺ macrophages. What is more, the expression was lower in disease related CD11c⁺MHCII⁻ cells in *Sle1*TLR9^{-/-} compared to *Sle1* controls (Figure 3.18). TLR7 was over expressed in these cells too in the previous finding [49] (Figure 3.17). This can be one of phenotypes in pre-disease of *Sle1*TLR9^{-/-} mice.

4.1.2.2 Impact of Abx treatment on *Sle1*TLR9^{-/-} at pre-disease stage

In contrast to several studies reporting the effect of antibiotics treatment to autoimmune diseases, we did not see improvement in severe disease in *Sle1*TLR9^{-/-} mice.

Unlike the change in T_h and GC B cell populations in TLR9^{-/-} mice, antibiotic treatment to TLR9-deficient mice on *Sle1* background did not have impact on frequencies of these populations in the spleen (Figure 3.11). Consistent with the comparison of B6 and TLR9^{-/-} mice, the populations of splenic neutrophils and CD11b⁺Gr1⁺ cells were decreased in Abx treated mice regardless of strain (Figure 3.11).

The Abx treatment seemed to have more influence on immune cells in PPs. There were significant decreases in CD8⁺ T cells and CD4⁺ T in PPs in treated mice regardless of strain (Figure 3.14). Furthermore, antibiotic treatment reduced IgA-switched B cells in GC in both strains. This was reflected in less circulating IgA in serum. IgA level was reduced in the treated mice regardless of strains (Figure 3.15). However, as it was difficult to obtain enough number of cells, some analysis had only little sample numbers therefore we need to accumulate more data for immunological traits in PPs.

These changes may be due to an effect of the Abx treatment, however, qPCR analysis showed that gut bacteria were not completely depleted in all *Sle1* and most of *Sle1*TLR9^{-/-}. Therefore, the change could be caused by bacteria that were survived from the antibiotics. We then assessed the bacteria sub-strains in the DNA sample by qPCR in order to find which bacteria survived from the treatment. Results showed that the all 4 sub-strains of bacteria were completely eliminated. Investigation of genome sequencing in the DNA sample from treated mice revealed that there was *Clostridiales* left (99.26%) in treated *Sle1* mice. In treated *Sle1*TLR9^{-/-} mice, there were also *Clostridiales* (55.22%) and *Anaeroplasmatales* (38.58%) left in the gut.

The further analysis of the sequencing also revealed that *Sle1* background mice had significantly high abundance of *Oscillospira* (*Osc*), and remarkably low abundance of *Rikenellaceae* (*Rik*) and *S24.7* bacteria compare to B6 background mice. This was confirmed by qPCR (Figure 3.23). This may indicate that those bacteria species are related to the disease in our mouse model.

4.2 Gut bacteria profile and disease, environment and mothers

The sequencing analysis also revealed that *Sle1* background mice had significantly high abundance of *Oscillospira* (*Osc*), and remarkably low abundance of *Rikenellaceae* (*Rik*) and *S24.7* compare to B6 background mice.

Significant differences in bacteria profile in our genome sequencing data showed that *Osc* was high in *Sle1* background mice while *Rik* and *S24.7* were low in *Sle1* background compared to B6 background mice. This was confirmed by qPCR (Figure 3.23). This result seemed to suggest that genetic background alter gut microbiota profile as it was shown.

The DNA samples in this analysis were all from one facility; BRC2, but origin of the mice was different. B6 and TLR9^{-/-} mice were transferred from another facility; BRC2. Therefore, we thought that the separation of the bacteria profile between *Sle1* and B6 background could be due to the facility difference. Analysis of bacteria relative abundance on different faecal DNA samples from B6 and *Sle1* mice revealed that *Rik* and *S24.7* are influenced by environment and mothers whereas *Osc* was not influenced by those.

There are several study suggesting relationships between *Oscillospira* and some diseases showing that this bacteria genus was reduced in patients and mouse models. For instance, Walters *et al.* found that *Oscillospira* was reduced in the gut in Crohn's disease patients [129]. *Oscillospira* was also reduced in paediatric non-alcoholic steatohepatitis, an inflammatory disease of the liver characterized by deposition of large amounts of fat in the liver [130]. Recently Giorgi *et al.* reported that abundance of *Clostridiales* in type 1 diabetes (T1D) mouse model was reduced and it was negatively associated with a frequency of iNKT17, which predominantly secrete IL-17 and have an effector and pathogenic role in autoimmune diseases such as T1D [131, 132]. *Clostridiales* is an only order of *Clostridia* which is a class of bacteria often but not always Gram-positive. *Oscillospira* is under this class [132]. Although this study did not go down to changes in genus level, *Oscillospira* may also follow this reduction. These studies suggest that

inflammatory or autoimmune disease is related to lower abundance of *Oscillospira*. However, our data contradict these findings and our analysis showed that *Oscillospira* was high in mice with *Sle1* background. In this study, this bacteria strain was not influenced by facility, mothers or timings of sample collection therefore this may be a good candidate to check strain difference. There was no difference in abundance of this bacteria between B6 and TLR9^{-/-} regardless of facility, and abundance of *Oscillospira* was higher in *Sle1* mice compared to B6 mice. This was consistent to the finding in genome sequencing analysis. Yet, association of SLE and *Oscillospira* is not studied therefore its relationship is still unclear.

Rikenellaceae was decreased in Vancomycin treated NOD mouse, which is a mouse model of T1D, together with massive elimination of IL-17 producing CD4⁺ T cells in lamina propria (LP) [133]. This may associate with our Immunological traits in PPs. Although we did not investigate LP, there was a significant reduction in a population of CD4⁺ T cells in PPs in *Sle1*/TLR9^{-/-} mice (Figure 3.14).

S24.7 is a family of *Bacteroides* known to be highly abundant bacteria in the gut in mice and human. Several studies have reported the altered abundance of *S24.7* family members in relationships with different environmental circumstances. For instance, abundance of *S24.7* was higher in diabetes-sensitive mice given a high fat diet than diabetes-resistant mice given the same chow [134]. *S24.7* was also more abundant in the mice following Gentamicin treatment-induced remission of colitis [135]. On top of these, our data implicated that changes in abundance of *S24.7* was influenced by time when the samples were collected regardless of strain. This bacteria family seems to be sensitive to environmental and other factors.

We did not assess an abundance of *Prevotellaceae* since we did not managed to design specific primer, however, there are multiple studies suggesting that a member of this family - *Prevotella* can be pro-inflammatory bacteria as abundance of *Prevotella* was high in RA patients and murine arthritis model [136-138]. What is more, Palm *et al.* found that gut bacteria highly coated by IgA were pathogenic and *Prevotellaceae* was one of the bacteria highly coated by secreted IgA in the gut in mice with inflammasome-mediated intestinal dysbiosis [139]. Our analysis did not show significant difference in

the comparison of B6 and *Sle1* background, but it can be another candidate bacteria specie to look into, especially for an investigation of gut microbiome in *Sle1*/TLR9^{-/-} mice.

Despite those reports showing relationships between relationships and diseases, our data suggested that environment and mothers also greatly affect microbiota profiles in mice rather than *Sle1* background. However, abundance of the bacteria species *Oscillospira* was high in *Sle1* background and it was not affected by mothers and environment change. Therefore, the bacteria may be a good indication to show an impact of *Sle1* on gut microbiome profile. To ascertain associations between SLE and gut microbiota, we need to increase the number of mice and need to investigate more about characteristic of gut microbiota in sever disease; *Sle1*/TLR9^{-/-}. Moreover, the Abx treatment need to be changed to a different mixture which were used in other studies in order to investigate precise immunological changes in absence of gut microbiota. For example, Vancomycin, Neomycin, Ampicillin, and Metronidazole were used to create germ free like condition in mice [112, 114, 115].

5 Conclusion

In summary, our data determined that gut microbiota has an impact on several immunological phenotypes, but it does not improve autoimmune disease such as autoantibody production and kidney pathology. However, this might have been led by inefficient antibiotic treatment especially in disease models of SLE; *Sle1* and *Sle1*/TLR9^{-/-} mice. Therefore, it is still unclear how the immune responses were altered by gut microbiome in SLE mouse.

Furthermore, DNA sequencing analysis revealed that the *Sle1* background influences gut microbiota. Some of bacteria, that were significantly low or high in *Sle1* background mice, were associated with autoimmune diseases. However, the differences of bacteria profile were also dominated by the impact of different animal facilities and their mothers.

Taken together, this research highlights the importance of gut microbiota in the disease, and how much environment effects on bacteria abundance.

6 References

1. Van Parijs, L. and A.K. Abbas, *Homeostasis and self-tolerance in the immune system: turning lymphocytes off*. Science, 1998. **280**(5361): p. 243-8.
2. Cooper, G.S., M.L. Bynum, and E.C. Somers, *Recent insights in the epidemiology of autoimmune diseases: improved prevalence estimates and understanding of clustering of diseases*. J Autoimmun, 2009. **33**(3-4): p. 197-207.
3. Ines, L., et al., *Classification of Systemic Lupus Erythematosus: Systemic Lupus International Collaborating Clinics Versus American College of Rheumatology Criteria. A Comparative Study of 2,055 Patients From a Real-Life, International Systemic Lupus Erythematosus Cohort*. Arthritis Care Res (Hoboken), 2015. **67**(8): p. 1180-5.
4. Mackay, I.R., *Science, medicine, and the future: Tolerance and autoimmunity*. BMJ, 2000. **321**(7253): p. 93-6.
5. Schmidt, C.W., *Questions persist: environmental factors in autoimmune disease*. Environ Health Perspect, 2011. **119**(6): p. A249-53.
6. McGrogan, A., S. Sneddon, and C.S. de Vries, *The incidence of myasthenia gravis: a systematic literature review*. Neuroepidemiology, 2010. **34**(3): p. 171-83.
7. Gonzalez, L.A., S.M. Toloza, and G.S. Alarcon, *Impact of race and ethnicity in the course and outcome of systemic lupus erythematosus*. Rheum Dis Clin North Am, 2014. **40**(3): p. 433-54, vii-viii.
8. Jacobson, D.L., et al., *Epidemiology and estimated population burden of selected autoimmune diseases in the United States*. Clin Immunol Immunopathol, 1997. **84**(3): p. 223-43.
9. Guerra, S.G., T.J. Vyse, and D.S. Cunninghame Graham, *The genetics of lupus: a functional perspective*. Arthritis Res Ther, 2012. **14**(3): p. 211.
10. Hochberg, M.C., *Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus*. Arthritis Rheum, 1997. **40**(9): p. 1725.
11. Tan, E.M., et al., *The 1982 revised criteria for the classification of systemic lupus erythematosus*. Arthritis Rheum, 1982. **25**(11): p. 1271-7.
12. Mohan, C. and C. Putterman, *Genetics and pathogenesis of systemic lupus erythematosus and lupus nephritis*. Nat Rev Nephrol, 2015. **11**(6): p. 329-341.

13. Kaul, A., et al., *Systemic lupus erythematosus*. Nat Rev Dis Primers, 2016. **2**: p. 16039.
14. Amezcua-Guerra, L.M., et al., *Performance of the 2012 Systemic Lupus International Collaborating Clinics and the 1997 American College of Rheumatology classification criteria for systemic lupus erythematosus in a real-life scenario*. Arthritis Care Res (Hoboken), 2015. **67**(3): p. 437-41.
15. Petri, M., et al., *Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus*. Arthritis Rheum, 2012. **64**(8): p. 2677-86.
16. Kuhn, A., et al., *The Diagnosis and Treatment of Systemic Lupus Erythematosus*. Dtsch Arztebl Int, 2015. **112**(25): p. 423-32.
17. Celhar, T., R. Magalhaes, and A.M. Fairhurst, *TLR7 and TLR9 in SLE: when sensing self goes wrong*. Immunol Res, 2012. **53**(1-3): p. 58-77.
18. Mok, C.C. and C.S. Lau, *Pathogenesis of systemic lupus erythematosus*. J Clin Pathol, 2003. **56**(7): p. 481-90.
19. Tsokos, G.C., *Systemic lupus erythematosus*. N Engl J Med, 2011. **365**(22): p. 2110-21.
20. Concetta Ferretti, A.L.C., *Systemic Lupus Erythematosus Basic, Applied and Clinical Aspects*. 2012, Department of Medicine, University of California Los Angeles, Los Angeles, CA, USA.
21. Du, J., et al., *DNA methylation pathways and their crosstalk with histone methylation*. Nat Rev Mol Cell Biol, 2015. **16**(9): p. 519-32.
22. J., W.D., *The Lupus Book: A Guide for Patients and Their Families.*, in 1st edition New York: Oxford University Press. 1995. p. 159-63.
23. Reeves, W.H., et al., *Induction of autoimmunity by pristane and other naturally occurring hydrocarbons*. Trends Immunol, 2009. **30**(9): p. 455-64.
24. Cooper, G.S. and C.G. Parks, *Occupational and environmental exposures as risk factors for systemic lupus erythematosus*. Curr Rheumatol Rep, 2004. **6**(5): p. 367-74.
25. Cooper, G.S., et al., *Occupational risk factors for the development of systemic lupus erythematosus*. J Rheumatol, 2004. **31**(10): p. 1928-33.
26. Rivera, T.L., H.M. Belmont, and G. Weissmann, *Systemic lupus erythematosus in 6 male cocaine users at Bellevue hospital*. J Rheumatol, 2009. **36**(12): p. 2854-5.
27. Costenbader, K.H., et al., *Cigarette smoking and the risk of systemic lupus erythematosus: a meta-analysis*. Arthritis Rheum, 2004. **50**(3): p. 849-57.

28. Arnson, Y., Y. Shoenfeld, and H. Amital, *Effects of tobacco smoke on immunity, inflammation and autoimmunity*. J Autoimmun, 2010. **34**(3): p. J258-65.
29. Yadav, P., et al., *Antibodies elicited in response to EBNA-1 may cross-react with dsDNA*. PLoS One, 2011. **6**(1): p. e14488.
30. Wu, Z., et al., *Ultraviolet B enhances DNA hypomethylation of CD4+ T cells in systemic lupus erythematosus via inhibiting DNMT1 catalytic activity*. J Dermatol Sci, 2013. **71**(3): p. 167-73.
31. Kuhn, A., V. Ruland, and G. Bonsmann, *Photosensitivity, phototesting, and photoprotection in cutaneous lupus erythematosus*. Lupus, 2010. **19**(9): p. 1036-46.
32. Mouse Genome Sequencing, C., et al., *Initial sequencing and comparative analysis of the mouse genome*. Nature, 2002. **420**(6915): p. 520-62.
33. Morel, L., et al., *Functional dissection of systemic lupus erythematosus using congenic mouse strains*. J Immunol, 1997. **158**(12): p. 6019-28.
34. Mohan, C., et al., *Genetic dissection of SLE pathogenesis. Sle1 on murine chromosome 1 leads to a selective loss of tolerance to H2A/H2B/DNA subnucleosomes*. J Clin Invest, 1998. **101**(6): p. 1362-72.
35. Soni, C., et al., *B cell-intrinsic TLR7 signaling is essential for the development of spontaneous germinal centers*. J Immunol, 2014. **193**(9): p. 4400-14.
36. Keszei, M., et al., *A novel isoform of the Ly108 gene ameliorates murine lupus*. J Exp Med, 2011. **208**(4): p. 811-22.
37. Limaye, N., et al., *Prevalence and evolutionary origins of autoimmune susceptibility alleles in natural mouse populations*. Genes Immun, 2008. **9**(1): p. 61-8.
38. Nickerson, K.M., et al., *TLR9 regulates TLR7- and MyD88-dependent autoantibody production and disease in a murine model of lupus*. J Immunol, 2010. **184**(4): p. 1840-8.
39. Blanco, P., et al., *Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus*. Science, 2001. **294**(5546): p. 1540-3.
40. Yasuda, K., et al., *Endosomal Translocation of Vertebrate DNA Activates Dendritic Cells via TLR9-Dependent and -Independent Pathways*. The Journal of Immunology, 2005. **174**(10): p. 6129-6136.
41. Christensen, S.R., et al., *Toll-like receptor 9 controls anti-DNA autoantibody production in murine lupus*. J Exp Med, 2005. **202**(2): p. 321-31.
42. Wu, X. and S.L. Peng, *Toll-like receptor 9 signaling protects against murine lupus*. Arthritis Rheum, 2006. **54**(1): p. 336-42.

43. Lartigue, A., et al., *Role of TLR9 in Anti-Nucleosome and Anti-DNA Antibody Production in lpr Mutation-Induced Murine Lupus*. The Journal of Immunology, 2006. **177**(2): p. 1349-1354.
44. Yu, P., et al., *Toll-like receptor 9-independent aggravation of glomerulonephritis in a novel model of SLE*. Int Immunol, 2006. **18**(8): p. 1211-9.
45. Santiago-Raber, M.L., et al., *Evidence that Yaa-induced loss of marginal zone B cells is a result of dendritic cell-mediated enhanced activation*. J Autoimmun, 2010. **34**(4): p. 349-55.
46. Christensen, S.R., et al., *Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus*. Immunity, 2006. **25**(3): p. 417-28.
47. Nickerson, K.M., et al., *Exacerbated autoimmunity in the absence of TLR9 in MRL.Fas(lpr) mice depends on Ifnar1*. J Immunol, 2013. **190**(8): p. 3889-94.
48. Fukui, R., et al., *Unc93B1 biases Toll-like receptor responses to nucleic acid in dendritic cells toward DNA- but against RNA-sensing*. J Exp Med, 2009. **206**(6): p. 1339-50.
49. Celhar, T., et al., *TLR9 deficiency breaks tolerance to RNA-associated antigens and upregulates TLR7 protein in Sle1 mice*. Arthritis Rheumatol, 2018.
50. Nickerson, K.M., et al., *TLR9 promotes tolerance by restricting survival of anergic anti-DNA B cells, yet is also required for their activation*. J Immunol, 2013. **190**(4): p. 1447-56.
51. Genestier, L., et al., *TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses*. J Immunol, 2007. **178**(12): p. 7779-86.
52. Rubtsov, A.V., et al., *TLR agonists promote marginal zone B cell activation and facilitate T-dependent IgM responses*. J Immunol, 2008. **180**(6): p. 3882-8.
53. Satpathy, A.T., et al., *Re(de)fining the dendritic cell lineage*. Nat Immunol, 2012. **13**(12): p. 1145-54.
54. Schlitzer, A. and F. Ginhoux, *Organization of the mouse and human DC network*. Curr Opin Immunol, 2014. **26**: p. 90-9.
55. Edwards, A.D., et al., *Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines*. Eur J Immunol, 2003. **33**(4): p. 827-33.
56. Fujimoto, K., et al., *A new subset of CD103+CD8alpha+ dendritic cells in the small intestine expresses TLR3, TLR7, and TLR9 and induces Th1 response and CTL activity*. J Immunol, 2011. **186**(11): p. 6287-95.

57. Boule, M.W., et al., *Toll-like receptor 9-dependent and -independent dendritic cell activation by chromatin-immunoglobulin G complexes*. J Exp Med, 2004. **199**(12): p. 1631-40.
58. Celhar, T., et al., *RNA sensing by conventional dendritic cells is central to the development of lupus nephritis*. Proc Natl Acad Sci U S A, 2015. **112**(45): p. E6195-204.
59. Hwang, S.H., et al., *B cell TLR7 expression drives anti-RNA autoantibody production and exacerbates disease in systemic lupus erythematosus-prone mice*. J Immunol, 2012. **189**(12): p. 5786-96.
60. Caton, M.L., M.R. Smith-Raska, and B. Reizis, *Notch-RBP-J signaling controls the homeostasis of CD8⁺ dendritic cells in the spleen*. J Exp Med, 2007. **204**(7): p. 1653-64.
61. Guillems, M., et al., *Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny*. Nat Rev Immunol, 2014. **14**(8): p. 571-8.
62. Maraskovsky, E., et al., *Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified*. J Exp Med, 1996. **184**(5): p. 1953-62.
63. Gilliet, M., W. Cao, and Y.J. Liu, *Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases*. Nat Rev Immunol, 2008. **8**(8): p. 594-606.
64. Banchereau, J. and V. Pascual, *Type I interferon in systemic lupus erythematosus and other autoimmune diseases*. Immunity, 2006. **25**(3): p. 383-92.
65. Friedman, R.M., et al., *Interferon production in patients with systemic lupus erythematosus*. Arthritis Rheum, 1982. **25**(7): p. 802-3.
66. Ronnblom, L. and M.L. Eloranta, *The interferon signature in autoimmune diseases*. Curr Opin Rheumatol, 2013. **25**(2): p. 248-53.
67. Rodriguez-Pla, A., et al., *IFN priming is necessary but not sufficient to turn on a migratory dendritic cell program in lupus monocytes*. J Immunol, 2014. **192**(12): p. 5586-98.
68. Svenson, J.L., et al., *Impact of estrogen receptor deficiency on disease expression in the NZM2410 lupus prone mouse*. Clin Immunol, 2008. **128**(2): p. 259-68.
69. Cunningham, M.A., et al., *Estrogen receptor alpha modulates Toll-like receptor signaling in murine lupus*. Clin Immunol, 2012. **144**(1): p. 1-12.
70. Palmer, C., et al., *Development of the human infant intestinal microbiota*. PLoS Biol, 2007. **5**(7): p. e177.

71. Dominguez-Bello, M.G., et al., *Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns*. Proc Natl Acad Sci U S A, 2010. **107**(26): p. 11971-5.
72. Biasucci, G., et al., *Mode of delivery affects the bacterial community in the newborn gut*. Early Hum Dev, 2010. **86 Suppl 1**: p. 13-5.
73. Chu, D.M., et al., *Maturation of the infant microbiome community structure and function across multiple body sites and in relation to mode of delivery*. Nat Med, 2017. **23**(3): p. 314-326.
74. Human Microbiome Project, C., *Structure, function and diversity of the healthy human microbiome*. Nature, 2012. **486**(7402): p. 207-14.
75. Arumugam, M., et al., *Enterotypes of the human gut microbiome*. Nature, 2011. **473**(7346): p. 174-80.
76. Aagaard, K., et al., *A metagenomic approach to characterization of the vaginal microbiome signature in pregnancy*. PLoS One, 2012. **7**(6): p. e36466.
77. Costello, E.K., et al., *Bacterial community variation in human body habitats across space and time*. Science, 2009. **326**(5960): p. 1694-7.
78. Seymour, G.J., et al., *Relationship between periodontal infections and systemic disease*. Clin Microbiol Infect, 2007. **13 Suppl 4**: p. 3-10.
79. Joshipura, K.J., et al., *Poor oral health and coronary heart disease*. J Dent Res, 1996. **75**(9): p. 1631-6.
80. Beck, J.D. and S. Offenbacher, *Systemic effects of periodontitis: epidemiology of periodontal disease and cardiovascular disease*. J Periodontol, 2005. **76**(11 Suppl): p. 2089-100.
81. Joshipura, K.J., et al., *Periodontal disease, tooth loss, and incidence of ischemic stroke*. Stroke, 2003. **34**(1): p. 47-52.
82. Offenbacher, S., et al., *Potential pathogenic mechanisms of periodontitis associated pregnancy complications*. Ann Periodontol, 1998. **3**(1): p. 233-50.
83. Genco, R.J., et al., *A proposed model linking inflammation to obesity, diabetes, and periodontal infections*. J Periodontol, 2005. **76**(11 Suppl): p. 2075-84.
84. Awano, S., et al., *Oral health and mortality risk from pneumonia in the elderly*. J Dent Res, 2008. **87**(4): p. 334-9.
85. Scharschmidt, T.C. and M.A. Fischbach, *What Lives On Our Skin: Ecology, Genomics and Therapeutic Opportunities Of the Skin Microbiome*. Drug Discov Today Dis Mech, 2013. **10**(3-4).
86. Belkaid, Y. and J.A. Segre, *Dialogue between skin microbiota and immunity*. Science, 2014. **346**(6212): p. 954-9.

87. Grice, E.A., *The intersection of microbiome and host at the skin interface: genomic- and metagenomic-based insights*. Genome Res, 2015. **25**(10): p. 1514-20.
88. Honda, K. and D.R. Littman, *The microbiome in infectious disease and inflammation*. Annu Rev Immunol, 2012. **30**: p. 759-95.
89. Frank, D.N., et al., *Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases*. Proc Natl Acad Sci U S A, 2007. **104**(34): p. 13780-5.
90. Mackie, R.I., A. Sghir, and H.R. Gaskins, *Developmental microbial ecology of the neonatal gastrointestinal tract*. Am J Clin Nutr, 1999. **69**(5): p. 1035S-1045S.
91. Mandar, R. and M. Mikelsaar, *Transmission of mother's microflora to the newborn at birth*. Biol Neonate, 1996. **69**(1): p. 30-5.
92. Erwin G. Zoetendal, A.D.L.A.W.M.A.-v.V.J.A.G.M.d.V.W.M.d.V., *The Host Genotype Affects the Bacterial Community in the Human Gastrointestinal Tract*. Microbial Ecology in Health and Disease, 2001. **13**(3): p. 129-134.
93. Yurkovetskiy, L., et al., *Gender bias in autoimmunity is influenced by microbiota*. Immunity, 2013. **39**(2): p. 400-12.
94. Benson, A.K., et al., *Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors*. Proc Natl Acad Sci U S A, 2010. **107**(44): p. 18933-8.
95. Liu, Z., et al., *Culture of human intestinal epithelial cell using the dissociating enzyme thermolysin and endothelin-3*. Braz J Med Biol Res, 2010. **43**(5): p. 451-9.
96. Cerf-Bensussan, N. and V. Gaboriau-Routhiau, *The immune system and the gut microbiota: friends or foes?* Nat Rev Immunol, 2010. **10**(10): p. 735-44.
97. Abreu, M.T., *Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function*. Nat Rev Immunol, 2010. **10**(2): p. 131-44.
98. Bouskra, D., et al., *Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis*. Nature, 2008. **456**(7221): p. 507-10.
99. Macpherson, A.J. and N.L. Harris, *Interactions between commensal intestinal bacteria and the immune system*. Nat Rev Immunol, 2004. **4**(6): p. 478-85.
100. Wu, H.J., et al., *Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells*. Immunity, 2010. **32**(6): p. 815-27.
101. Ivanov, II, et al., *Induction of intestinal Th17 cells by segmented filamentous bacteria*. Cell, 2009. **139**(3): p. 485-98.

102. Lee, Y.K., et al., *Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis*. Proc Natl Acad Sci U S A, 2011. **108 Suppl 1**: p. 4615-22.
103. Zhang, H., et al., *Dynamics of gut microbiota in autoimmune lupus*. Appl Environ Microbiol, 2014. **80**(24): p. 7551-60.
104. Hemmi, H., et al., *A Toll-like receptor recognizes bacterial DNA*. Nature, 2000. **408**(6813): p. 740-5.
105. Morel, L., et al., *Production of congenic mouse strains carrying genomic intervals containing SLE-susceptibility genes derived from the SLE-prone NZM2410 strain*. Mamm Genome, 1996. **7**(5): p. 335-9.
106. Koh, A.Y., et al., *Mucosal damage and neutropenia are required for Candida albicans dissemination*. PLoS Pathog, 2008. **4**(2): p. e35.
107. Barman, M., et al., *Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract*. Infect Immun, 2008. **76**(3): p. 907-15.
108. Savage, D.C. and R. Dubos, *Alterations in the mouse cecum and its flora produced by antibacterial drugs*. J Exp Med, 1968. **128**(1): p. 97-110.
109. Koh, D.R., et al., *Murine lupus in MRL/lpr mice lacking CD4 or CD8 T cells*. Eur J Immunol, 1995. **25**(9): p. 2558-62.
110. Morrison, L.J., et al., *Immunological abnormalities in BXSB mice at the onset of SLE development*. J Clin Lab Immunol, 1990. **32**(1): p. 5-11.
111. Rosser, E.C. and C. Mauri, *Regulatory B cells: origin, phenotype, and function*. Immunity, 2015. **42**(4): p. 607-12.
112. Mu, Q., et al., *Antibiotics ameliorate lupus-like symptoms in mice*. Sci Rep, 2017. **7**(1): p. 13675.
113. Lichtman, J.S., et al., *Host-Microbiota Interactions in the Pathogenesis of Antibiotic-Associated Diseases*. Cell Rep, 2016. **14**(5): p. 1049-1061.
114. Rosser, E.C., et al., *Regulatory B cells are induced by gut microbiota-driven interleukin-1beta and interleukin-6 production*. Nat Med, 2014. **20**(11): p. 1334-9.
115. Rogier, R., et al., *Alteration of the intestinal microbiome characterizes preclinical inflammatory arthritis in mice and its modulation attenuates established arthritis*. Sci Rep, 2017. **7**(1): p. 15613.
116. Jung, C., J.P. Hugot, and F. Barreau, *Peyer's Patches: The Immune Sensors of the Intestine*. Int J Inflam, 2010. **2010**: p. 823710.
117. Fagarasan, S., et al., *Adaptive immune regulation in the gut: T cell-dependent and T cell-independent IgA synthesis*. Annu Rev Immunol, 2010. **28**: p. 243-73.

118. Slack, E., M.L. Balmer, and A.J. Macpherson, *B cells as a critical node in the microbiota-host immune system network*. Immunol Rev, 2014. **260**(1): p. 50-66.
119. Reboldi, A., et al., *IgA production requires B cell interaction with subepithelial dendritic cells in Peyer's patches*. Science, 2016. **352**(6287): p. aaf4822.
120. Barreau, F., et al., *CARD15/NOD2 is required for Peyer's patches homeostasis in mice*. PLoS One, 2007. **2**(6): p. e523.
121. Stone, M., et al., *Should tetracycline treatment be used more extensively for rheumatoid arthritis? Metaanalysis demonstrates clinical benefit with reduction in disease activity*. J Rheumatol, 2003. **30**(10): p. 2112-22.
122. Johnson, B.M., et al., *Impact of dietary deviation on disease progression and gut microbiome composition in lupus-prone SNF1 mice*. Clin Exp Immunol, 2015. **181**(2): p. 323-37.
123. Ewaschuk, J.B., et al., *Surface expression of Toll-like receptor 9 is upregulated on intestinal epithelial cells in response to pathogenic bacterial DNA*. Infect Immun, 2007. **75**(5): p. 2572-9.
124. Lee, J., et al., *Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells*. Nat Cell Biol, 2006. **8**(12): p. 1327-36.
125. Gribar, S.C., et al., *Reciprocal expression and signaling of TLR4 and TLR9 in the pathogenesis and treatment of necrotizing enterocolitis*. J Immunol, 2009. **182**(1): p. 636-46.
126. Gopal, R., D. Birdsell, and F.P. Monroy, *Regulation of toll-like receptors in intestinal epithelial cells by stress and Toxoplasma gondii infection*. Parasite Immunol, 2008. **30**(11-12): p. 563-76.
127. Rumio, C., et al., *Degranulation of paneth cells via toll-like receptor 9*. Am J Pathol, 2004. **165**(2): p. 373-81.
128. Nakano, V., et al., *Antimicrobial resistance and prevalence of resistance genes in intestinal Bacteroidales strains*. Clinics (Sao Paulo), 2011. **66**(4): p. 543-7.
129. Walters, W.A., Z. Xu, and R. Knight, *Meta-analyses of human gut microbes associated with obesity and IBD*. FEBS Lett, 2014. **588**(22): p. 4223-33.
130. Zhu, L., et al., *Characterization of gut microbiomes in nonalcoholic steatohepatitis (NASH) patients: a connection between endogenous alcohol and NASH*. Hepatology, 2013. **57**(2): p. 601-9.
131. Ronchi, F. and M. Falcone, *Immune regulation by invariant NKT cells in autoimmunity*. Front Biosci, 2008. **13**: p. 4827-37.
132. De Giorgi, L., et al., *Increased iNKT17 Cell Frequency in the Intestine of Non-Obese Diabetic Mice Correlates With High Bacteroidales and Low Clostridiales Abundance*. Front Immunol, 2018. **9**: p. 1752.

133. Candon, S., et al., *Antibiotics in early life alter the gut microbiome and increase disease incidence in a spontaneous mouse model of autoimmune insulin-dependent diabetes*. PLoS One, 2015. **10**(5): p. e0125448.
134. Serino, M., et al., *Metabolic adaptation to a high-fat diet is associated with a change in the gut microbiota*. Gut, 2012. **61**(4): p. 543-53.
135. Rooks, M.G., et al., *Gut microbiome composition and function in experimental colitis during active disease and treatment-induced remission*. Isme j, 2014. **8**(7): p. 1403-17.
136. Bernard, N.J., *Rheumatoid arthritis: Prevotella copri associated with new-onset untreated RA*. Nat Rev Rheumatol, 2014. **10**(1): p. 2.
137. Moreno, J., *Prevotella copri and the microbial pathogenesis of rheumatoid arthritis*. Reumatol Clin, 2015. **11**(2): p. 61-3.
138. Ogrendik, M., *Rheumatoid arthritis is an autoimmune disease caused by periodontal pathogens*. Int J Gen Med, 2013. **6**: p. 383-6.
139. Palm, N.W., et al., *Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease*. Cell, 2014. **158**(5): p. 1000-10.

7 *Appendix*

7.1 *Appendix I Antibodies for Flow cytometry*

[target, conjugate, clone]

Anti- CD3, PE, HIT3a	Becton Dickinson (Franklin Lakes, NJ, USA)
Anti- CD3, BUV395, UCHT1	Becton Dickinson (Franklin Lakes, NJ, USA)
Anti- CD4, FITC, RPA-T4	Biolegend (San Diego, CA, USA)
Anti- CD4, V500, RPAT4	Becton Dickinson (Franklin Lakes, NJ, USA)
Anti- CD8, BV421, RPA-T9	Becton Dickinson (Franklin Lakes, NJ, USA)
Anti- CD8, APC-Cy7, SK1	Biolegend (San Diego, CA, USA)
Anti- CD11b, PE, BM8	BD Pharmingen™ (Franklin Lakes, NJ, USA)
Anti- CD16, APC-Cy7, 3G8	Biolegend (San Diego, CA, USA)
Anti- CD19, PE-Cy7, H1B19	Biolegend (San Diego, CA, USA)
Anti- CD19, PE TxRed, J3-119	Beckman coulter (Brea, CA, USA)
Anti- CD25, FITC, BC96	Biolegend (San Diego, CA, USA)
Anti- CD28, PE, CD28.2	Biolegend (San Diego, CA, USA)
Anti- CD34, APC, MEC14.7	Biolegend (San Diego, CA, USA)

Anti- CD45, BrilliantViolet 605™,30-F11	Biolegend (San Diego, CA, USA)
Anti- CD45, AF700, HI30	Biolegend (San Diego, CA, USA)
Anti- CD45RA, PECy7, HI100	Biolegend (San Diego, CA, USA)
Anti- CD45R/B220, FITC, RA3-6B2	eBioscience (San Diego, CA, USA)
Anti- CD45.1, PE-Cy7, A20	Biolegend (San Diego, CA, USA)
Anti- CD62L, FITC, MEL-14	BD Pharmingen™ (Franklin Lakes, NJ, USA)
Anti- CD66L, FITC, B1.1/CD66	BD Pharmingen™ (Franklin Lakes, NJ, USA)
anti- CD69, APC, FN50	eBioscience (San Diego, CA, USA)
Anti- CD86, FITC, GL-1	eBioscience (San Diego, CA, USA)
Anti- CD115, PE, AFS98	Biolegend (San Diego, CA, USA)
Anti- EpCAM (CD326), BrilliantViolet605, G8.8	Biolegend (San Diego, CA, USA)
Anti- CD95 (APO-1/Fas), biotin, 15A7	eBioscience (San Diego, CA, USA)
Anti- GL7, BV421, GL7	BD Pharmingen™ (Franklin Lakes, NJ, USA)
Anti- ICOS, PE-eFluor 610, ISA-3	eBioscience (San Diego, CA, USA)
Anti- IgA, FITC, C10-3	BD Pharmingen™ (Franklin Lakes, NJ, USA)
Anti- IgM, APC (, polyclonal)	Jackson ImmunoResearch Laboratories Inc. (USA)
Anti- Ly108, PE, 330-AJ	Biolegend (San Diego, CA, USA)

Anti- Ly-6A/E (Sca-1), FITC, D7	BD Pharmingen™ (Franklin Lakes, NJ, USA)
Anti- Ly6G, PerCPCy5.5, 1A8	BD Pharmingen™ (Franklin Lakes, NJ, USA)
Anti- Ly6G, AlexaFluor700, GR1	BD Pharmingen™ (Franklin Lakes, NJ, USA)
Anti- CD317 (mPDCA1), FITC, eBio927	eBioscience (San Diego, CA, USA)
Anti- MHC II, PerCPCy5.5, 551	Biolegend (San Diego, CA, USA)
Anti- MHC II (IA/IE), FITC, M5/114.15.2.	Biolegend (San Diego, CA, USA)
Anti- PD-1 (CD279), FITC, J43	eBioscience (San Diego, CA, USA)
Anti- Ter-119, AlexaFluore700, Ter119	Biolegend (San Diego, CA, USA)
Anti- SiglecH, PerCPCy5.5, 551	Biolegend (San Diego, CA, USA)
Fc-block antibody	AMF lab (Singapore)

7.2 Appendix II Primer sequences

Bacteria name	Primer name	Primer sequence	Tm	Product size (bp)
f.Rikenellaceae	f.Rike1_FWD	GGTAATACGGGGGATGCGAG	60.04	220
	f.Rike1_REV	TGCGGATTTGTGAGATGCCT	60.04	
f.S24.7	f.S24.7-1_FWD	CCGAAGGAACATTCCCCGAA	60.04	340
	f.S24.7-1_REV	AGCTTCATGGAGTCGGGTTG	60.04	
g.Oscillospira (f.Ruminococcacea	g.Oscil1_FWD	AAGGAGTTTTTCGGACAACGG	58.41	408-418
	g.Oscil1_REV	ATTCAAGGGGTACCGTCTTC	56.93	
f.Prevotellaceae	f.Prevo1_FWD	GGAAGGTCCGGGCGTTATC	60.23	217-220
	f.Prevo1_REV	GTCAGTGCCGCTACGGT	59.36	
g.Bacteroides	HuBac566f (forward)	GGGTTTAAAGGGAGCGTAGG	(60°C for qPCR reactions)	120-130
	HuBac692r (reverse)	CTACACCACGAATTCCGCCT	(60°C for qPCR reactions)	

7.3 Appendix III Pathogens screened in each facility

	BRC2	BRC3
SEROLOGY	Sendai virus	Sendai virus
	Pneumonia virus of mice (PVM)	Pneumonia virus of mice
	Mouse hepatitis virus (MHV)	Mouse hepatitis virus (MHV)
	Minute virus of mice (MVM)	Minute virus of mice (MVM)
	Mouse parvovirus (MPV)	Mouse parvovirus (MPV)
	Theiler's murine encephalomyelitis virus (TMEV orbGDVII)	Theiler's murine encephalomyelitis virus
	Reovirus Type 3	Reovirus Type 3
	Mouse rotavirus (Epizootic diarrhea of infant mice, EDIM)	Mouse rotavirus (Epizootic diarrhea of infant mice, EDIM)
	Mouse adenovirus type 1 and 2 (MAV)	Mouse adenovirus type 1 and 2 (MAV)
	Mouse polyoma virus	Mouse polyoma virus
	Lymphocytic choriomeningitis virus (LCMV)	Lymphocytic choriomeningitis virus (LCMV)
	Mouse cytomegalovirus (MCMV)	Mouse cytomegalovirus (MCMV)
	Hantaan virus	Hantaan virus
	Ectromelia Virus	Ectromelia Virus
	Mycoplasma pulmonis	Mycoplasma pulmonis
BACTERIOLOGY	Bordetella bronchiseptica	Bordetella bronchiseptica
	Citrobacter rodentium	Corynebacterium kutscheri
	Clostridium piliforme	Citrobacter rodentium
	Salmonella spp	Helicobacter hepaticus, bilis and rodentium
	Streptobacillus moniliformis	Clostridium piliforme
		Salmonella spp
		Streptobacillus moniliformis
		Streptococcus pneumoniae
		Pasteurella pneumotropica
		Pseudomonas aeruginosa
		Streptococci beta-haemolytica (Groups A, B & C)
PARASITOLOGY	Encephalitozoon cuniculi	Klebsiella pneumoniae
	Ectoparasites – e.g. fleas, fur mites and lice	Encephalitozoon cuniculi
	Endoparasites – e.g. pinworms, tapeworms, roundworms and other helminthes	Ectoparasites – e.g. fleas, fur mites and lice
		Endoparasites – e.g. pinworms, tapeworms, roundworms and other helminthes
		Protozoa – Tritrichomonas muris and Entamoeba muris
PATHOLOGICAL LESIONS	External	External
	Necropsy	Necropsy
*All the pathogens are regularly screened every 3 or 6 months.		

7.4 Appendix IV Reagent recipe

Complete RPMI (cRPMI)

450 mL RPMI 1640 plain RPMI – pRPMI)

10% FBS (HyClone) Characterised

1 mM sodium pyruvate

15 mM HEPES

0.1 mM MEM NEAA

1% PenStrep

2 mM L-glutamine

0.16 μ M 2-mercaptoethanol

FACS buffer

5% FBS

1.5% HEPES

DPBS

1X PBS, pH 7.4

137 mM NaCl

2.7 mM KCl

10 mM Na₂HPO₄

2 mM KH₂PO₄

LB medium

120 ug/ml Penicillin

25 ug/ml Kanamycin (optional)

2% glucose (optional)

1.5% Agar (optional)

LB

7.5 Appendix V Software

Photoshop elements 9.0

Adobe (USA)

FACS Diva Becton Dickinson (Fanklin Lakes, NJ, USA)

FloJo X10 Tree Star (Ashland, Oregon, USA)

MS Office 2010 Microsoft (Redmond, WA, USA)

Graphpad Prism La Jolla (CA, USA)

8 Author's publications

RNA sensing by conventional dendritic cells is central to the development of lupus nephritis (*PNAS*, 2015)

TLR9 deficiency breaks tolerance to RNA-associated antigens and upregulates TLR7 protein in Sle1 mice (*Arthritis Rheumatol.* 2018)

9 Posters, awards

Singapore International Graduate Awards (SINGA)

Keystone Symposia 2018, Microbiome, Host Resistance and Disease