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SYSTEMIC VH4-34-ENCODED ANTIBODY RESPONSES AGAINST COMMENSAL BACTERIA IN PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

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ETHICAL DECLARATION

In this thesis study, I declare that all the information and documents have been obtained in the base of the academic rules and all audio-visual and written information and results have been presented according to the rules of scientific ethics. I did not do any distortion in data set. In case of using other works, related studies have been fully cited in accordance with the scientific standards. I also declare that my thesis study is original except cited references. It was produced by myself in consultation with supervisor Prof. Eric MEFFRE, PhD and Assoc. Prof. Hande CANPINAR, PhD and written according to the rules of thesis writing of Hacettepe University Institute of Health Sciences.

Fatma Naz Cemre KALAYCI

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ABSTRACT

Kalayci, FNC., Systemic VH4-34-encoded antibody responses against commensal bacteria in patients with systemic lupus erythematosus, Hacettepe University Graduate School of Health Sciences, Tumor Biology and Immunology Program, Doctor of Philosophy Thesis, Ankara, 2023. The germline immunoglobulin variable heavy chain 4–34 encoding B lymphocytes (known as VH4–34 or 9G4 B cells) are endowed with inherent self-reactivity due to the presence of a germline AVY motif in their framework region 1 of the VH4–34 gene segment. Germline self-reactive VH4-34 undergoes negative selection in the memory and plasmablast compartments of healthy donors. However, the VH4-34 censorship is compromised in systemic lupus erythematosus (SLE) patients (1), resulting in higher proportions of VH4-34 expression among plasmablasts and memory B cells.(1-3) Given the fact that gut dysbiosis and disturbances to the gut barrier integrity can set off inflammatory immune responses, and also that the association between circulating 9G4 memory B lymphocytes isolated from individuals with SLE and the gut bacteria has not been investigated to date, we seek to determine the anti-commensal bacteria reactivity of VH4-34+ IgG antibodies in SLE subjects. VH4-34 $CD27^{+}IgG^{+}$ B lymphocytes from individuals with SLE exhibit aberrant VH4-34 gene usage and display a reduction in somatic hypermutation frequencies, often with an unmutated AVY site, suggesting that their antibodies continue to possess intrinsic self-reactivity unlike their healthy donor (HD) counterparts as well as a propensity for reactivity towards commensal microorganisms. Herein, we observed that SLE VH4-$34^{+}IgG^{+}$ B lymphocytes with an unmutated AVY site exhibited an elevated level of reactivity towards gut bacteria present in the fecal samples of control groups and SLE patients, but to a smaller extent in HD stools and to a greater extent in SLE stools. Since SLE VH4-34 clonal expansion is associated with disease flare, our discovery of the SLE VH4-34 clonal expansion of gut bacteria-reactive circulating 9G4+IgG+ B cells in one SLE patient suggests that commensal bacteria translocating from the gut to the bloodstream are therefore responsible for the VH4-34 expansion in SLE. Lastly, we identified bacteria strains recognized by circulating unmutated AVY motif-encoding VH4-34-encoded

antibodies, which could be attributed to a compromised intestinal barrier function in SLE patients.

Keywords: Systemic lupus erythematosus, VH4-34 gene, 9G4, AVY/ NHS motif, commensal bacteria

ÖZET

Kalaycı FNC., Sistemik Lupus Eritematozuslu (SLE) Hastalarda Kommensal Bakterilere Karşı Sistemik VH4-34 Kodlu Antikor Yanıtlarının Değerlendirilmesi, Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü, Tümör Biyolojisi ve İmmünolojisi Programı Doktora Tezi, Ankara, 2023. Germ hattı immünoglobulin değişken ağır zinciri 4-34'ü kodlayan B lenfositleri (VH4-34 veya 9G4 B hücreleri olarak bilinir), VH4-34 geninin segmentinin çerçeve bölgesi 1'deki bir germ hattı AVY motifinin varlığına bağlı olarak otoreaktiviteye sahiptir. Germ hattı kodlayan otoreaktif VH4-34, sağlıklı donörlerin hafıza B hücresi ve plazmablast kompartmanlarında negatif seçime uğrar. Ancak bu negative seçim ile sistemik lupus eritematozus (SLE) hastalarında VH4-34 geninin kontrolü tehlikeye girer (1), bu durum plazmablastlar ve hafıza B hücreleri arasında daha yüksek oranlarda VH4-34 ekspresyonuna neden olur**.**(1-3) Bağırsak disbiyozu ve bağırsak bariyeri bütünlüğündeki bozukluklar inflamatuar bağışıklık yanıtlarını tetikleyebilmektedir. SLE'li hastalardan izole edilen dolaşımdaki 9G4 hafıza B hücreleri ile bağırsak bakterileri arasındaki ilişkinin bugüne kadar araştırılmamış bir konu olması nedeniyle bu tez çalışmasında SLE hastalarında, VH4-34 kodlu IgG antikorlarının anti-kommensal bakteri reaktivitesini belirlemeyi amaçlanmıştır. Çalışma bulgularımız, SLE hastalarından izole edilen VH4-34 CD27+IgG+ B hücreleri, anormal VH4-34 gen kullanımı sergilediğini ve sıklıkla mutasyona uğramamış bir AVY motifiyle somatik hipermutasyon frekanslarında bir azalma gösterdiğini ve bu antikorların, sağlıklı donörlerden farklı olarak hala içsel özreaktiviteye sahip olduğunu göstermiştir. Ayrıca çalışma sonuçlarımızda, SLE hastalarından elde edilmiş bu VH4-34 CD27+IgG+ B hücrelerinın kommensal mikroorganizmalara karşı reaktivite eğilimi bulunduğunu da tespit ettik. Çalışmamızda, mutasyona uğramamış bir AVY bölgesine sahip SLE VH4-34+IgG+ B hücrelerinin, hem sağlıklı bireylerin hem de SLE hastalarının dışkı örneklerinde mevcut olan bağırsak bakterilerine karşı yüksek seviyede reaktivite gösterdiğini, ancak sağlıklı donorlerin dışkılarında daha az ve SLE hastalarının dışkılarında daha büyük ölçüde reaktivite gösterdiğini de gözlemledik. SLE VH4-34 klonal genişlemesi hastalığın alevlenmesiyle ilişkili olduğundan, bir SLE hastasında bağırsak bakterilerine reaktif dolaşımdaki 9G4+IgG+ B hücrelerinin SLE VH4-34

klonal genişlemesini keşfetmemiz, bağırsaktan kan dolaşımına yer değiştiren kommensal bakterilerin SLE'deki VH4-34 genişlemesinden sorumlu olabileceğine işaret etmektedir. Son olarak, çalışmamızda SLE hastalarında bozulmuş bağırsak bariyer fonksiyonunundan sorumlu dolaşımda bulunan mutasyona uğramamış AVY motifini kodlayan VH4-34 kodlu antikorlarını tanıyan bakteri suşları tespit edilmiştir.

Anahtar Kelimeler: Sistemik lupus eritematozus, VH4-34 geni, 9G4, AVY/ NHS motifi, kommensal bakteri

CONTENTS

ABBREVIATIONS

ACR	American College of Rheumatology
AID	Activation-induced cytidine deaminase
AIRE	Autoimmune regulator
ANA	Anti-nuclear antibodies
anti-dsDNA	Anti-double-stranded deoxyribonucleic acid
anti-Sm	Anti-Smith
APRIL	A proliferation-inducing ligand
ASCs	Antibody-secreting cells
ASV	Amplicon sequence variant
ATR	Ataxia-telangiectasia and Rad3-related
AVY	Ala-Val-Tyr
BAFF	B-cell activating factor
BCR	B cell receptor
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
BTK	Bruton’s tyrosine kinase
C4	Complement component 4
CARs	Chimeric antigen receptors
CD	Custer of differentiation
CDR	Complementarity determining region
CO2	Carbon dioxide
CT	Connectıcut
Cy5	Cyanine5
D	Diversity
dH20	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
dNTP	Deoxynucleotide triphosphate
DPBS	Dulbecco's phosphate-buffered saline
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme-linked immunosorbent assay
EULAR	European Alliance of Associations for Rheumatology
FITC	Fluorescein isothiocyanate
FR1	Framework region 1
FWR	Framework region
GALTs	Gut-associated lymphoid tissues
GC	Germinal center
H	Histidine
HD	Healthy donor
Hep-2	Human epithelial cell tumor
hex N6	Hexamer
Id	Idiotype
IFN-α	Interferon-alpha
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IL	Interleukin
J	Joining
L	light (chain)
LB	Lysogeny broth
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
NHS	Asn-X-Ser N-glycosylation site
NP40	Tergitol-type NP-40 and nonyl phenoxypolyethoxylethanol
OD	Optical density
P1 Buffer	Resuspension buffer
P2 Buffer	Lysis buffer
PB	Binding buffer
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction

PE	R-Phycoerythrin
PE buffer	Wash buffer
PEI	Polyethylenimine
PTPN22	Protein tyrosine phosphatase non-receptor type 22
R	Arginine
RAG	Recombination activating genes
RBC	Red blood cells
Ro60	Ro protein 60
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SCFA	Short-chain fatty acid
SHM	Somatic hypermutation
SLE	Systemic Lupus Erythematosus
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
SLEDAI-2K	Systemic Lupus Erythematosus Disease Activity Index-2000
Th17	T helper 17 cells
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T-lymphocyte
V	Variable
VH4–34	Variable heavy chain 4–34
W	Tryptophan
Y	Tyrosine
κ	kappa
λ	lambda
µ	Micro
16S rRNA	16S ribosomal ribonucleic acid
2xYT	2x Yeast Extract Tryptone medium

FIGURES

TABLES

1. INTRODUCTION

The pathomechanism of SLE remains incompletely elucidated; nonetheless, the role of B lymphocytes and self-reactive antibodies on the pathogenesis of this autoimmune disorder is well recognized. Aside from impaired early B lymphocyte tolerance checkpoint controls (4), individuals with SLE also exhibit a modified subset of IgG+ memory B lymphocytes expressing the VH4-34 gene fragment that is rarely seen in healthy controls or individuals with other autoimmune disorders.(5-7) Presence of unmutated and weakly mutated VH4-34 antibodies, particularly in the VH4-34 AVY and NHS motifs, has been implicated in disease flare; however, the specific antigens that are recognized by these antibody clones have yet to be identified.(2) VH4-34-expressing antibodies with unmutated AVY exhibit inherent self-reactivity and detect I/i carbohydrates present on hematopoietic cells (8, 9), and we have previously shown that these clones can bind to commensal gut bacteria as well, implying that SLE flares may arise due to disruptions in the gut microbiota, which could then potentially trigger the VH4-34-encoding IgG+ B lymphocyte generation from naïve B cells.(7)

Herein, we first examined is the link between intrinsically autoreactive VH4-34 CD27+IgG+ B lymphocytes and gut bacteria in patients with SLE through the presence of a germline AVY motif expressing the VH4-34 gene segment.

Secondly, we assessed the reactivity of recombinant IgG VH4-34 antibodies derived from both healthy participants and individuals with SLE towards fecal gut bacteria obtained from both healthy subjects and SLE patients.

Thirdly, we sought to explore certain taxonomic groups present in the stool of SLE patients that bind to VH4-34 IgG recombinant antibodies with a stronger affinity.

Finally, the present investigation may be reconceptualized as an exploration into the underlying reasons for the high occurrence of V4-34-encoded B cell expansions in SLE. Additionally, it aims to investigate the potential association between these expansions and immunological responses that are elicited by the existence of intestinal bacteria in the bloodstream, which may be attributed to a compromised intestinal barrier function.

2. LITERATURE REVIEW

2.1. B Lymphocyte Development

2.1.1. Developmental Stages of B Lymphocytes

Lymphopoiesis occurs within specialized lymphoid tissues. These tissues include the bone marrow, which is responsible for the development of the majority of B lymphocytes, and also the thymus, where T lymphocytes grow. Afterwards, B lymphocytes undergo migration to populate the peripheral lymphoid sites, thereby allowing us to mount responses of adaptive immunity to effectively counteract the diverse array of pathogens we confront throughout our lifespan. (10)

In the initial physiologic stages of B cell development, pre-pro-B lymphocytes represent the earliest stage of committed B lymphocyte precursors (11) , which exhibit reduced expression levels of the recombination activating genes (RAG) (12, 13), and lack the expression of B lymphocyte antigen receptor (BCR) components.(14) Several factors associated with transcription are being discovered as vital regulators of B lymphocyte growth, as represented in Figure 2.1.(15, 16) Pax-5 is considered exceptional due to its vital function in preserving the commitment of the B lineage.(15)

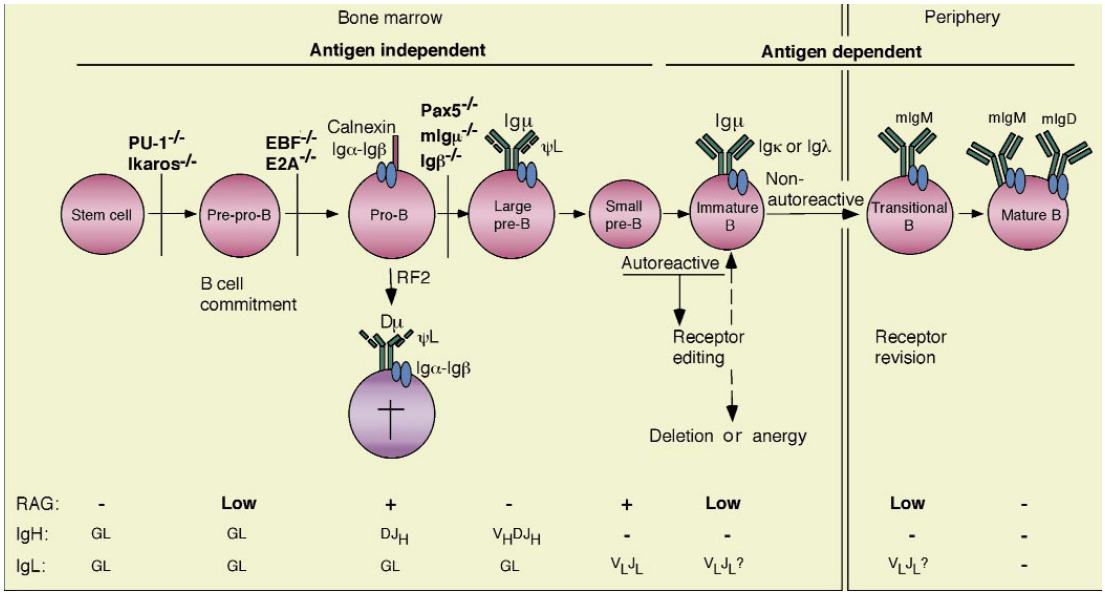


Figure 2.1: Schematic representation of B cell–differentiation. The B-lineage cell undergoes a sequence of progressive phases of growth and maturation (Adapted from (15))

Pro-B lymphocytes, where recombination initiation starts (11), are the first B lineage lymphocytes to manifest a precursor BCR, which comprises immunoglobulin α (Igα), Igβ, and calnexin (pro-BCR).(15, 17) The process of immunoglobulin gene recombination starts with the rearrangement of diversity (D) and junction (J_H) segments in pro-B lymphocytes.(18, 19) Also, pro-B lymphocytes are the primary site where antibodies exert their regulatory role in the B lymphocyte development.(15) Following the DJ_H reorganizing process, the genes of V_H are made accessible to the V(D)J recombinase, allowing the assembly of complete heavy chain transcription units.(20)

When the $V(D)J_H$ rearrangement is successful, the mIgμ expression results in the formation of the pre-BCR which signals via Bruton's tyrosine kinase (BTK) and signifies the progression towards the pre-B lymphocyte developmental phase.(10, 15) The pre-BCR complex, which consists of mIgm, surrogate light (L) chain (V-pre-B, ψ L and λ5), Iga and Igb (15, 21, 22), is a crucial checkpoint regulator in B lymphocyte growth and development. Its principal roles include promoting clonal expansion, selecting the VH repertoire, stimulating B cell differentiation, repressing the recombination machinery, enforcing heavy chain allelic exclusion, and suppressing the surrogate L chain genes. (15, 23) Lymphocytes that lack the capacity to generate a pre-BCR complex experience developmental arrest and undergo apoptosis.(15) Pre-B cells produce germline RAG and Igκ transcripts, then subsequently go through the process of recombination of L chain genes.(24-26)

The assembly of the BCR is achieved through an effective L chain gene rearrangement and the substitution of yLs with Igk or Igl.(15) During this phase, research has revealed the occurrence of L chain allelic exclusion is only partially achieved, in contrast to heavy chain allelic exclusion.(27, 28) Also, roughly 10% of total mature B lymphocytes possess more than one in-frame L chain gene.(29) The entrapment of growing B lymphocytes in a compartment where they endure secondary rearrangement is anticipated in cases where self-reactive BCRs undergo cross-linking or when BCR assembly fails to initiate signaling.(30, 31) Autoreactive B lymphocytes were engaged in secondary L chain gene recombination, referred to as receptor editing, by substituting their receptors.(30, 32, 33)

The process of selecting antibodies is triggered by the L chain gene expression in the early immature B lymphocyte phase.(34) Immature B lymphocytes are the first cells of the B lineage to exhibit surface BCR, and they express surface IgM but minimal or no IgD.(15) Within this stage, autoreactive B lymphocytes that are unable to modify their receptors are subject to deletion or anergy.(35) Indeed, immature B lymphocytes present a greater susceptibility to apoptosis induced by BCR as compared with mature B lymphocytes (35), and the extent of this removal is contingent upon the level of receptor cross-linking.(36) Anergy, also known as a hyporesponsive state, which is a different strategy to induce self-tolerance in immature B lymphocytes, is characterized by an abolished normal function of B cells as evidenced by a reduction in proliferation and antibody production following exposure to mitogens.(37) Anergic B cells have a limited lifespan and encounter challenges in transitioning from the immature to the long-lived B lymphocyte population within the spleen.(38, 39) Anergic cells have reduced expression of BCR upon prolonged exposure to antigens.(40) Overall, BCR complex plays a crucial role in regulating the development of immature B lymphocytes. For instance, the regulatory role of Igα and Igβ in the development of immature B lymphocytes is believed to involve BTK and Lyn, as well as BCR-modulating coreceptors like CD19, CD22, or CD45. Therefore, the malfunction of these genes has been observed to impede the establishment of anergy and the evolution of immature B lymphocytes.(41-47) Autoreactivity screening is conducted on immature B lymphocytes prior to their departure from the primary lymphoid tissue. (10)

Transitional B lymphocytes are referred to as immature B lymphocytes that relocate from the bone marrow to the peripheral regions. (15) Transitional B lymphocytes, which can be differentiated from mature B cells through a set of surface markers (48) and their relatively low expression of RAG mRNA (49, 50), have a limited lifespan, and just 10–30% of these transitional B lymphocytes have the capability to reach the peripheral compartment of long-lived mature B lymphocytes.(48, 51) The transitional B cell compartment exhibits variability in size, while the mature B cell compartment demonstrates a consistent size.(15, 39, 52) It has been found that the shift of transitional B lymphocytes to the mature B lymphocyte compartment requires BCR signaling, which was proven by an experiment where a

lack of BCR resulted in the cessation of the transition of new emigrant or transitional B lymphocytes into mature B-cell lymphocytes.(53) Overall, the regulation of various checkpoints in the B lymphocyte pathway, which contribute to the formation of the antibody repertoire through cellular and molecular selection, is controlled by signaling through membrane immunoglobulin.(15)

2.1.2. Tolerance mechanisms of B lymphocytes

In the course of B lymphocyte evolution, a diverse array of specificities is generated through the process of random gene segment reassortment and V(D)J rearrangement, resulting in the assembly of antibodies. One possible drawback of this random process is that certain antibodies formed may exhibit self-reactivity. A large number of antibodies produced by early immature B lymphocytes show self-reactivity, which encompasses both polyreactive and anti-nuclear specificities. The majority of those self-antibodies underwent elimination in humans at two distinct checkpoints in the course of B lymphocyte development. If the regulation of checkpoints is inefficient, it could result in significant elevation of autoantibodies in circulation. Pre-B lymphocytes and early immature B lymphocytes exhibit an enrichment of antibodies possessing long and/or highly positively charged IgH CDR3s, which have been linked to autoreactivity or polyreactivity. The aforementioned characteristics are deliberately removed from the B lymphocyte repertoire as B lymphocytes develop. It is demonstrated that substantial amounts of self-reactive antibodies are eliminated from the repertoire throughout the immature B lymphocyte stage in the bone marrow as well as transitioning from the new emigrant to the mature naive B lymphocyte phase in the peripheral tissue through normal human B lymphocyte development. (54) The induction of immunological tolerance (the state of unresponsiveness to antigens) towards distinct self-antigens can occur through two mechanisms: central tolerance, which involves the encounter of these antigens by developing lymphocytes in the generative (central) lymphoid organs, and peripheral tolerance, which happens when mature lymphocytes come across self-antigens in peripheral lymphoid organs or peripheral tissues.(55)

Three main mechanisms have been identified for B cell central tolerance, namely apoptosis, anergy, and receptor editing.(56, 57) Receptor editing refers to the

strategy by which the recombination of secondary Ig gene segments modifies the self-reactive immature B cell BCR specificity. The developing B lymphocytes in the bone marrow displayed evidence of receptor editing, which encompassed the downregulation of BCR, elevated expression of RAG genes, and a rise in secondary L-chain gene rearrangement events, including the utilization of downstream Jκ gene segments and L-chain λ-locus excision circles.(58) It is still believed that autoreactive BCR signaling controls secondary rearrangements in the L-chain loci.(56, 58) In cases where the editing process becomes unsuccessful, immature B lymphocytes that exhibit a high degree of affinity towards self-antigens are subjected to apoptotic death signals and subsequently undergo deletion.(55) Synergistic signaling between BCR and Toll-like receptor (TLR) signals elicits adequate levels of activation-induced cytidine deaminase (AID) expression to trigger apoptotic cell death, thereby mediating deletional tolerance.(56) B lymphocytes that specifically bind certain self-antigens with low avidity survive; however, the expression of their antigen receptors is diminished, leading to a state of functional unresponsiveness, also known as anergy.(55) Overall, intrinsic B-cell pathways are responsible for regulating central B-cell tolerance.

The human body employs a second checkpoint to eliminate self-reactive antibodies, which occurs during the transition from new emigrant to mature B cells in the periphery. (59) In cases where B lymphocytes in the periphery identify an autoantigen, B lymphocytes, in the absence of T lymphocyte assistance, can undergo functional inactivation, rendering them unable to react to the antigen (anergy) and eventually dying owing to an insufficiency in crucial signals necessary for survival. Alternatively, the B lymphocyte may go through apoptosis (deletion), or its activation may be suppressed through the engagement of inhibitory receptors.(55) However, it is noteworthy that a proportion of 20% of antibodies generated by mature human B lymphocytes exhibit reactivity towards HEp-2, while 4.3% of these antibodies are polyreactive.(54) The prevention of self-reactive naive B cells detecting peripheral self-antigens is typically facilitated by the regulatory T-lymphocyte (Treg) via the autoimmune regulator (AIRE) mechanism.(60) The latest research reveals that a peripheral checkpoint that occurs at the new emigrant/transitional B lymphocyte stage requires positive and negative selection. These two important mechanisms mold the

naive B lymphocyte repertoire. In fact, the positive selection of expanding naive B lymphocytes in a humanized mouse model occurred in an autologous thymic tissue-independent manner. Whereas the elimination of self-reactive B lymphocytes through negative selection was found to be dependent on thymus derived Treg cells and the B lymphocyte presentation of autoantigens with MHC class II restriction.(61)

Moreover, low-affinity or self-reactive germinal center (GC) B lymphocytes endure apoptosis and are eliminated by macrophages.(62) The regulation of B lymphocyte selection and maintenance within the GC is highly stringent in order to effectively prevent the generation of self-reactive plasma cells and memory B lymphocytes.(63)

To sum up, the generation of autoantibodies is primarily attributed to the random insertion and deletion of nucleotides in the CDR3 of the immunoglobulin heavy chain (IgH), which is also responsible for generating a significant portion of the diversity in the heavy chain repertoire. Healthy people necessitate two important B cell developmental checkpoints for the control of autoantibodies and even minor alterations in the efficiency of self-reactive antibody regulation may result in heightened vulnerability to autoimmunity.(54) Therefore, autoimmune disorders are characterized by defective B-lymphocyte tolerance checkpoints that are unable to counterselect the emerging self-reactive B lymphocytes.(57)

Development of B lymphocyte and representation of the percentage of self-reactivity across developing B cell stages, which encompassed both poly-reactive and anti-nuclear specificities are represented in Figure 2.2.

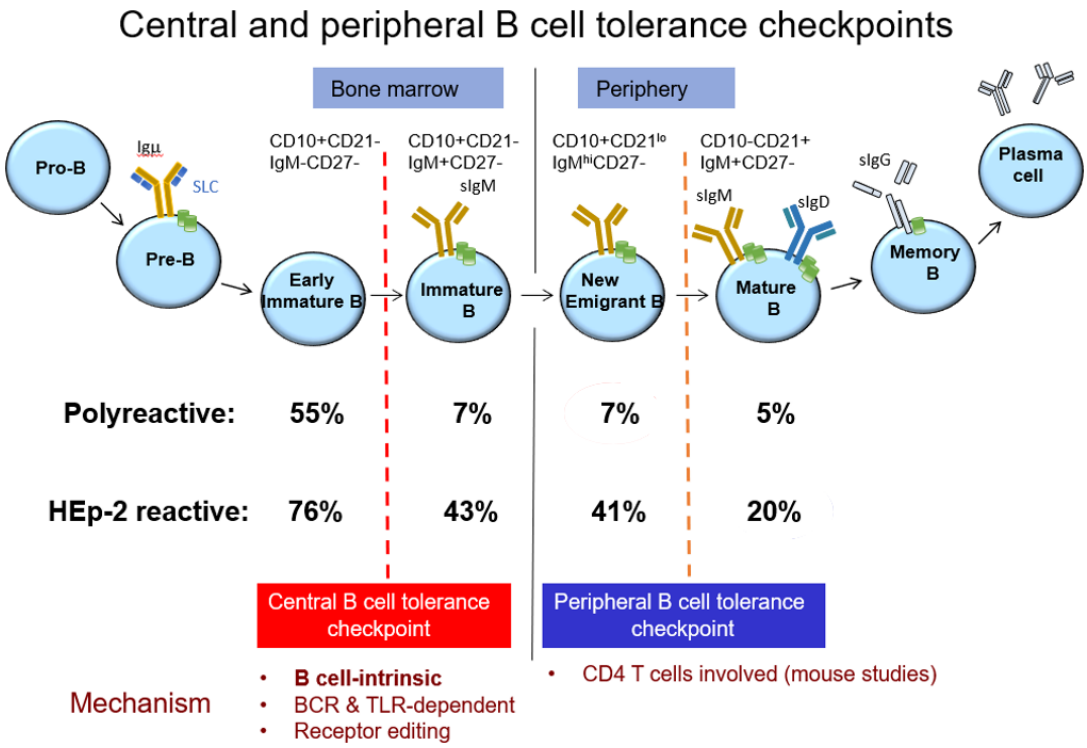


Figure 2.2: Development of B lymphocytes. (Adapted from (54) and Meffre lab presentations)

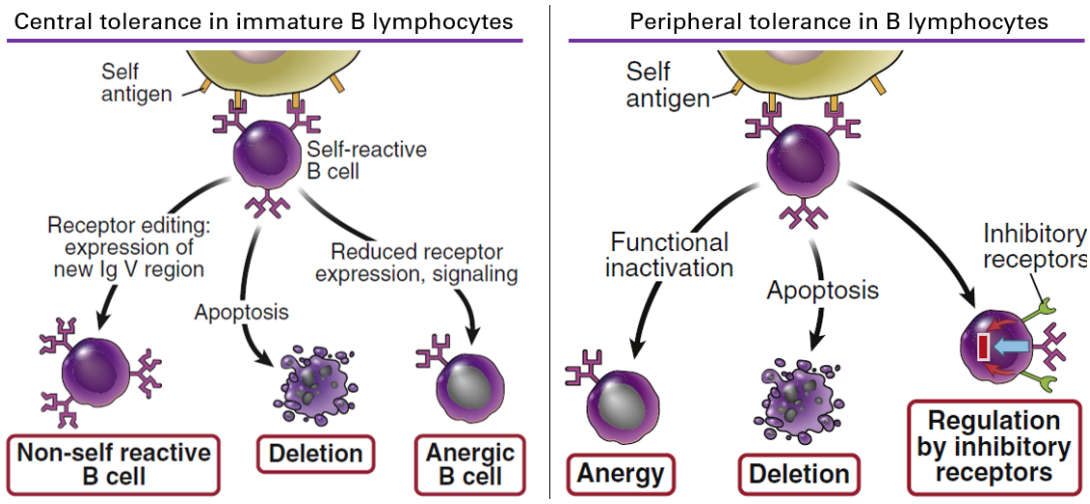


Figure 2.3: Examining the mechanisms underlying B lymphocyte tolerance to self-antigens. (Adapted from (55))

2.1.3. Memory B lymphocytes

B-cell memory is established following the initial exposure to a pathogen and is propagated by two separate lineages. These include long-lived plasma cells, which produce protective antibodies throughout an individual's lifespan, and memory B cells, which can evoke heightened and improved responses upon subsequent antigenic challenges. It has been widely acknowledged that the retention of memory is a distinguishing characteristic of B lymphocytes that have gone through isotype switching along with affinity maturation as a result of T-dependent GC reactions.(64)

Antibody-producing cells are a type of long-lived plasma cells that migrate to the bone marrow, release antibodies over an extended duration, and presumably do not undergo turnover; thereby, they do not necessitate additional antigen stimulation.(65) In contrast to antibody-forming cells, memory B cells possess this unique capacity to retain their reactivity to antigens, allowing them to initiate a rapid response when exposed to a specific challenge.(66) Furthermore, memory B cells have the ability to re-enter GCs, in which they endure subsequent cycles of somatic mutation.(67) Nevertheless, it is essential to acknowledge that memory B cells constitute a relatively minor fraction of the cellular population within secondary GCs.(68)

Memory B cells, which enables an accelerated antibody response following re-exposure to an anticipated antigen (69), possess distinctive characteristics such as long life ranging from months to years in the lack of any further exposure to antigens and undergo slow cycling (55), high sensitivity to minimal amounts of antigen, expeditious and vigorous proliferation (mounting a swift response upon re-exposure to the antigen) (55), and prompt differentiation into plasma cells, which generate antibodies of elevated affinity in the secondary response.(70) The CD27 molecule serves as a distinctive identifier for memory B cells in humans, which can account for approximately 40-60% of peripheral blood human B lymphocytes.(71) For instance, long-lived classical memory B lymphocytes are progressively produced in reaction to an infectious agent.(72) The CD27+ memory B lymphocytes exhibit co-expression of IgM or isotype-switched Ig.(73) Memory B lymphocytes do not appear to produce antibodies; however, they are present in the bloodstream and are also found in a multitude of tissues, encompassing mucosal tissues.(55) The spleen and tonsils are

recognized as the primary storage sites for antigen-specific human memory B cells.(74) Typically, memory B lymphocytes that express high-affinity and class-switched BCRs are generated within the GC.(75) The generation of the majority of class-switched memory B cells is reliant upon the GC response and the assistance of T lymphocytes. The process of Ig isotype switching is accompanied by a significant degree of somatic hypermutation of Ig genes, which has the potential to alter the affinity of antibodies or yield auto-reactive antibodies.(76) However, new discoveries suggest that there are also memory B lymphocytes that are developed independently of the GC.(77)

IgA-positive memory B lymphocytes are mostly found in mucosal tissues, where they play a vital part in maintaining the balance of the intestinal microbiota and providing defense against pathogens. Moreover, the presence of IgG-positive memory B lymphocytes are generated upon systemic exposure to antigens.(78, 79)

Over 50% of developing B lymphocytes in the human body initially exhibit autoreactive antibodies. Nevertheless, the majority of these self-antibodies are eliminated from the repertoire during two checkpoints prior to reaching maturity as naive B cells. The third checkpoint removes any residual self-antibodies from the population of antigen-encountered IgM+ memory B lymphocytes. Unexpectedly, it is observed that IgG+ memory B lymphocytes in normal controls can frequently produce self-reactive antibodies, such as anti-nuclear antibodies. The majority of these antibodies were generated through somatic hypermutation during the transition from mature naive B cells to IgG+ memory B lymphocytes. The findings suggest a potential association between autoimmunity and the breach of autoreactive IgG+ memory B lymphocyte control.(76)

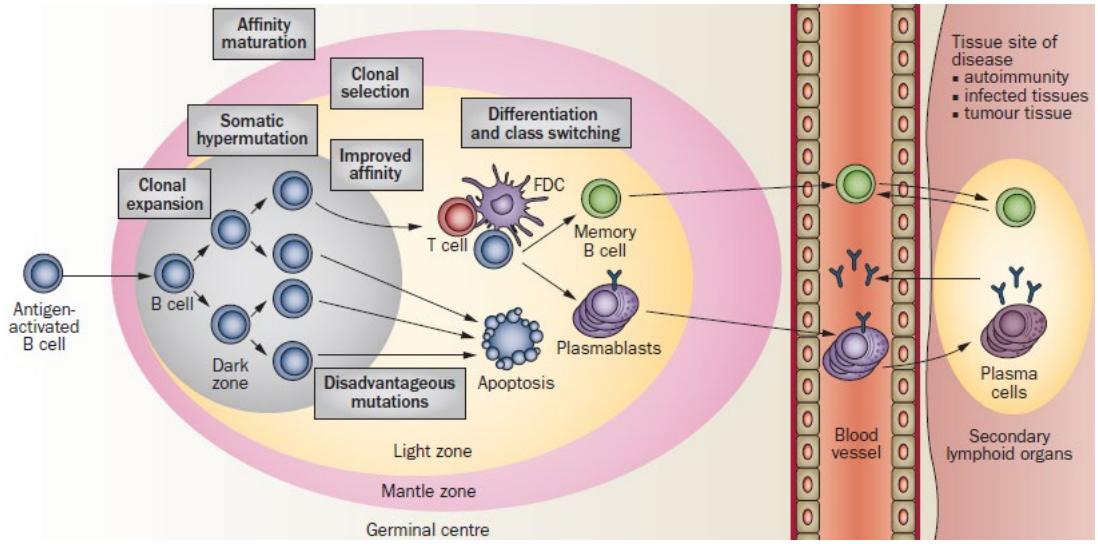


Figure 2.4: The response of B-cells. Upon encountering antigens and receiving cell help or co-stimulatory signals, B lymphocytes go through affinity maturation and clonal expansion. (Adapted from (69))

2.2. SLE

2.2.1. A Snapshot of SLE

SLE, frequently referred to as lupus, is a prototypical example of a persistent autoimmune disease defined by the production of self-reactive antibodies by the immune system and cytokine dysregulation, which in turn results in inflammation in various organs, encompassing the cutaneous, renal, cardiovascular, musculoskeletal, neurological, and gastrointestinal systems. SLE has the potential to cause severe debilitation and negatively impact a person's quality of life, and frequently leading to relapses.(80-83) It is anticipated that the prevalence of SLE will fall within the range of 13 to 7713.5 per 100,000 individuals globally, with a general incidence rate estimated to fluctuate between 1.5 and 11 per 100,000 person-years worldwide.(84) Lupus is more common among African American and other ethnic minority women.(80) A multitude of factors, encompassing genetics, estrogen, and environmental exposures may lead to the development of the SLE disease, whose severity can range from mild to severe.(81, 83)

While ongoing research aims to comprehend the precise lupus pathomechanism, current evidence suggests that SLE is distinguished by the levels of the type I interferon signature, enhanced T and B lymphocyte immune reactions, and the inability of immunological tolerance checkpoints to prevent the formation of pathologic self-reactive antibodies.(80, 83) The formation of immune complexes such as nucleic acids, nucleic acid-binding proteins, and autoantibodies targeting these components initiates the process of inflammation and subsequent damage to organs, as portrayed in Figure 2.5.(83)

The SLE diagnosis is typically established through the evaluation of clinical and laboratory evidence, following the exclusion of other potential diagnoses. Additionally, the identification of specific antibodies, such as anti-dsDNA and anti-Sm, through serologic analysis is a significant indicator of the SLE likelihood. The lupus clinical variability and the absence of pathognomonic characteristics or diagnostics appear to be a challenge in terms of diagnosis for physicians. The 2019 criteria, as described in Figure 2.6, established by the EULAR guidelines for SLE, were formulated with the aim of enhancing the identification of early- or new-onset SLE. To classify a patient SLE, it is necessary to establish the presence of a positive antinuclear antibody (ANA) as a prerequisite criterion. The additional points encompass a total of ten categories, which are further divided into seven clinical categories (namely constitutional, neuropsychiatric, hematologic, serosal, mucocutaneous, renal, and musculoskeletal) and three immunologic categories (namely SLE-specific antibodies, antiphospholipid antibodies, and complement proteins). Each of these categories is assigned a weight ranging from 2 to 10. As per the EULAR/ACR criteria, the diagnosis of SLE can be established when a patient exhibits a positive ANA result of ≥1:80 and achieves a score of 10 or higher.(85)

Assessing disease activity poses a significant challenge due to the intricate complexity of clinical manifestations and their temporal variability.(83) It is generally accepted that the presence of severe disease activity upon initial presentation, as demonstrated by a SLEDAI-2K score of 20 or higher, is a prognostic factor that is linked to mortality.(83, 86)

Despite the absence of a cure, SLE can be successfully managed with the appropriate medications.(80) Management strategies must be customized to suit the specific organ system under consideration to achieve remission or a state of low disease activity.(81) SLE patients may receive treatment involving the administration of various immunosuppressive drugs.(87) As a result of the prevalence of altered B-lymphocyte pathways in lupus, the reduction of B lymphocytes has been found to be beneficial to some lupus patients.(81) Novel therapeutic approaches, including biological agents and small-molecule drugs, are currently under development to rectify dysfunctional immune-cell activity, which in turn holds promise for enhanced efficacy and reduced toxicity compared to existing treatment modalities. (87)

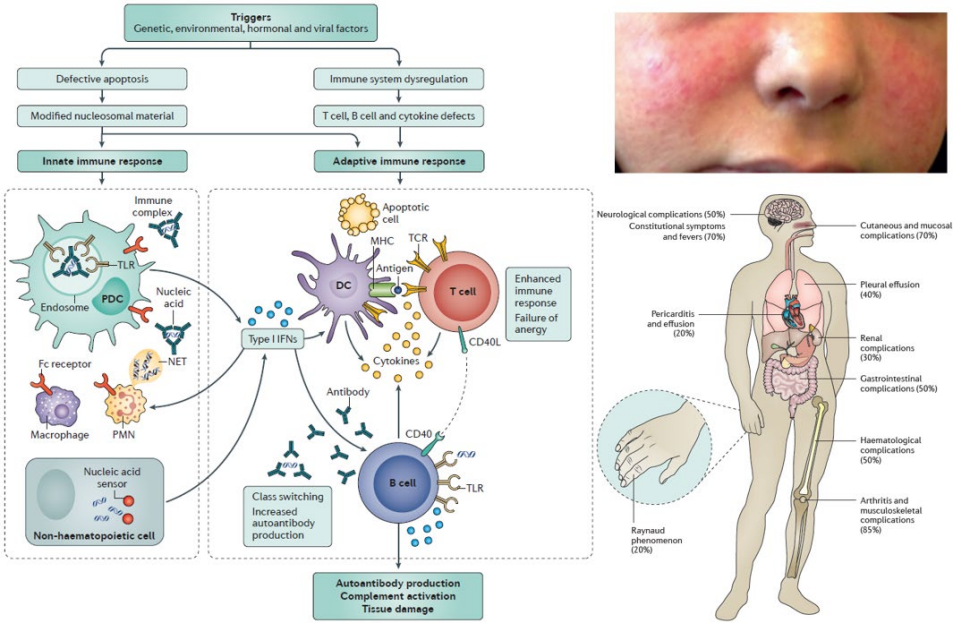


Figure 2.5: SLE immune dysfunction and its disease manifestations. (Adapted from(83))

2019 European Alliance of Associations for Rheumatology (EULAR)/American College of Rheumatology (ACR) classification criteria for systemic lupus erythematosus

The entry criterion is necessary to classify SLE.	
Entry criterion: ▪ ANA at a titer of ≥1:80 on HEp-2 cells or an equivalent positive test (ever).*	
At least 1 clinical criterion required to classify SLE. Additional additive (clinical or immunology) criteria are counted toward the total score.	
Additive criteria: ▪ Do not count a criterion if there is a more likely explanation than SLE. ▪ Occurrence of a criterion on ≥1 occasion is sufficient. ▪ Criteria need not occur simultaneously. ▪ Within each domain (eg, mucocutaneous, complement proteins), only the highest-weighted criterion is counted toward the total score if more than 1 is present.¶	
Clinical domains and criteria	**Weight**
Constitutional	
Fever	2
Hematologic	
Leukopenia	3
Thrombocytopenia	4
Autoimmune hemolysis	4
Neuropsychiatric	
Delirium	2
Psychosis	3
Seizure	5
Mucocutaneous	
Nonscarring alopecia	2
Oral ulcers	2
Subacute cutaneous or discoid lupus	4
Acute cutaneous lupus	6
Serosal	
Pleural or pericardial effusion	5
Acute pericarditis	6
Musculoskeletal	
Joint involvement	6
Renal	
Proteinuria >0.5 g per 24 hours	4
Renal biopsy Class II or V lupus nephritis	8
Renal biopsy Class III or IV lupus nephritis	10
Immunology domains and criteria	**Weight**
Antiphospholipid antibodies	
Anti-cardiolipin antibodies **or** anti-beta-2GP1 antibodies **or** lupus anticoagulant	2
Complement proteins	
Low C3 **or** low C4	3
Low C3 **and** low C4	4
SLE-specific antibodies	
Anti-dsDNA antibodyΔ **or** anti-Smith antibody	6
A total score of ≥10 and ≥1 clinical criterion are required to classify SLE.	
Total score	

SLE: systemic lupus erythematosus; ANA: antinuclear antibody; HEp-2: human epithelial type 2; anti-beta-2GP1: anti-beta-2 glycoprotein 1; anti-dsDNA: anti-double-stranded DNA.

* If ANA is absent, do **not** classify as SLE.

¶ Additional criteria within the same domain will not be counted.

Δ In an assay with 90% specificity against relevant disease controls.

Figure 2.7: Revised guidelines for the SLE classification: the 2019 EULAR/ACR criteria (Adapted from UpToDate (85)

2.2.2. B lymphocytes associated with SLE

Autoimmune diseases characterized by a strong association between B-cell functions and disease activity encompass SLE, scleroderma, rheumatoid arthritis, multiple sclerosis, and type 1 diabetes. In particular, SLE is distinguished by the existence of elevated autoantibody titers and the involvement of numerous organ systems.(88) Indeed, the levels of serum anti-dsDNA antibodies exhibit a positive correlation with disease activity in SLE patients, with a particular emphasis on those diagnosed with lupus nephritis.(89) Moreover, the presence of autoantibodies tends to occur several years prior to the diagnosis of lupus when patients remain without any noticeable signs of the disease.(90) This observation may indicate a significant involvement of B lymphocytes in the advancement of SLE. On top of that, it has been observed that MRL/lpr mice lacking B-cells exhibit no pathological manifestations at a stage in their development when MRL/lpr mice with intact B-cells display full-blown disease.(91) Furthermore, environmental insults could cause the release of nuclear autoantigens from dying cells. This release subsequently triggers the generation of pro-inflammatory cytokines, like interferons, which are crucial in facilitating autoimmune B cell responses and SLE progression.(92)

There is a substantial body of research that has established the existence of B lymphocyte phenotype abnormalities in individuals with lupus. Specifically, a reduction in CD27+ switched memory B lymphocytes has been observed (93), while there may be an elevation in the frequency and quantity of naive B cells (94). Additionally, there is a variable expansion of transitional B lymphocyte and plasma cell populations in patients with SLE.(95, 96) Moreover, individuals with SLE are prone to exhibiting impaired central and peripheral B cell tolerance checkpoint mechanisms (seen in Figure 2.8), resulting in the presence of autoreactive B lymphocyte in their bloodstream.(4) Additionally, there have been a report indicating that B cells that secrete IL-10 and possess regulatory capabilities exhibit functional impairment in lupus, referring to a skewed SLE B cell repertoire.(97)

A comparable breach in tolerance shows that dysregulation of TLR and BCR networks, along with impaired co-stimulation, can disrupt the mechanisms responsible for preserving self-tolerance at both the antibody-forming cell and GC checkpoints,

consequently leading to the emergence of self-reactive B lymphocyte, the generation of autoantibodies, and the subsequent development of SLE.(92) In the context of SLE, it has been observed that autoreactive B lymphocytes have the ability to endure as long-lasting plasma cells or memory B lymphocytes. Certain investigations indicate that a majority of autoantibody-producing lymphocytes found in individuals with lupus originate from non-autoreactive B lymphocyte precursors, demonstrating the contribution of somatic mutations to the specificity and reactivity of autoantibodies.(6)

The deficiency of C4, which SLE has been found to be potentially linked to (87), has been connected to a reduction in the elimination of self-reactive B lymphocytes, which compromises the process of negative selection.(87, 98) The presence of the R620W polymorphism in the PTPN22/Lyp gene, which is linked to lupus development, results in a reduction in BCR signaling, thereby influencing the development of human B cell tolerance. It has been previously documented that individuals carrying the PTPN22 risk allele exhibit a higher prevalence of self-reactive clones in transitional and mature naive B lymphocytes as compared to donors who do not carry this allele.(99) Also aberrant CD19 expression and the diminished tolerogenic function of TLR9 in B cells in SLE patients could potentially result in a breach in B lymphocyte tolerance checkpoints in these patients.(100) On the other hand, it has recently been observed that the aberrant function of B lymphocytes in lupus is attributed to ATR-mediated DNA injury responses.(101) It remains to be determined what the other different cascades or means are that affect B lymphocytes and cause the emergence of SLE.

Aberrant B-cell pathways frequently occur in lupus. B lymphocytes participate in antigen presentation to T lymphocytes, producing autoantibodies, and releasing cytokines such as interleukin 6, interleukin 10, interferon α, APRIL, BAFF, and TNF-α. Therefore, the B-lymphocyte depletion has been observed to yield beneficial outcomes in certain SLE patients. For instance, Rituximab specifically binds to CD20 receptors on B lymphocytes, leading to diminished B-cell precursors and mature B lymphocytes.(81) Based on an experimental lupus mouse model, the presence of cytotoxic T lymphocytes that express CD19-targeted chimeric antigen receptors (CARs) led to a sustained decline in CD19+ B lymphocytes, cessation of autoantibody generation, reversal of disease symptoms in affected organs, and a significant

extension of lifespan beyond the expected norm.(102) Also, the administration of bortezomib, a proteasome inhibitor, resulted in the depletion of plasma cells and improved the clinical symptoms associated with refractory SLE.(103) The modulation of B lymphocyte function or quantities can be very promising. Cytokine treatments targeting B-cells encompass various approaches for lupus management options. (87) The expression of BTK is significantly elevated in individuals diagnosed with lupus nephritis. The kinase plays an essential role in mediating intracellular signaling networks within B lymphocytes as well as facilitating the activation of macrophages and monocytes. The blockade of BTK in SLE murine models has demonstrated encouraging findings. Overall, in spite of the progress made in therapeutic interventions, the morbidity associated with SLE continues to be significant, so hopes for tomorrow involve a strategic focus on the utilization of pharmaceuticals that impede the functionality of B-cells.(81)

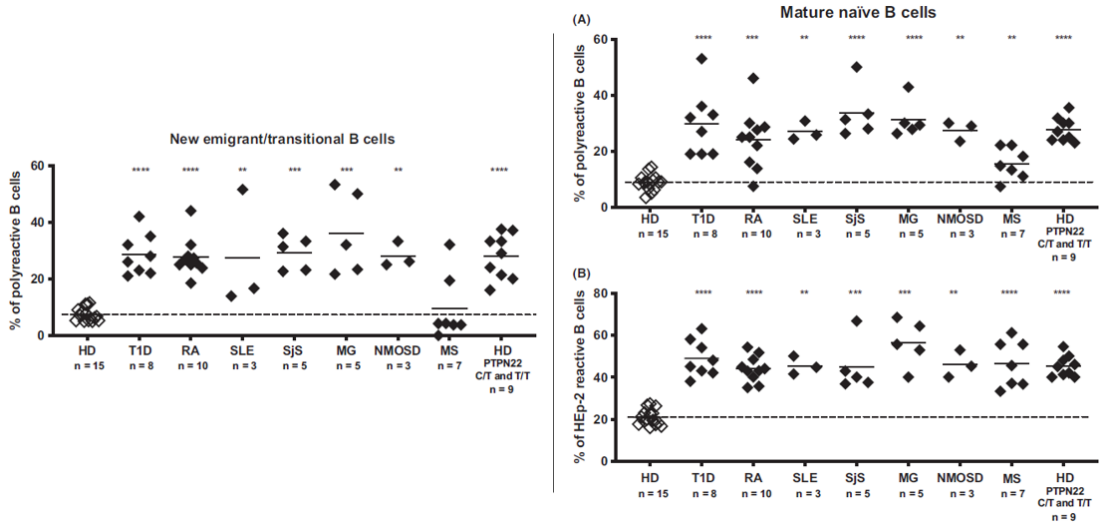


Figure 2.8: Patients with autoimmune disease exhibit compromised central B and peripheral B lymphocyte tolerance checkpoints. (Adapted from (57))

2.3. Intestinal Microbiota Alterations Linked To SLE

2.3.1. Gut Flora and Mucosal Immunity

The concept of "microbiota" denotes the assemblage of microorganisms that reside harmoniously within their mammalian hosts; on the other hand, the microbiome encompasses the entirety of the genetic material possessed by the microbiota of humans. (104-106) Afterwards, dissecting human microbiota has been significantly

assisted by the implementation of high-throughput sequencing tools. (105, 107-112) Commensal and symbiotic microorganisms colonize various anatomical locations. However, the intestinal tract, particularly the colon, serves as the principal habitat, hosting an array of over 400 bacterial species.(104, 113-117) Various types of microbial organisms, such as viruses, archaea, protozoa, and fungi, can be found within the mammal gastrointestinal tracts. However, bacteria exhibit the highest prevalence among all these indigenous microbial cells in the digestive tract and the most densely colonized in the colon.(118, 119) The Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria phyla are considered fundamental constituents of the microbiome community, playing a crucial role in fostering bodily homeostasis within the intestines. (120, 121) The establishment of a mutually beneficial connection between the host and its symbiotic intestinal microbial inhabitants has been facilitated by their long-term evolutionary cooperation and adaptability. In this relationship, the resident gut microbiota actively contributes to various host physiological processes, while the host, in turn, provides suitable habitats as well as the required resources for the survival of these microbial lives.(108, 118, 122) Moreover, there is significant variation in the composition and complexity of the indigenous commensal intestinal flora among individuals, apart from substantial differences between different regions of the intestinal tract.(123, 124) Given the intricate nature of the gut microbiota, numerous variables, such as age, sex, genetic makeup, dietary patterns, stress levels, exposure to pathogens, environmental conditions, sanitation practices, and the use of pharmaceuticals, notably antibiotics, all exert an influence on the composition and diversity of the gut flora.(123, 125, 126) The wide array of variations observed in the gastrointestinal system can be attributed to a process of meticulous host selection and coevolution (119), and the fundamental characteristics of human nature are shaped by communities of commensal microorganisms.

Over the course of the past twenty years, a considerable amount of research has extensively documented the profound impact exerted by the gut microbial communities on the physiological processes and immunological reactions of the host organism. (123, 127, 128) The microbial communities in the intestines perform an important duty in various metabolic processes as well as impact the development and functioning of the host's innate and adaptive immune systems. The human body relies

on a well-functioning gut microflora to facilitate various physiological processes, which encompass food digestion, energy extraction, synthesis of vitamins, regulation of host metabolism, development and maturation of immune cells, facilitation of gut-associated lymphoid tissues (GALTs), production of antimicrobial molecules, protection against pathogens (seen in Figure 2.9), metabolism of pharmaceutical and xenobiotic compounds, modulation of neurotransmitters, and release of hormones.(111, 112, 118, 124, 129-132) The establishment of a harmonious microbiota that is unique to the individual is crucial for maintaining optimal immune system functionality.(133) The nature and arrangement of the gut flora have an immense effect on various aspects of the human immune system. These involve the formation of the B cell repertoire, the production of polyreactive and autoreactive antibodies, the balance of helper T cell populations, the sustaining of harmony between the Th17 and Treg subsets, the management of the Th17 cell pool, and the control of cytokines; thereby, the gut microbiota acts an indispensable part in shaping and maintaining the individual's immunity.(125) In addition, the gut symbionts mold a repertoire of pre-immune BCRs and enhance the systemic immune response towards microbial entities by boosting the production of IgG. Furthermore, they contribute to the amplification of T cell-independent commensal reactive IgA input.(134) Overall, the bidirectional communication between microbial organisms and their human niches serves as an important factor in preserving the host's health.(123)

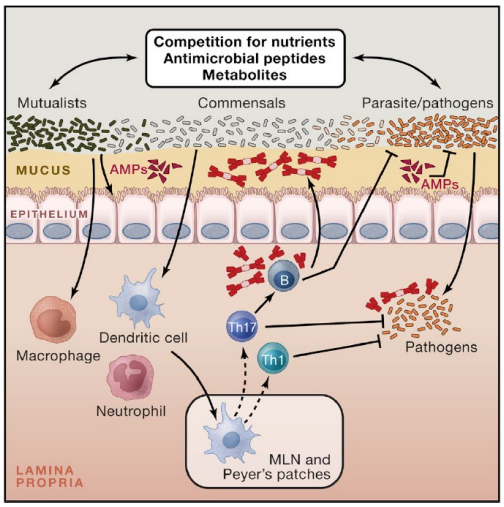


Figure 2.9: Protective Immunity Promotion by the Gut Microbial Community. (Adapted from(132))

2.3.2 SLE and Gut Dysbiosis

Various environmental factors, such as dysbiosis of microbiota, have the ability to trigger the activation of immune receptors or stress-mediated cell responses, which subsequently results in the secretion of interferons, hallmarks of SLE.(92) The principal site of symbiosis, the gut microbiome, has been reported to control how the host immunity develops and is probably connected to genetic alterations that may lead to systemic autoimmune disease.(135) In fact, the development of tolerance to both the host's own antigens and commensal microorganisms depends on this reciprocal relationship between the immune systems of individuals and the residing commensal gut communities.(115, 118, 136)

Intestinal dysbiosis, a disequilibrium state in the composition of gut microflora, is typically identified by a transition from a state of healthy equilibrium to a pathological condition. (137, 138) This phenomenon has been connected to the formation of autoimmune illnesses, such as lupus.(139, 140) Given the fact that one of the immunological tolerance preservation strategies relies on the gut micro-ecosystem in the host, intestinal flora dysbiosis can result in shifts in the characteristics and function of immune cells in SLE subjects. Consequently, this disruption may lead to breaches in the checkpoints, which may be one of the causes of the broken tolerance in individuals with SLE.(126)

Despite the extensive research conducted on gut bacteria over the years, there has been a notable surge in interest in the past decade regarding the association between intestinal dysbiosis and SLE. The initial publication documenting the presence of intestinal dysbiosis in relation to SLE by Hevia et al. examined the microbiota interaction with the host in autoimmune diseases, specifically focusing on identifying intestinal pathological alterations associated with SLE. This study utilized 16S rRNA gene analysis on stool samples to demonstrate that SLE patients with inactive SLE exhibited a significantly reduced ratio of Firmicutes to Bacteroidetes. Additionally, a decrease in certain families of Firmicutes was observed.(139) Following investigations provided further evidence for a reduced ratio of Firmicutes to Bacteroidetes.(141-150) However, it is important to acknowledge that a few investigations examining alterations in the Firmicutes to Bacteroidetes ratio among

individuals with lupus have produced inconsistent results across various contexts. In the study conducted by Luo et al., it was observed that the human cohort exhibited no significant disparity in the relative abundance of Firmicutes to Bacteroidetes when comparing the microbiota of individuals with SLE and those without SLE.(151) Contrary to the findings of previous studies, J. He et al. observed an increase in the Firmicutes/Bacteroidetes ratio and a notable decrease in the abundance of Bacteroidetes among SLE patients.(152) Nevertheless, alterations in the proportion of Firmicutes to Bacteroidetes are not limited to SLE and have been seen in other disorders like Crohn's disease (153), obesity (154), and type 2 diabetes (155) as well. This ratio may therefore serve as an indicator of a more widespread dysregulated state.

The effects SLEDAI and intestinal dysbiosis may potentially constitute a reinforcing two-way cycle. A dysbiotic gut bacteria species' adjustment might be a reaction to a localized human gut's response to the pathophysiology of lupus, which showed expansion and diminution of specific microorganisms that could be either the major or secondary causes of lupus pathology.(156) Indeed, the functions of resident gut flora in the development of SLE remain puzzling. Consequently, interpreting the microbiological profiles linked to SLE has been undertaken lately in an effort to gain more insight into the disease's pathology.

One of the predominant factors through which gut microorganism modulate the immunological equilibrium of the host is by modifying the composition of metabolites, thereby exerting an impact on the immune response.(157) For instance, the presence of changes in lipid panels has been observed to be linked to autoimmune mechanisms and the level of disease activity in SLE.(158-161) Indeed, individuals diagnosed with SLE exhibit an atypical lipid profile in both their serum and stool samples.(162) Furthermore, SCFA biosynthesis from the intestinal symbiotic microorganisms facilitates the augmentation of anti-inflammatory cytokines, aside from promoting the development of Treg cells (163, 164), is impaired in lupus.(141) The reported change has been associated with a decline in the ratio of Firmicutes to Bacteroidetes and a climb in SCFA levels.(141) SLE patients from a study exhibited a decreased prevalence of the Ruminococcaceae family, known for its ability to produce SCFAs. Consequently, a reduction in the number of such bacteria may result

in a drop in SCFA synthesis, thereby contributing to metabolic dysregulation in individuals with SLE.(165) Interestingly, the shrinking of Ruminococcaceae has also been connected to leaky gut syndrome, which pertains to an elevated permeability of the intestinal barrier.(165)

In addition, in various autoimmune scenarios, the availability of tryptophan may regulate the population of self-reactive helper T lymphocytes, and an elevated tryptophan metabolism rate contributes to the advancement of lupus disease. In fact, the restriction of dietary tryptophan exhibited a preventive effect on autoimmune disorders in a SLE murine model.(166)

Moreover, Chen et al. reported that Lactobacillus salivarius, Shuttleworthia satelles, Clostridium leptum, Actinomyces massiliensis, Atopobium rimae, Bacteroides fragilis, Clostridium species ATCC BAA-442 and an unclassified Escherichia were found to be elevated in the gut microbiota of individuals diagnosed with SLE, and these microbial abundances were observed to decrease after therapeutic intervention.(167)

According to research, the colonization of commensal bacteria with orthologs of human Ro60 has the potential to induce long-lasting autoimmunity in individuals with a genetic predisposition to lupus through cross-reactivity and epitope spreading mechanisms.(143)

Also, the murine model has gained significant appeal as a platform for investigating the relationship between the SLE and gut microbiome due to its ability to facilitate precise manipulation and examination of specific microbiota. In a study, mouse lupus models demonstrated a substantial reduction in lactobacilli and a rise in Lachnospiraceae in female mice predisposed to lupus.(127) Moreover, the fecal microbiome derived from mice with SLE has the ability to elicit the anti-dsDNA antibody production, induce an inflammatory state, and modulate lupus susceptibility gene expression in germ-free mice.(168) In general, prior studies investigating dysbiosis in relation to SLE provide evidence that intestinal dysbiosis is a substantial contributor to the pathogenesis of SLE.

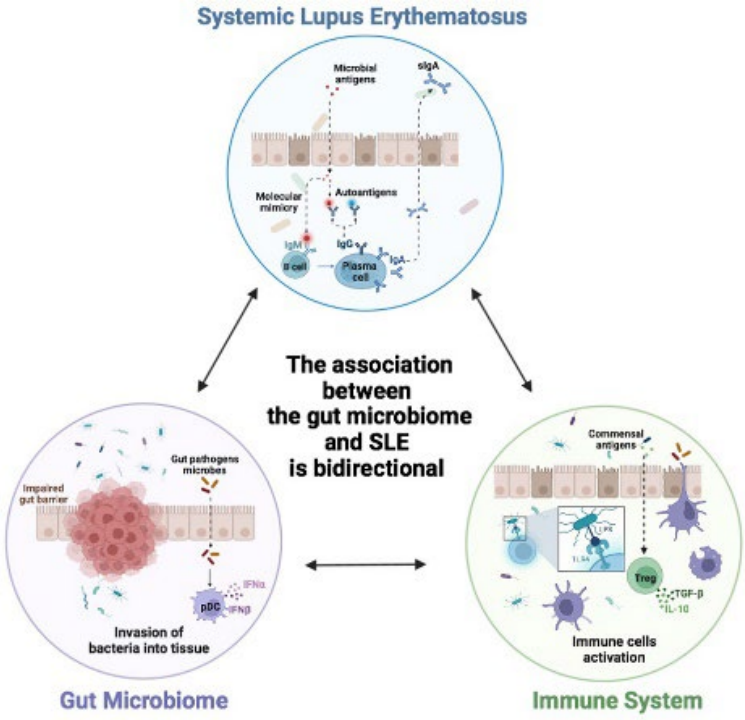


Figure 2.10: The phenomenon of reciprocal communication between SLE and gut dysbiosis. (Adapted from (126))

2.4. Importance of VH4-34 B cells on SLE

2.4.1. VH4-34 gene segment and its AVY/NHS motif

The VH locus in humans is located on chromosome 14, and human VH germline genes have been divided into at least six families (which are designated VH1 through VH6 and range in size) with 80% similarity in their nucleotide sequences.(169, 170) Our research has mostly concentrated on the VH4 gene family, in particular the VH4-34 gene, which has been shown to be overrepresented in B lymphocytes of patients with SLE (5, 171) and has often been paired with pathologic autoantibodies.(172)

The V4-34 gene segment shares a homology of 96% with the germline.(173) The V4-34 gene segment encodes immunoglobulin heavy chains, which are the distinctive feature of all monoclonal IgM cold agglutinins that specifically attach to the I/i carbohydrate antigens found on the surface of red blood cells (RBCs).(8) The I/i antigen is comprised of iteratively occurring N-acetyllactosamine units, which

exhibit a linear arrangement in the i structure and a branched configuration in I.(174) Thus far, all antibodies that have been characterized as exhibiting reactivity towards B-cell/i-antigens have been derived from the V4-34 gene. The binding of antibodies to B-cell/i-antigens is not observed in antibodies produced from different human VH families, such as other members of the VH4 family.(175) The robust correlation between a single VH gene portion and its specificity for the I/i antigen suggests that the unique configuration established by the V4-34-encoded segment of the antibody is necessary for binding to the I/i antigen. (8) In fact, the observed inhibition of red blood cell agglutination by the monoclonal antibody led to initial speculation that the framework region 1 (FR1) might potentially contribute to the identification of the I Ag.(8, 176) The mutational analysis displayed that the 9G4 idiotype (Id) is positioned within FR1 of the immunoglobulin encoded by the V4-34 gene. Indeed, the 9G4 idiotope is associated with the amino acid positions 23–25 of the FR1 protein, possessing the specific amino acid sequence AVY, which is essential for I carbohydrate antigen binding.(8, 177, 178) In addition, the previous findings provide compelling evidence that both the FR1 and CDRH3 regions of V4-34-encoded immunoglobulins play a crucial role in facilitating binding to the I antigen.(178) The binding of I carbohydrate antigen to V4-34-encoded antibodies also occurs outside of the typical combining site, which supports the notion that the surface-exposed CDRH3 residue adjacent to FR1 can affect the ability of the I Ag to associate with both CDRH3 and FR1, potentially due to their shape and/or charge characteristics. Based on the analysis of the crystal structure, it is observed that the AVY motif, defining the 9G4 idiotope (177), collectively constitutes a hydrophobic region within FR1, which simultaneously interacts with complementarity-determining region (CDR) H3 and I carbohydrate antigen.(8) In fact, the same research group also suggests that the FR1 of the heavy chain is potentially significant in facilitating the necessary interaction between the antibody and antigen, whereas the CDR3 of the heavy chain determines the precise specificity and binding strength.(178) Moreover, due to the absence of amino acid variations that differentiate V4-34 from other members of the VH4 family, excluding FR1, it is challenging to establish the involvement of sequences beyond FR1 in the restriction of V4-34 among cold agglutinins.(8)

Also, all of the antibodies derived from VH4-34, in the past designated as VH4-21 (172), were found to be reactive with 9G4 with few exceptions, while immunoglobulins utilizing gene segments from other VH families lacked reactivity with 9G4, indicating that the 9G4 antibody shows a specific reactivity with antibodies that are encoded by the VH4-34 gene segment. Besides, the 9G4 reactive idiotype is specifically limited to the heavy chain component only.(177) In addition, all immunoglobulins (Igs) that exhibit reactivity with 9G4 possess the AVY motif in the FR1 region. However, the reactivity of 9G4 is eliminated when the amino acid alanine at position 23 is substituted with threonine. The significance of the valine residue at position 24 becomes evident when it gets replaced with alanine in several antibodies, resulting in their loss of reactivity with 9G4. In parallel, the replacement of the VH4-21 amino acids AVY at positions 23-25 with TVS results in the antibody becoming unresponsive to 9G4. The restoration of reactivity with 9G4 is observed when the AVY sequence is reintroduced into the FR1 region. Although there is a chance that a spontaneous mutation will occasionally appear in the FR1 segment that the 9G4 antibody recognizes, sequence analysis of a number of cold agglutinins suggests that this is not a frequent occurrence.(177) Moreover, it has been reported that the anti-i-secreting B lymphocytes display the VH4-34 gene in its unmutated germline-encoded form.

Furthermore, it should be noted that the immunoglobulins encoded by the V4-34 and VH4 families exhibit distinct amino acid compositions at positions 52 and 53 within the CDR of the heavy chain 2 loop (specifically, residues 52-56). The V4-34 gene segment is responsible for encoding the amino acid sequence NH at specific positions, on the other hand, the rest of the VH4 family members exhibit either YY or YH at those same positions.(8) In the study conducted, it was observed that the substitution of the V4-34 CDR H2 sequence with the CDR H2 of a V4-61-encoded Ig resulted in the replacement of NH with YY, which did not have any discernible impact on the binding of I. (178) This observation suggests that the amino acids NH within CDR H2 do not contribute significantly to I binding. The CDRH2 substitution resulted in the elimination of a putative N-linked glycosylation segment at the NHS motif, a characteristic exclusive to V4-34-encoded immunoglobulins within the VH4 family.(8)

2.4.2. Autoreactive VH4-34 B lymphocytes

A particular subset of B lymphocytes in humans, known as VH4–34 or 9G4 B lymphocytes, has been deemed to possess a role that operates at the junction of both adaptive and innate immune reactions.(5) The 9G4 B lymphocyte examination provides invaluable insight regarding the intricate equilibrium between advantageous and harmful immune reactions.(1) Autoreactive clones that express inherently autoreactive VH, namely VH4–34, undergo development and accumulation within the adult naive B lymphocyte compartment.(179) The earlier evaluations demonstrated a high prevalence of the VH4-34 gene in the autoimmune repertoire.(180) In one study, the fact that two human monoclonal antibodies detect lipid A and a carbohydrate epitope on B lymphocytes suggested that the highly conserved VH4-34 gene plays a dual role in both the immune response to bacteria and the B cell formation.(181) Specifically, the unmutated VH4-34 genes possess the FR1 AVY sites, which are recognized for their ability to bind to I/i self-antigens found on RBC antigens.(182, 183) These self-reactive antibodies might be involved in removing dying erythrocytes.(179) It has been shown that antibodies produced from the VH4-34 gene possess distinctive autoreactive characteristics. It includes a variety of anti-Rh antibodies (184) as well as polyreactive antibodies that exhibit binding affinity towards DNA, bacterial lipid A, rheumatoid factor and cardiolipin. (172, 180, 181) Antibodies containing the IGHVH4-34 gene have been demonstrated to identify commensal bacteria.(7)

The identification and isolation of this specific group of B lymphocytes may be achieved utilizing a rat monoclonal antibody known as 9G4.(185) Apart from exhibiting specific inhibitory effects on cold agglutination (176), the anti-idiotypic antibody (9G4) has particular binding affinity towards a distinct epitope that is encoded within FR1 of the human VH4-34; thereby, it may be inferred that every 9G4 B cell inherently possesses the VH4-34 gene.(1) Strikingly, the 9G4 monoclonal antibody demonstrated reactivity exclusively with V4-34 expressing immunoglobulins, irrespective of the D, JH, or L chain sequence and isotype.(186) It is noteworthy that the identical FR1 sequence, which is responsible for encoding the 9G4 epitope, also performs an important function in the superantigen binding characteristic and 9G4 B lymphocyte autoreactivity.(8) The 9G4 epitope

disappearance could manifest as a consequence of the accumulation of somatic mutations in the FR1 responsible for encoding the 9G4 epitope.(1) In addition, it has been demonstrated by mutational studies that the 9G4 epitope loss leads to a concomitant autoreactivity disappearance.(187) Hence, the identification of the 9G4 epitope can be regarded as indicative of autoreactivity, and the examination of 9G4 expression provides a means to ascertain the destiny of autoreactive B lymphocytes and explore the underlying processes governing their control.(1)

The VH4-34 gene has a higher frequency in normal adults at the level of BCR. According to the findings of 9G4 monoclonal antibodies, a small proportion ranging from 3 to 8% of B lymphocytes in both human peripheral lymphoid tissues and peripheral blood exhibit VH4-34 positivity. Nevertheless, circulating VH4-34 immunoglobulins are present at minimal to undetectable concentrations in the healthy individuals' peripheral blood.(176, 188) Furthermore, the presence of VH4-34 is not detected in the plasma cells of healthy donors' tonsils and bone marrow samples.(5) Negative selection, a process that often results in the suppression or tolerance of VH4-34-bearing B lymphocytes, seems to occur during their differentiation. (180) Although there is a high prevalence of 9G4 B lymphocytes in the primary normal repertoire, they are rarely found in healthy sera, regardless of whether it is IgM or IgG.(171, 176, 189-191) In fact, it is shown that less than 1% of the repertoire of circulating IgM and IgG proteins is derived from the VH4-34-expressing cell population. Moreover, the findings presented in a study provide evidence for the presence of a mechanism in humans that facilitates the occurrence of mutations leading to reduced self-reactivity. The efficiency of clonal redemption of anergic cells during healthy human antibody responses is evident due to the use of IGHV4-34*01 by 2.5% of switched memory B cells, with over 43% of these cells exhibiting mutations that eliminate I/i binding.(9) In the event of dysregulation, VH4-34-bearing B lymphocytes can undergo expansion.(180) With the evidence supporting B-cell tolerance breaches and pathogenic involvement of B lymphocytes, polygenic autoimmune disorders, namely SLE, multiple sclerosis and rheumatoid arthritis show considerably skewed B-cell receptor repertoires in comparison to healthy individuals. Remarkably, these disorders have shared repertoire defects, including an increased use of the IGHV4-34 gene.(183) However, this reference is likely not supported by other publications that showed that

VH4-34+IgG+ B lymphocytes are restricted to lupus, and the detection of elevated IgG-9G4 autoantibody levels is highly indicative of this particular autoimmune disorder.(1, 171, 189-192)

2.4.3. Significant role of VH4-34 B lymphocytes in SLE

In some studies, the 9G4 B lymphocytes may be classified as innate B lymphocytes due to their significant presence in the primary pre-antigenic repertoire, comprising approximately 5-10% of mature naïve B lymphocytes. Furthermore, these cells undergo expansion during certain infections, such as Mycoplasma pneumoniae and Infectious mononucleosis, suggesting their potential involvement in protective immune responses.(193, 194) Crucially, for the categorization of 9G4 B lymphocytes as innate like B lymphocytes, their antigenic reactivities towards autoantigens and pathogens are assigned by the germline VH4-34 sequence without the need for antigen-selected affinity maturation or somatic hypermutation, although further investigation is necessary.(8) The 9G4 B lymphocytes are also found in the IgM/IgD memory population and the marginal zone B cell compartment; however, they are not present in the T lymphocyte-dependent IgG memory repertoire, which means they typically do not contribute to adaptive B lymphocyte responses.(1) The 9G4 B lymphocyte sequestration within the innate immune system can be disrupted under specific circumstances, like in SLE patients, whose 9G4 B lymphocytes are significantly increased within the IgG memory subset and involve lupus pathogenesis.(5, 171) It has been demonstrated that VH4-34-expressing antibodies are present in fifty-five percent of SLE patients. (191) Interestingly, enhanced VH4-34 immunoglobulin production is particularly specific to SLE.(5)

The sequencing investigations on BCR regarding variable gene analysis for SLE patients have consistently shown a notable increase in the use of the IGHV4 gene family, particularly the IGHV4-34 gene.(2, 183) The VH4-34 gene, which is recognized for its inclination towards autoreactivity in its germline configuration, experiences negative selection in the memory and plasmablast subsets of normal subjects, free of disease. It is claimed that the act of censoring at the GC reaction stage, to some extent, might be attributed to anergy brought about by prolonged exposure to self-antigens.(1) However, the regulatory mechanisms responsible for censoring 9G4

IgG antibodies are faulty in SLE; consequently, VH4-34 is observed in greater proportions among activated naive B lymphocytes, plasmablasts, and memory B lymphocytes of lupus patients.(2, 3, 190, 195)

SLE, a condition characterized by polyclonal B-lymphocyte hyperreactivity (196), has been linked to a multitude of autoantibody targets exceeding 100. Additionally, ANAs have been found to be present in over ninety-five percent of SLE patients. As stated earlier, the expression of 9G4 was found to be considerably elevated in the blood levels of IgM and IgG among SLE patients in comparison to healthy individuals. Nevertheless, previous studies have demonstrated that there was no discernible disparity in the degree of 9G4 expression on IgM, IgG, or anti-dsDNA antibodies among patients who experienced a disease flare within one year after rituximab-based B lymphocyte depletion therapy and those who had a more prolonged response to the same therapy.(197)

Considering the significance of autoreactive VH4-34 antibodies in SLE pathogenesis, a study employed a novel strategy to produce anti-VH4-34 antibodies, specifically utilizing the compound HMBD-011. The objective was to eliminate VH4-34 auto-antibodies and diminish the VH4-34+ B cell population, thereby exploring the therapeutic prospects of HMBD-011 for individuals afflicted with VH4-34-associated autoimmune disorders.(198) It is worth mentioning that CD19-positive B cell lymphopenia, which impacts more on naïve B lymphocytes compared to memory B lymphocytes (199), has been observed among SLE patients, leading to a reduction in the quantity of both naive and memory cells. But still, SLE patients in the study exhibit a higher frequency of CD27+ early plasma cells.(193)

Acute SLE episodes course with the manifestation of a diverse array of antibody-secreting cells (ASCs) marked by clones expressing the variable heavy-chain region VH4-34, which are responsible for the production of prominent autoantibodies seen in the serum. The circulating ASCs, whose source was a specific fraction of activated naive B lymphocytes, seen during SLE flares exhibit polyclonality and have a reduced frequency of somatic hypermutation.(2) The VH4-34 segment exhibiting a high degree of autoreactivity has been shown to have a positive correlation with active

SLE in serological investigations.(3, 190) The self-reactivity of VH4-34 monoclonal antibodies obtained from both naïve and memory lymphocytes of SLE patients were reported.(200)

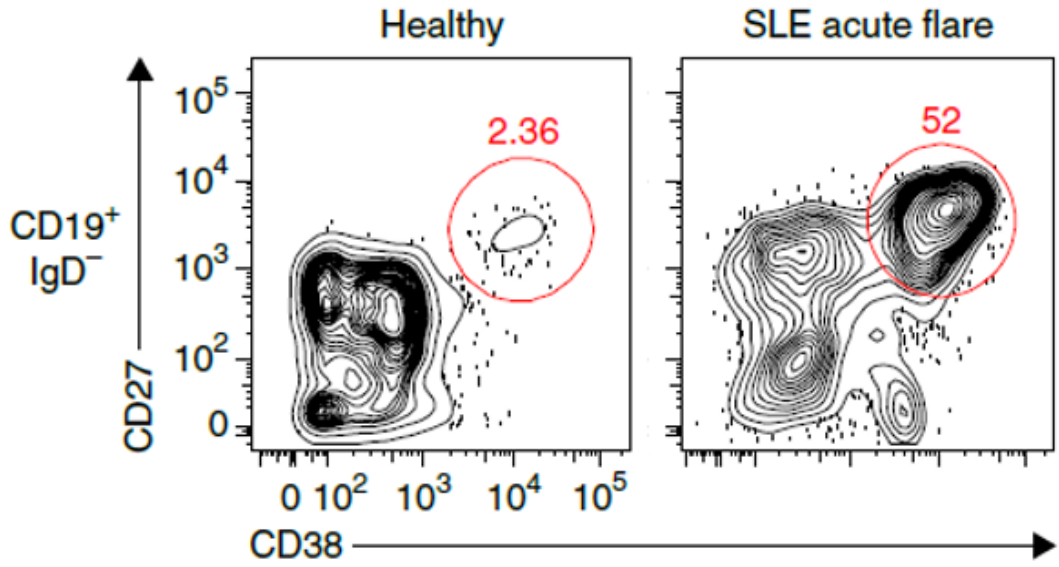


Figure 2.11: Acute SLE episodes with substantial ASC polyclonal expansions (Adapted from (2))

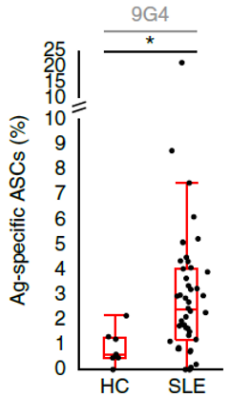


Figure 2.12: SLE ASCs in flares are distinguished by intricate VH4-34+ clonal expansion. (Adapted from (2)).

Overall, the exclusion of VH4-34-encoding self-reactive B lymphocytes from GC and the memory B lymphocytes pool has been seen in healthy individuals. Nevertheless, it has been shown that in individuals with SLE, B lymphocytes expressing VH4–34 are attracted inside germinal centers, potentially leading to the production of autoantibodies with pathogenic properties.(5, 179) Indeed, there is a

correlation between levels of serum VH4-34 antibodies and lupus disease activity.(179, 191)

3. METHODS

Between September 2020 and September 2022, this research was conducted in Dr. Eric Meffre's laboratory in the Yale Immunobiology Department in New Haven, Connecticut.

Jean-Nicolas Schickel, a former Meffre lab member, conducted part of the early experiments and analyzed the bacterial lysates. He also performed the initial VH4-34 analysis regarding the mutational status of memory B cells. Additionally, we express our gratitude to the former members of the Meffre Lab who were responsible for the initial part of the cloning of some samples.

3.1. Healthy controls and patients' samples for VH4 clones

All samples were gathered after patients and healthy volunteers completed informed consent in line with methods established by Yale University's institutional review board.

Hedda Wardemann from the German Cancer Research Center generously gave us their previously studied VH4-34 miniprep sequences that were cloned from single CD27+IgG+ memory B lymphocytes from healthy participants and lupus patients. Some of her clones were re-expressed by me, and we added them to our study for further analysis of our hypotheses.

3.2. Staining and sorting B lymphocytes

Blood mononuclear cells were collected from the peripheral venous system and purified according to manufacturer guidelines utilizing Ficoll-Paque density gradient centrifugation. Next, the B lymphocytes were enriched in HDs and SLE patients' samples by employing magnetic separation via CD20 microbeads (Miltenyi Biotec). CD20+ enriched B cells were stained with anti-human CD markers like CD10, CD19, CD21, and CD27, anti-human IgG-PE, and anti-9G4-FITC (IGM Biosciences). On a FACS Aria flow cytometer, single CD19+CD27+CD21+IgG+ or CD19+CD27+CD21+IgG+9G4+ memory B cells were sorted onto 96-well PCR plates and promptly frozen on dry ice and stored in a -80-degree freezer.

3.3. Methods to generate monoclonal antibodies from single human B lymphocytes

Single CD27+ IgG+ B cells were sorted to evaluate B cell tolerance in the Meffre laboratory. Using a single-cell RT-PCR technique, 20 to 30 Ig genes for each B cell fraction were then cloned. After transient transfections of A293 fibroblasts with plasmids expressing Ig heavy and light chains, supernatants containing recombinant antibodies would be titrated, and their reactivity would be assessed by ELISA for binding to polyreactivity, Hep-2 cell lysates, and anti-nuclear reactivity (anti-nuclear antibody slides). In this study, I focused on commensal bacteria reactivity immunoassays rather than autoreactivity and polyreactivity assays.

The cloning strategy employed for this study is illustrated in Figure 3.1. The components of the illustration were rendered with images obtained from Servier Medical Art. Servier Medical Art, created by Servier, is subject to a licensing agreement under the Creative Commons Attribution 3.0 Unported License. (https://creativecommons.org/licenses/by/3.0/).

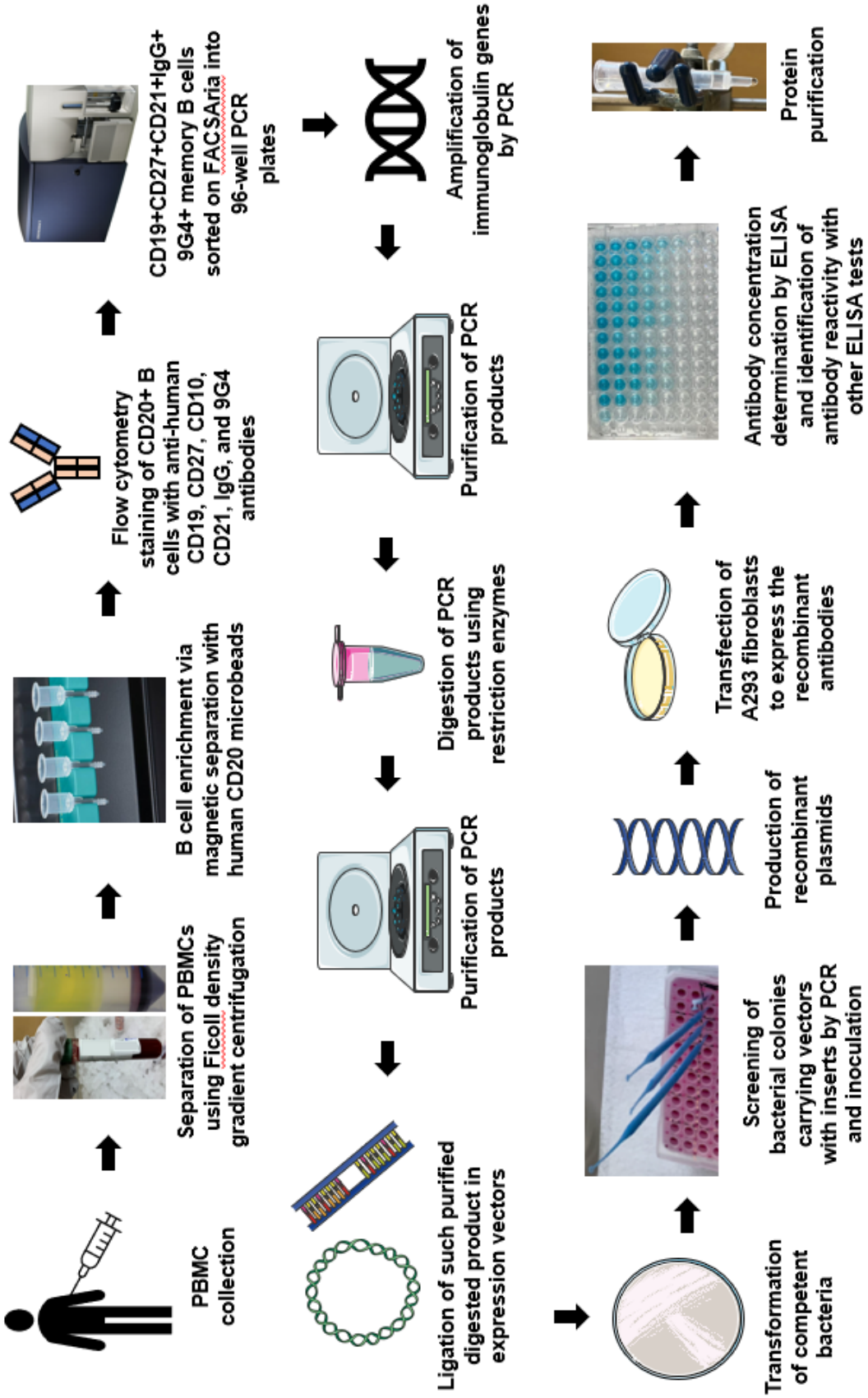


Figure 3.1: Methodology for cloning antibodies from single B lymphocytes

3.3.1. cDNA / rtPCR Protocol

1- Preparation

- Clean the PCR hood with DNA-ZAP
- Thaw buffers and reagents
- Leave a sorted 96-well PCR plate in the cold block

2- Make a reaction Mix1

- 718.3 uL dH20
- 30.8 uL random hex N6
- 66 uL 10% NP40
- 5.5 uL RNAse inhibitor
- 44 uL DTT

3- Add 7.2 uL of mix1 into each well. (The mixture should be washed over each side of the well several times from a height sufficient to obtain single-cell context.)

4- After covering the plate with sticky foil, put the plate at 65 °C for 1 minute, then immediately replace the plate back on the ice block.

5- Make a reaction Mix2

- 226uL dH20
- 330uL 5X Buffer
- 55uL dNTPs
- 110uL DTT
- 6uL Rnasin
- 15uL superscript III

6- Add 7 uL of mixture #2 to wells.

7- Incubate for 15 minutes at room temperature.

8- RT-PCR reaction (thermocycler)

42°C 5 min

25°C 10 min

42°C 55 min

94°C 10 min

Hold at 15°C

3.3.2. Single-cell PCR Protocol

1- Prepare PCR mix (a full plate requires three times the volume.)

- 1225.4 uL dH20
- 165 uL 10X Buffer
- 17.6 uL dNTPs
- 5.5 uL primer1
- 5.5 uL primer2
- 11 uL Taq polymerase

2. Arrange a 96-well PCR plate on a cold block, then add 38 uL of mix into each well.

3. Add 3.5 uL of cDNA or 4 uL of PCR1 product per well.

4. Start the PCR (1 or 2) thermocycler program (50 cycles).

94°C 5 min

94°C 30 sec

57°C (PCR1) or 59°C (PCR2) 30 sec

72°C 55 sec (PCR1) or 45 sec (PCR2)

72°C C 5 min

Hold at 4°C, 15°C if overnight.

After performing one RT PCR for each fraction, then two consecutive 2 PCRs per chain, we run the gel to find matching pairs. An example of PCR2 gel images from my previous work in the lab is seen in Figure Figure 3.2.

Figure 3.2: A representation of a PCR2 gel image.

3.3.3. Sequencing

Following the selection of matching pairs with the appropriate reverse primer, aliquots of the second PCR products from the heavy (H) and lambda (λ) chains were sequenced by the Yale Keck Sequencing Facility. Finally, the sequences were examined using igBLAST against GenBank to determine the segments of germline V(D)J genes with the highest degree of identity.

Primers

PCR products

- H (5'AgeV$_H$mix)
- λ (3'XhoC$_{\lambda 1}$)

3.3.4. Purification

Prior to cloning, the purification of all matching PCR products was conducted using the Qiagen Purification Kit with PE buffer, PB buffer, and elution buffer.

An example of my purification experiment snapshot is seen in Figure 3.3.

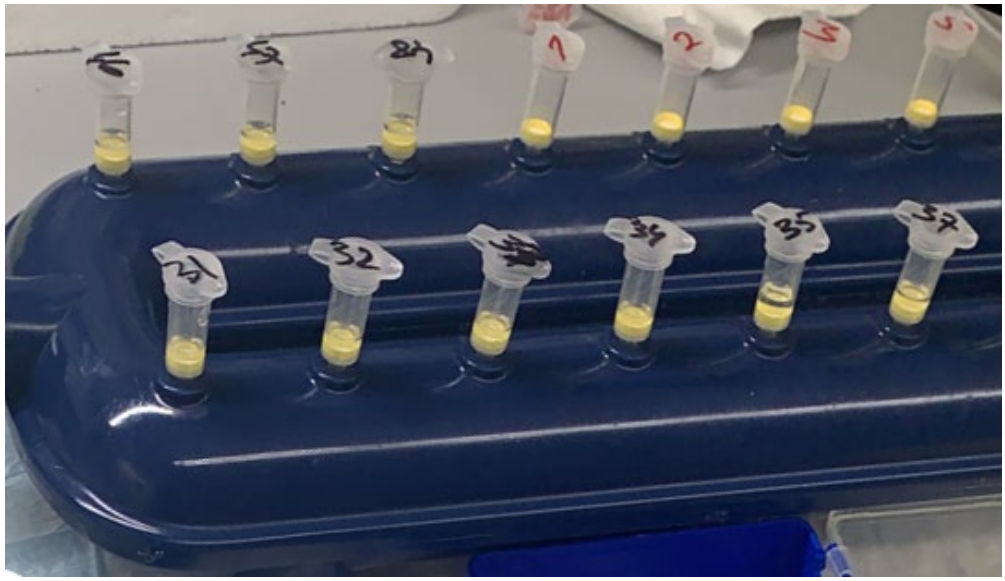


Figure 3.3: Purification step

3.3.5. Digestion

In a total volume of 40–50 µl, digestions were performed using the corresponding restriction enzymes, namely SalI, AgeI, BsiWI, and XhoI.

For heavy chain clones: AgeI, SalI

For kappa chain clones: AgeI, BsiWI

For lambda chain clones: AgeI, XhoI

3.3.6. Purification

Purification procedures for digested PCR products were followed as previously indicated.

3.3.7. Ligation

PCR-digested products were inserted into expression vectors for human Igγ1,

Igκ, and Igλ with numerous cloning sites upstream of the human Ig constant regions. The ampicillin resistance gene in the vector can be used to choose clones of interest.

In the ligation procedure, we used 1 µl T4-Ligase enzyme (NEB), 7.5 µl purified digested PCR product, and 0.2 µl respective vectors in 10 µl total volume.

3.3.8. Making an agar plate

1. Add 1 L of autoclaved water to a bottle.
2. Add 31 g of 2X YT , then 15 g of Bacterioagar.
3. Mix.
4. Autoclave.
5. Cool to 50–55°C, then add 500 µL of ampicillin (Sigma-Aldrich).
6. Pour into pedri dishes (Falcon, 351029) so that they just cover the bottom surface.
7. Let it cool on the bench until its temperature reaches room temperature.
8. Put them in a sleeve and store them in a 4°C fridge until the transfection experiment.

Figure 3.4: Preparation and storage of agar plates.

3.3.9. Transformation

A competent E. coli aliquot was thawed and quickly added on ice to each tube/well. Then, 3.5 uL of ligation products were added, respectively. Later, the samples were incubated on ice for half an hour before being heat-shocked at 42°C for 30 seconds and placed back on ice for 120 seconds. After adding 100 uL of 1X LB without ampicillin to each tube/well, we capped gently and taped the tubes/plate to the shaker for 30 minutes at 37C 300 rpm. Finally, the bacterial mixture was dispersed on labeled pedri dishes using blue hockey sticks and incubated overnight at 37 °C, upside down.

An example photo of the E. coli colonies I had after one of my transfection protocols is shown in Figure 3.5.

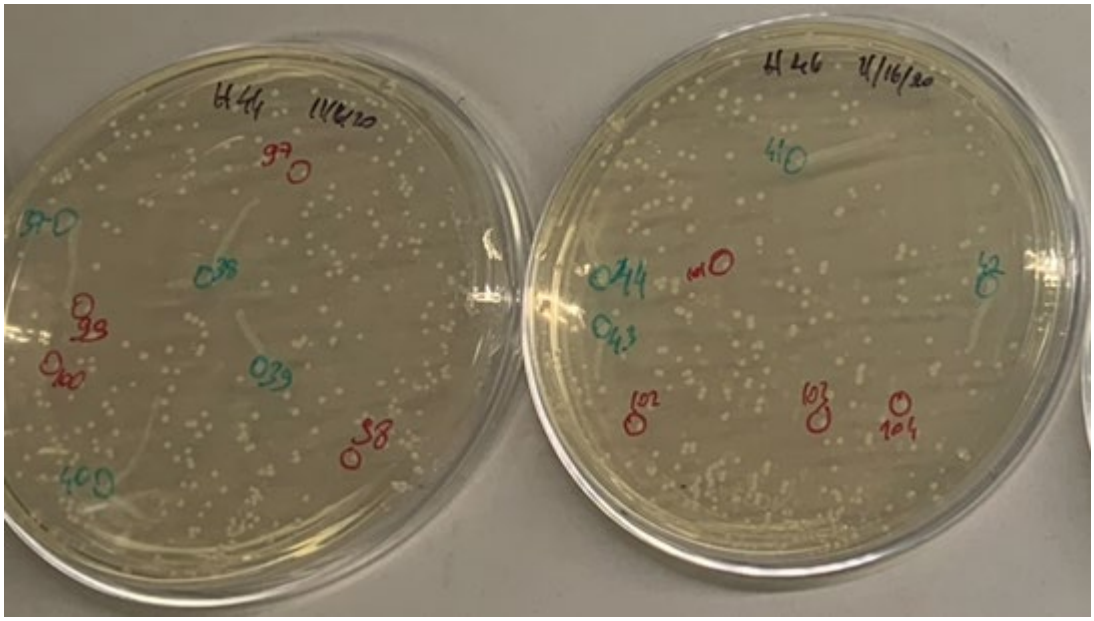


Figure 3.5: E. coli colonies carrying my insert.

3.3.10. Screening of colonies

After overnight incubation of agar plates with LB-ampicillin at 37 °C, 4-8 colonies for each plate and chain were screened by PCR with a 5′ Ab sense primer and corresponding reverse primers to select the plasmids with variable-region inserts.

PCR mix preparation (values for 10 colonies)

- 130 uL H20
- 50 uL screening dye
- 40 uL dNTPs
- 25 uL Taq 10X Buffer
- 2 uL Primer 1 (5’ Absense)
- 2 uL Primer 2 (3’ Primer: 3’IgG (H), 3’ Cκl, 3’ C λI)
- 2 uL Taq polymerase

Following the screening PCR, gel electrophoresis was conducted to determine whether the insertion of the ligated product was with the vector.

Figure 3.6 shows the screening process, and Figure 3.7 represents an example of me loading agarose gel electrophoresis for screening.

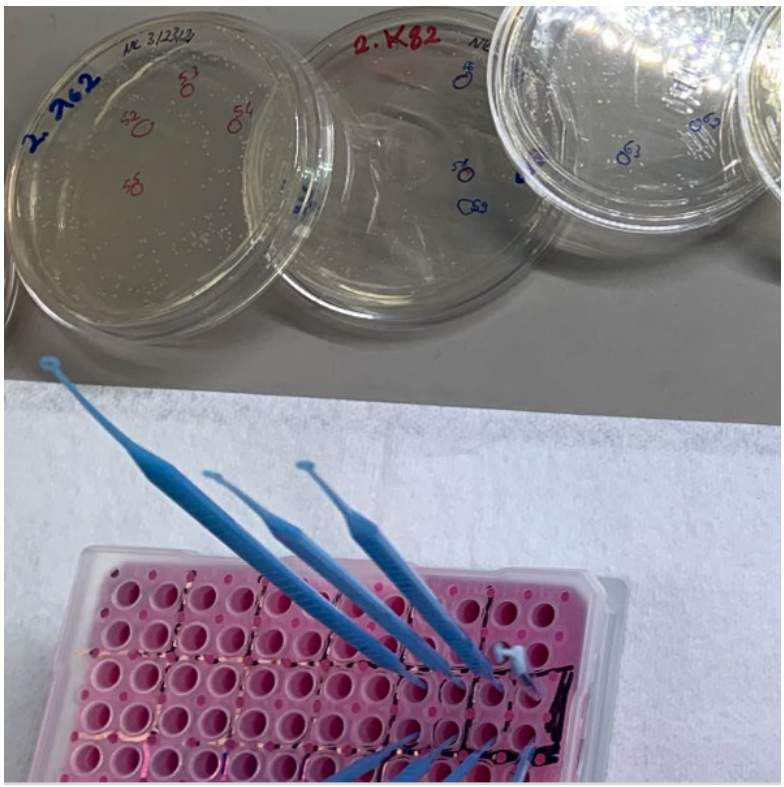


Figure 3.6: Screening process.

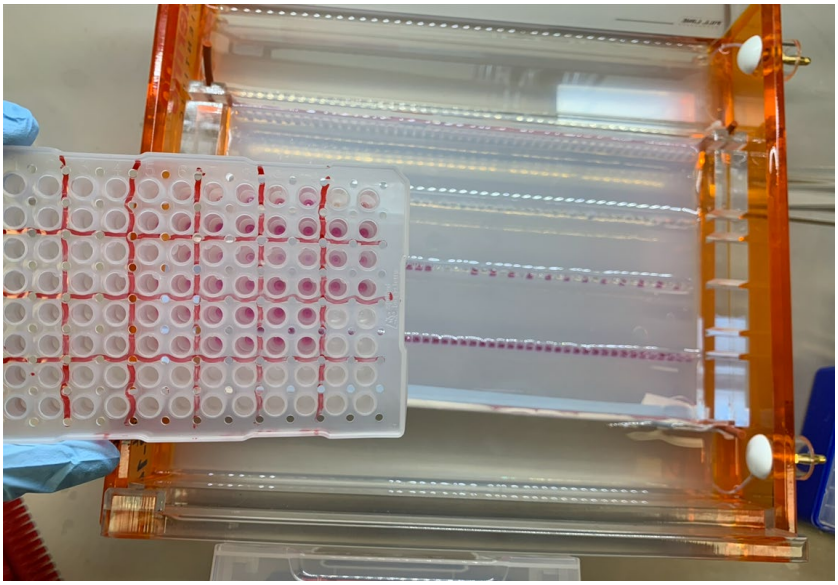


Figure 3.7: Agarose gel electrophoresis for screening.

3.3.11. Inoculation

Colonies having genes of interest were swabbed with inoculation sticks (Fisherbrand, 22363598) and put into respective 14 mL inoculation tubes (Corning™ 352059) with 4 mL of 1X LB with ampicillin and left in a shaker for 15-18 hours at 300 rpm and 37°C.

The preparation step for the inoculation experiments is shown in Figure 3.8**.**

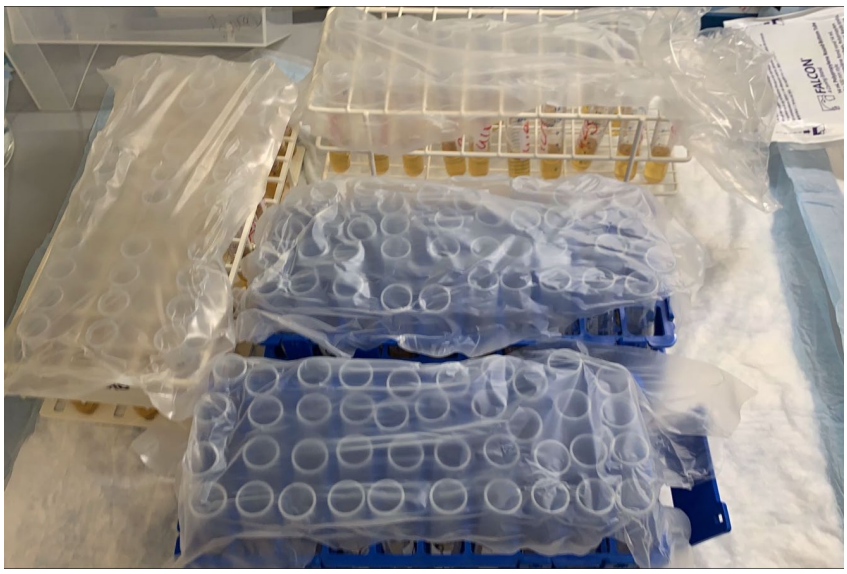


Figure 3.8: Preparation for the inoculation step.

3.3.12. Minipreps (Preparation of plasmid DNA)

Using QIAprep Spin columns from Qiagen (27106) with P1 Buffer, P2 Buffer, N3 Buffer, PE Buffer, and EB Buffer, the DNA of the plasmid was recovered from a 4 ml volume of bacterial cultures that were cultivated overnight at a temperature of 37 °C in LB with ampicillin.

3.3.13. Sequencing

With the appropriate reverse primer, aliquots of the minipreps from the VH, Vλ and also Vκ chains were sequenced. IgBLAST was utilized to analyze the sequences and detect the germline V(D)J gene segments with the best match.

The IgBLAST program allows for the analysis of germline V, D, and J genes and the identification of IG V domain framework regions, as well as the determination of rearrangement junction information and complementarity-determining regions.

One of my sequencing plates for miniprep samples is depicted in Figure 3.9 Also, the example of DNA sequence illustrated by SnapGene in Figure 3.10.

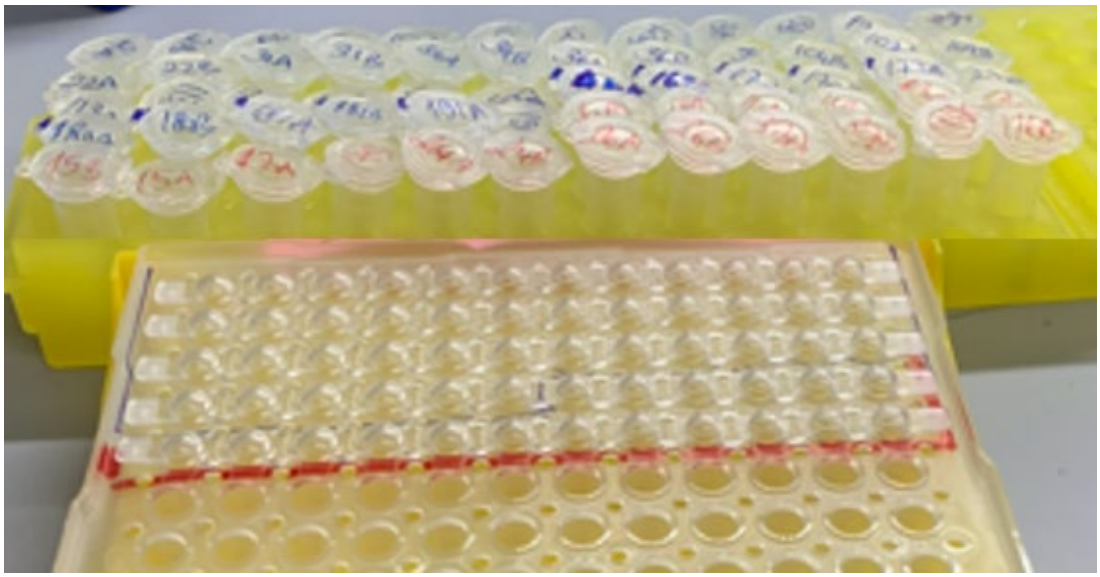


Figure 3.9: Sequencing plate for minipreps.

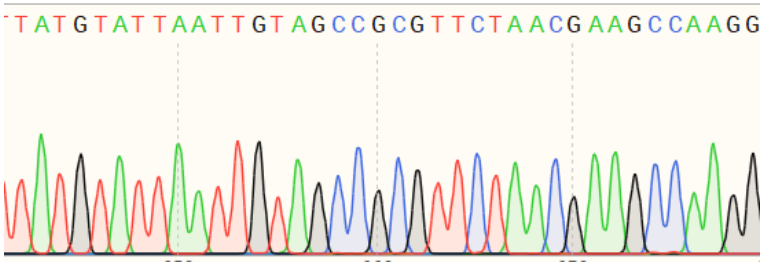


Figure 3.10: An example of electropherograms acquired through the use of Sanger sequencing.

3.3.14. Transfection Protocol

Preparation of the Nutridoma Media

- DMEM
- Sodium pyruvate 100 mM
- Antibiotic Antimycotic 100X
- L-Glutamine 200 mM 100X
- Nutridoma (Roche)

The transfection of matching clones was performed using Human Embryonic Kidney (HEK) 293A cells. Firstly, we grew HEK 293A in full DMEM (Dulbeccos Modification of Eagles Medium) on 150 mm cell culture dishes (Falcon® 353025). The 2.5–3 million cells were then transferred into 100mm x 20mm tissue culture dishes (Corning 353003) in full DMEM. Then, the Nutridoma medium was replaced with full DMEM when the cells reached 90% cell confluency, indicating they were ready to be transfected.

1.2 ml of a 150 mM NaCl solution was added into 14-mL round-bottom tubes, and equal amounts of matching heavy and light chain DNA were mixed with the solution. Afterward, 90 uL of linear PEI (1 mg/mL) was added dropwise while vortexing, and we waited for the incubation at room temperature. In sterile conditions, the mix was dispensed onto the HEK293A cell line with the Nutridoma medium. Lastly, the transfection plates were kept in an incubator for ten days at 37 °C, 5.0% CO2, and humid conditions. A photo from one of my transfection experiments is shown in Figure 3.11.

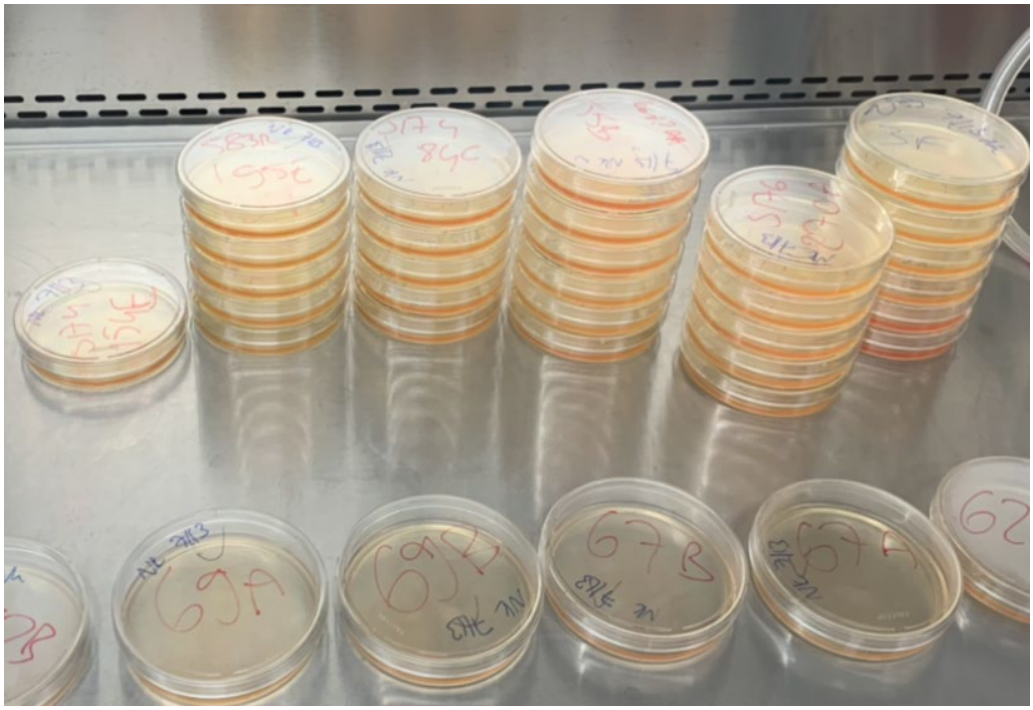


Figure 3.11: Transfection plate preparation.

3.3.15. Harvesting

After 10 days, the supernatants were spun at 1500 rpm for 10 minutes to get rid of any leftover cell debris. Subsequently, the antibodies were placed in a cold chamber maintained at a temperature of 4°C until they were used in the IgG ELISA.

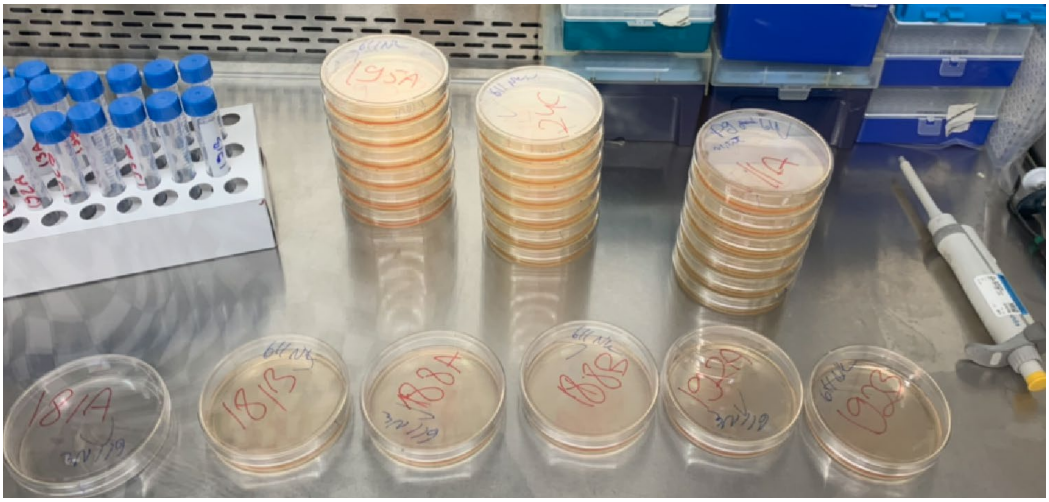


Figure 3.12: Transfected plates were ready to be collected after 10 days for the supernatants.

3.3.16. IgG ELISA for Antibody Concentrations

Preparation

Blocking Buffer (1L):

- 1L 1X DPBS (Gibco, 2430028))
- 2ml EDTA 0.5M (Invitrogen, 2059919)
- 0.5ml Tween-20 (Sigma Aldrich, SLCH5035)

Making plates

Flat-bottomed 96-well plates were coated with 1:500 goat antihuman IgG in blocking buffer and left overnight.

The coated plates were subjected to three rounds of washing using water, following which a blocking buffer was introduced into each well. Subsequently, the plates were subjected to incubation at room temperature for a duration of 1 hour.

Meanwhile, the antibodies from the supernatant were diluted 1:20 in PBS, and then 1:4 serial dilutions were prepared in the dilution plate, beginning with row A, adding to row B, and so on until row H.

The coated plates were subjected to three rounds of washing using water following the first incubation, and 50 uL of dilutions from the dilution plate were added to the coated plate. Plates were then left to incubate for a two-hour period at room temperature.

After three washes, a 1:1000 ratio of goat antihuman IgG secondary antibody in 5 mL blocking buffer was added. The plates underwent a one-hour wait at room temperature.

The coated plates were subjected to three rounds of washing using water, following which a blocking buffer was introduced into each well.They were incubated for 5 minutes at RT.

After final washes, HRP Substrate Kit (Bio-Rad, 1721064) was added per well, and multiple readings were recorded at 405 nm. Alternatively, 1-Step™Ultra TMB-

ELISA Substrate could be used for substrate, and the readings were captured at 450 nm.

The concentrations of antibodies were computed using Excel with the help of a positive control and the standard curve of an IgG1 isotype control.

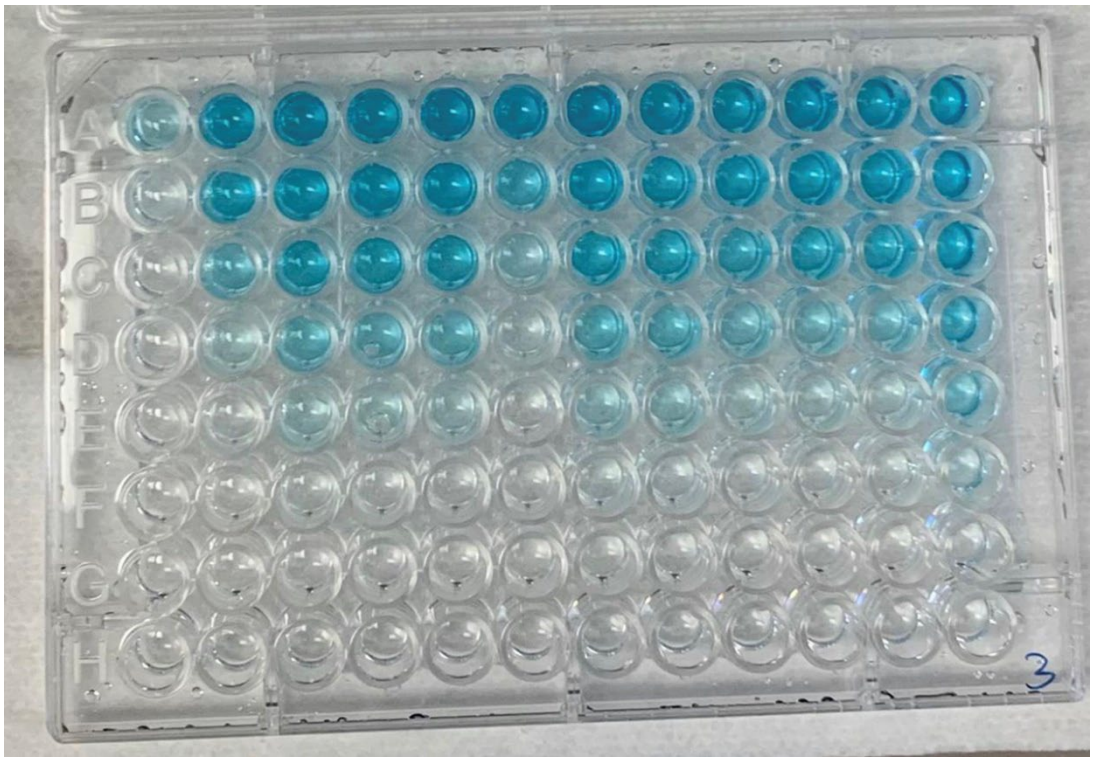


Figure 3.12: IgG ELISA plates following substrate exposure.

After this step, we could do a polyreactivity ELISA; however, I primarily focus on the commensal bacteria binding assay. Therefore, I directly purified the expressed antibodies.

3.3.17. Antibody Purification

Preparation

- The addition of Protein G Agarose Beads was performed in the expressed antibody tubes, after keeping an aliquot of each antibody supernatant.
- The recombinant antibodies were put on the spinning wheel in a 4°C cold room for at least 1-2 days.

After incubation, the antibodies were removed from the wheel, and spun at 2000 rpm for 15 minutes, and then resuspended in DPBS. At the same time, the Bio-Spin Chromatography Columns were subjected to activation.

The activated labeled columns were loaded with resuspended beads.

Once all of the media had been run through the column, DPBS was used to wash the beads three times.

The antibodies attached to beads were serially diluted with glycine (Sigma, SLCG7669) pH=3 in 20 uL Tris pH=8 (AmericanBio, AB14043-01000) containing Eppendorf tubes. The OD 450 of proteins was then determined using a bell curve.

The spinning of the expressed monoclonal recombinant antibodies, which have been bound to purification beads under a refrigerated environment, is represented in Figure 3.13.

We used a purification method using protein G agarose beads for the isolation of recombinant antibodies, as seen in Figure 3.14.

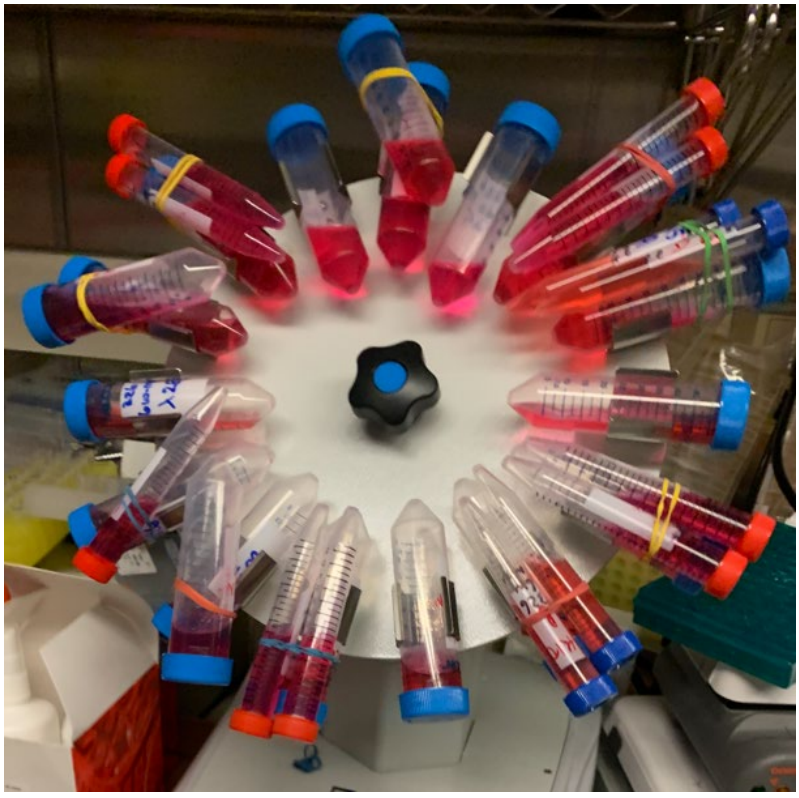


Figure 3.13: Spinning of the expressed antibodies with the purification beads in a cold room.

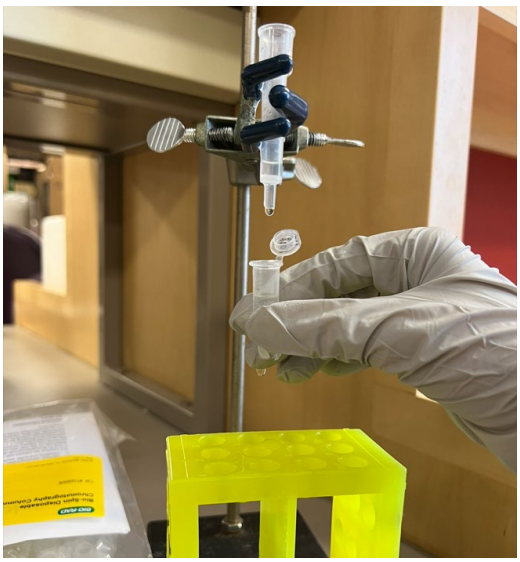


Figure 3.14: Purification of recombinant antibodies with protein G agarose beads.

3.4. Restoring the mutant AVY motif of HD VH4-34+IgG+ B cells to their germline unmutated form

The strategy to revert mutated antibodies to their unmutated counterparts involves only the AVY site in VH4-34 FWR1. After determining the mutated AVY motif harboring HD VH4-34 recombinant antibodies with NCBI IGBLAST software, we reverted the mutated AVY motifs into their unmutated counterparts in those antibodies miniprep DNA sequences according to the germline sequence VH4-34*01. Then we ordered updated miniprep DNA sequences as gBlocks Gene Fragments from the IDT company. Finally, we cloned that gene product, as it is a purified PCR product, and then tested its commencal gut bacteria reactivity with fecal staining flow experiments.

3.5. Cohort of human subjects for stool samples

The fresh aliquots of stool samples were generously given by Martin A. Kriegel and kept in a -80°C freezer until use. The stools I used for my project were originally studied by Greiling et al. and Ruff et al. (143, 201) All human subject procedures used in their research received approval from the Yale Human Investigations Committee.

3.6. Staining of Fecal Bacteria for Flow Cytometry

Preparation

- The concentration of purified recombinant antibodies previously cloned from memory B lymphocytes, called primary antibodies, was diluted to 20 μg/mL for normalization.

Fecal homogenates obtained from approximately 100 mg of frozen human stool in a 2 mL screw-cap tube were thawed on ice, then mixed with about 20 Lysing Matrix D beads (MP Biomedicals, 116913050) in 1 mL sterile Phosphate-Buffered Saline (PBS) as initially reported.(202) The stool sample was bead-beaten with a minibeadbeater (Biospec) for 12 seconds, then spun at 4 °C at 50 g for 15 minutes to eliminate big debris. Later, the stool aliquot was filtered through 40μm nylon to remove large particulates and normalize to approximately OD600 1.0. Fecal bacteria

suspensions were distributed equally in new 1.5 ml Eppendorf tubes per staining reaction and was rinsed using a 1 ml solution of PBS having 1% bovine serum albumin and was subjected to centrifugation at a force of 10,000 times the acceleration due to gravity (g), at a temperature of 4 °C, for a duration of 90 seconds.

The bacterial samples were subsequently incubated with cloned primary antibodies of memory B lymphocytes, preceded by secondary staining with anti-IgG Alexa Fluor 647 and anti-IgA PE. Of note, stool homogenate was incubated with primary and secondary antibodies in a 1% PBS/BSA buffer containing 10% normal goat serum and 10% normal mouse serum. Also, between the steps, the stool suspension underwent two rounds of washing, each involving the addition of 1 mL of 0.1% BSA in PBS.

Samples were acquired using a BD LSRII cytometer, as seen in Figure 3.14, and analyzed on FlowJo.

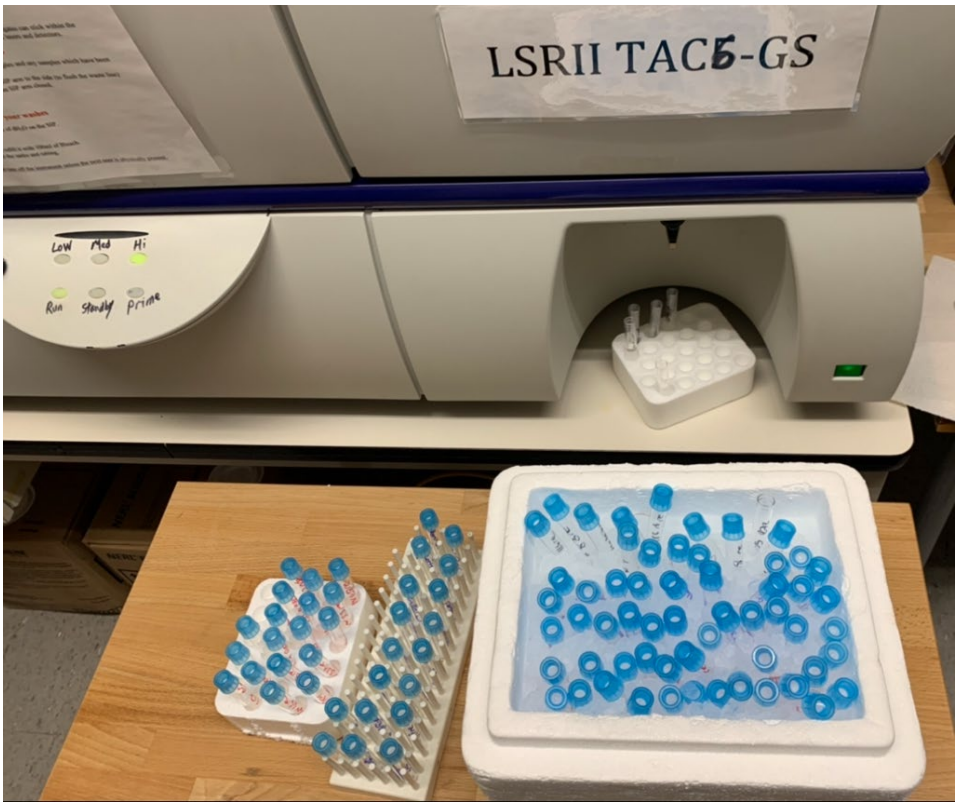


Figure 3.14: Samples prepared after fecal bacteria staining for flow cytometry.

3.7. Bacteria Sorting

The thawing of fecal material and normalization of 9G4 recombinant antibodies from IgG+ memory B lymphocytes, assigned as primary antibodies, were noted previously. The bacteria bound by primary antibodies were marked with anti-human IgG Alexa Fluor 647 and later incubated with 25 uL of Anti-Cy5/Anti-Alexa Fluor 647 MicroBeads in 1% BSA in PBS (staining buffer). After washing and elution steps by utilizing a 3D-printed inverted 96-well plate magnet, the positive fraction, referring to 9G4-bound bacteria isolation, was sorted. To isolate the negative fractions, a MultiMACS M Separator (Miltenyi Biotec, 130-091-937) as seen in Figure 3.15 and molecular-grade column strips (Multi-96 Column, Miltenyi Biotec, 130-092-445) were used. Following magnetic activated cell separation, the positive fraction (9G4 antibody-bound fecal samples) and the negative fraction (antibody-unbound stool samples), as well as presorted stool samples, were then frozen at -80 °C for further analysis.

Additionally, a detailed explanation of bacteria staining for feces and bacteria sorting was also demonstrated by Noah Palm, from whom we adapted our protocol for those experiments.(202)

Figure 3.15: Preparation for bacterial sorting.

3.8. DNA isolation and sequencing of 16S rRNA

Frozen 9G4 antibody-bound and 9G4 unbound fractions (an example seen in Figure 3.16), as well as a pre-sort stool aliquot, were sent to Zymo Research Microbiomics Services on dry ice for DNA isolation and 16S rRNA sequencing.

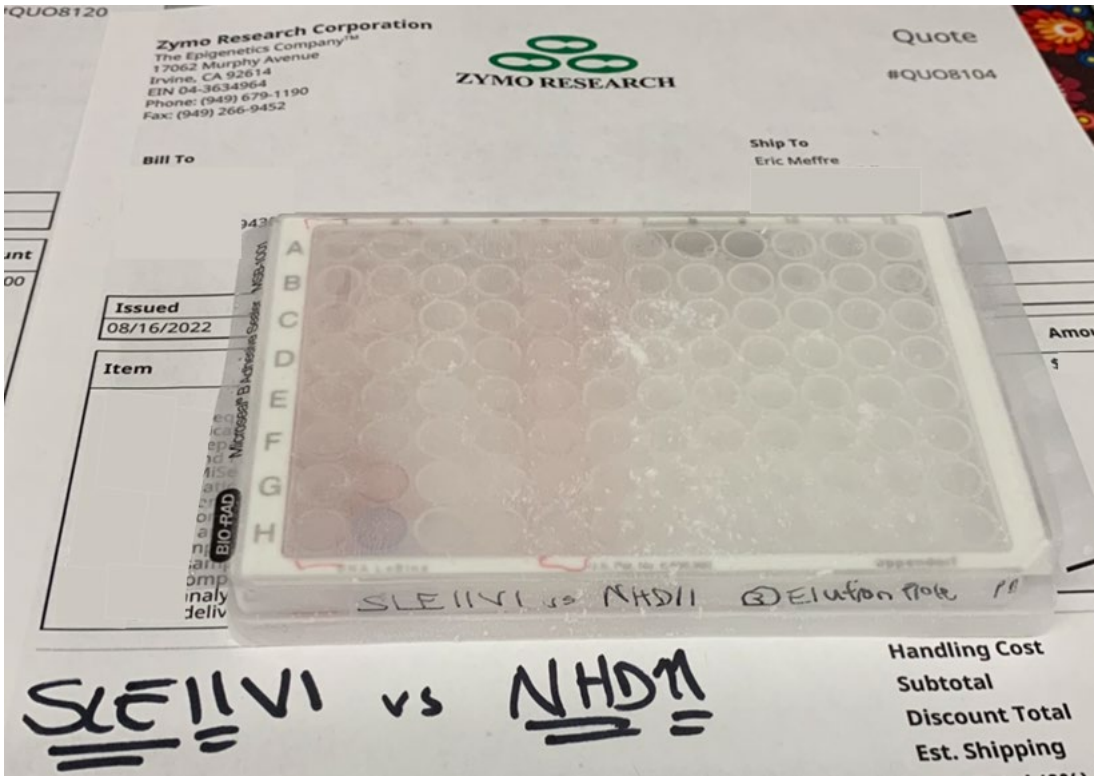


Figure 3.16: Example of frozen 9G4 antibody-bound and 9G4 unbound fractions.

3.9. Statistics

Statistical analysis was conducted using GraphPad Prism, version 7.03 or 9.40 (GraphPad Software). Differences between interest groupings were evaluated by Mann-Whitney U tests and in parallel with the Wilcoxon test when paired. A P value of 0.05 was chosen as the threshold for significance. In our research, every statistical assessment has been carried out via a two-sided test.

4. RESULTS

4.1. Key medical and demographic features of the subjects

Table 4.1 presents the clinical variables and disease activity index of SLE patients from whom we isolated VH4-34+IgG+ B cells to clone. (The disease and its corresponding therapy exhibited a state of stability for a minimum duration of six months.)

Table 4.1: Clinical characteristics and disease activity index of SLE patients.

Patient n°	Age	Sex	Duration of disease	SLEDAI	Ig levels (g/L)	ANA	Anti-dsDNA	Streroids mg/day	Hydroxy chloroquine
1	45	F	20y	<4	ND	1/640	-	-	-
2	32	F	5y	<4	25.1	1/1280	-	-	-
3 (SLESt1)	36	F	16y	<4	ND	1/640	-	-	-
4	31	F	2y	2	21	1/1280	+	-	-
5 (SLESt2)	40	F	6y	<4	ND	1/640	+	-	-
6 (SLE62)	35	F	unknown	8	ND	ND	-	12.5	-
7	38	M	10y	<5	ND	1/1280	-	-	+
8	33	F	unknown	<4	ND	1/640	+	-	-

The tables below present the demographic features of both the patients diagnosed with SLE and the control group who willingly contributed their stool samples.

Table 4.2: SLE stool cohort of human subjects with clinical features.

Subject	Diagnosis	Age (years)	Sex	Ro60	HLA-DR3	HLA-DR15	Immunosuppressive medications	HCQ
SLE01	SLE	40	F	+	–	–	Azathioprine	–
SLE03	SLE	49	F	–	–	–	Mycophenolate mofetil, Prednisone 7.5 mg daily	+
SLE11	SLE	31	F	–	+	+	Mycophenolic acid, Prednisone 10 mg daily	+
SLE14	SLE	32	F	–	–	–	—	–
SLE18	SLE	33	F	+	–	–	NA	NA

F, female; HCQ, hydroxychloroquine; Ro60, anti-Ro60 serum IgG; NA, not available.

*The collection of SLE stool samples, which was previously reported, was kindly provided by Dr. Martin A. Kriegel.(143)

Table 4.3: Healthy donor stool cohort of human subjects.

Subject	Diagnosis	Age (years)	Sex
HDLS	Healthy	30	F
NOR08	Healthy	55	F
NHD09	Healthy	32	F
NHD11	Healthy	48	F
NOR12	Healthy	NA	NA

F, female; NA, not available.

* The healthy donor stool cohort, which was previously published, was generously given by Dr. Martin A. Kriegel.(143, 201)

4.2. The VH4-34 Autoreactive Motif is Conserved in IgG+ B Lymphocytes from SLE Patients

To ascertain the potential recognition of commensal bacteria by VH4-34-encoding antibodies expressed by IgG+ B cells in subjects with SLE, we initially examined the VH4-34 sequences obtained from single CD27+ IgG+ B cells isolated from both SLE patients and healthy controls (HDs). Our analysis focused particularly on two VH4-34-specific motifs that have been linked to the recognition of commensal bacteria.(7) The first motif, The Ala-Val-Tyr (AVY) motif located in the VH4-34 FWR1 is accountable for the binding of I/i, consequently, any alterations or mutations in the AVY motif result in the loss of self-reactivity.(8, 9) Secondly, one more distinctive attribute of VH4-34-expressing antibodies is the existence of an Asn-X-Ser N-glycosylation site (NHS) inside the CDR2, which enables the adjustment of antibody avidity towards the corresponding antigen.(203) Therefore, in order to investigate the impact of SHM on the two VH4-34 motifs in single IgG^+ B cells, an analysis was conducted on IgG sequences obtained from healthy controls and SLE patients, and the mutated VH4-34 sequences were aligned with the germline VH4-34*01 sequence (Figure 4.1). This work used VH4-34 sequences obtained from 11 single-sorted IgG+, which had been previously isolated from different healthy donors.(76) In Figure 4.1, the amino acid sequence alignment of VH4-34 for IgG+ memory B lymphocytes derived from healthy donors and SLE subjects is shown.

In an earlier investigation from our lab, it was previously shown that a total of 11 out of 17 VH4-34-expressing antibodies generated by IgG+ B lymphocytes from healthy controls had mutations in their AVY motif, consequently leading to the cessation of self-reactivity. In fact, the majority of VH4-34 antibodies found in HD IgG+ B lymphocytes exhibited mutations in both AVY and NHS motifs, and furthermore, it was observed that these recombinant antibodies did not exhibit recognition towards commensal bacteria.(7) (Figure 4.1). On the other hand, it was observed that VH4-34-encoded antibodies produced by SLE IgG+ B lymphocytes frequently exhibited unmutated AVY sequences (six out of eight) and retained the ability for carbohydrate recognition and possibly autoreactivity. SHM frequently resulted in the loss of the NHS motif, consequently leading to the absence of CDR2 glycosylation in VH4-34-encoding IgG (twelve out of seventeen) clones derived from

HDs. Conversely, the glycosylation region exhibited preservation in 75% (six out of eight) of VH4-34 bearing IgG sequences that were obtained from SLE patients. SLE VH4-34-expressing antibodies had unmutated AVY and NHS at a frequency of 62.5%, whereas a mutation was present in both motifs in 12.5% of cases. Mutation was present only in one of the motifs in 12.5% cases for each motif. (Figure 4.1B).

Overall, it can be noted that in immunological responses involving VH4-34 clones in healthy donors, the presence of AVY and NHS motifs is typically suppressed by SHM; inversely, in SLE patients, antigenic selection allows for the preservation of VH4-34 germline-expressing AVY and NHS sequences which are linked to the responses against commensal bacteria.(7)

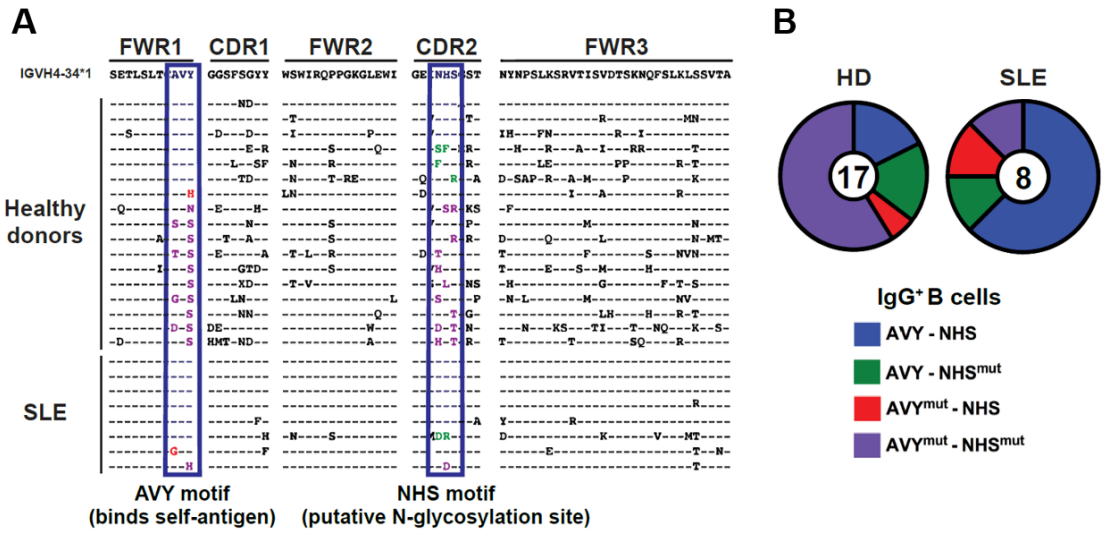


Figure 4.1: VH4-34+ IgG+ memory B cells of SLE patients exhibit atypical mutation patterns. **A.** The amino acid sequence of the germline IGHV4-34*01 is shown in the uppermost section. The identification of germline identity is shown by a dash, whereas replaced residues are represented in black. The germline AVY and NHS sequences in FWR1 and CDR2 are shown in blue. The color green is used to indicate mutations only in the NHS gene, while the color red is used to represent mutations solely in the AVY gene. Mutations occurring in clones that are mutated for both AVY and NHS are depicted in purple. **B.** Pie chart showing percentage of clones that have undergone mutations in the AVY and/or NHS VH4-34 sequences obtained from IgG+ B cells derived from healthy donors as well as SLE patients.

4.3. SLE VH4-34 IgG+ B cells show a higher level of anti-fecal gut bacteria reactivity

We then would like to assess if VH4-34-expressing antibodies cloned from single CD27+IgG+ B cells of SLE patients and HDs demonstrate an affinity for the gut bacterial populations that were obtained by stool samples of different HDs and SLE patients. Therefore, we tested purified VH4-34-expressing antibodies cloned from single CD27+IgG+ B cells of SLE patients and HDs, which have distinct mutation patterns for their AVY and NHS motifs, on stool samples from different HDs and SLE patients. HD VH4 non-VH4-34 antibodies derived from individual CD27+ IgG+ B cells of HD patients did not react to gut bacteria significantly; consequently, we decided to use those HD VH4 non-VH4-34 antibodies as a negative control for our flow cytometry analysis. Binding of VH4-34-encoded antibodies and the corresponding control clones was analyzed by flow cytometry with the utilization of secondary antibodies for IgA and IgG.

We observed that SLE VH4-34^{+}IgG^{+} B cells exhibited an elevated level of reactivity towards gut bacteria present in the fecal samples of both healthy individuals and SLE patients, but to a smaller extent in HD stools and to a greater extent in SLE stools. Our finding was that SLE VH4-34 IgG+ B cells showed a higher level of anti-commensal reactivity compared to healthy donor VH4-34 IgG+ B lymphocytes on SLE stools, with a significance less than 0.05, which was also consistent for the healthy donor stools when comparing the average values of HD and SLE VH4-34+IgG+ recombinant antibodies on HD and SLE stools**.** In fact, VH4-34 IgG+ B lymphocytes in SLE patients show elevated expression of anti-commensal reactivity at an average of 10.82% on all SLE stools when compared to HD stools at 5.87% (p-value 0.0156 (Figure 4.2D)). Moreover, HD VH4-34+IgG+ B cells, characterized by prevalent mutations in the AVY and/or the NHS motifs, demonstrated significantly reduced affinity towards fecal gut bacteria in comparison to SLE VH4-34+IgG+ B cells.

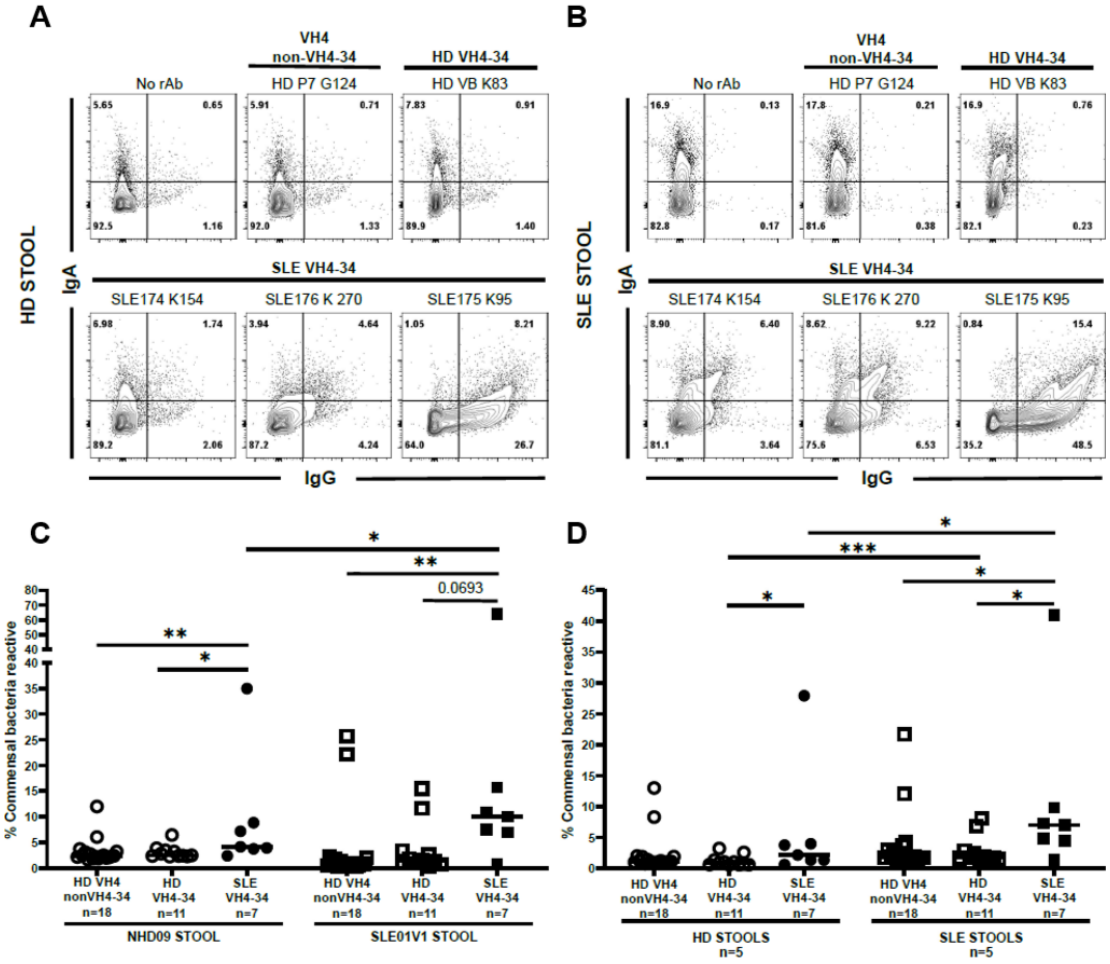


Figure 4.2: SLE VH4-34+IgG+ B cells have an increased level of responsiveness to fecal gut bacteria. A and B. Representative flow cytometry plots of IgG versus IgA on bacteria that were tested for VH4-34 IgG+ B cells binding to gut bacteria.Flow cytometry plots in Figure **A** show the binding of VH4-34 antibodies from CD27+ IgG+ B cells on a healthy donor stool. In contrast, Figure **B** depicts the binding of VH4-34-encoded antibodies expressed by IgG+ B cells to gut bacteria on an SLE stool. The percentages of gut bacteria reactivity for a representative healthy donor and SLE stool pair are illustrated in Figure **C**. The frequencies of gut bacteria reactivity for the average values of HD and SLE VH4-34+IgG+ as well as HD VH4 non-VH4-34 recombinant antibodies on HD and SLE stools are depicted in Figure **D**. Each circle represents the results that were tested on a healthy donor stool, whereas each square symbol shows the data obtained from a SLE patient stool sample, and horizontal bars denote medians. In addition, symbols denoting an unfilled gap indicate the HD IgG antibodies, whereas symbols filled with black represent the SLE VH4-34+IgG antibodies in Figures **C** and **D**. [**** p-value < 0.0001, *** p-value <0.001, ** p-value <0.01, * p-value <0.05]

Additionally, the percentages of gut bacteria reactivity for other four healthy donor and SLE stool pairs are illustrated in Figure 4.3. Overall, there is an increase in the degree of gut bacteria reactivity of SLE VH4-34+IgG antibodies on SLE stools

compared to the ones acquired from healthy donor stools. Among all the tested groups (HD VH4 non-VH4-34, HD VH4-34, and SLE VH4-34 recombinant monoclonal antibodies) evaluated for fecal bacteria staining, the binding of SLE VH4-34+IgG antibodies to gut bacteria yields the strongest signal when the means of each group are compared. For example, the average of SLE VH4-34 antibodies gives the highest signal on SLE stool SLE18, SLE14, SLE03, and SLE11 at 10.32, 14.68, 5.85, and 6.67, respectively.

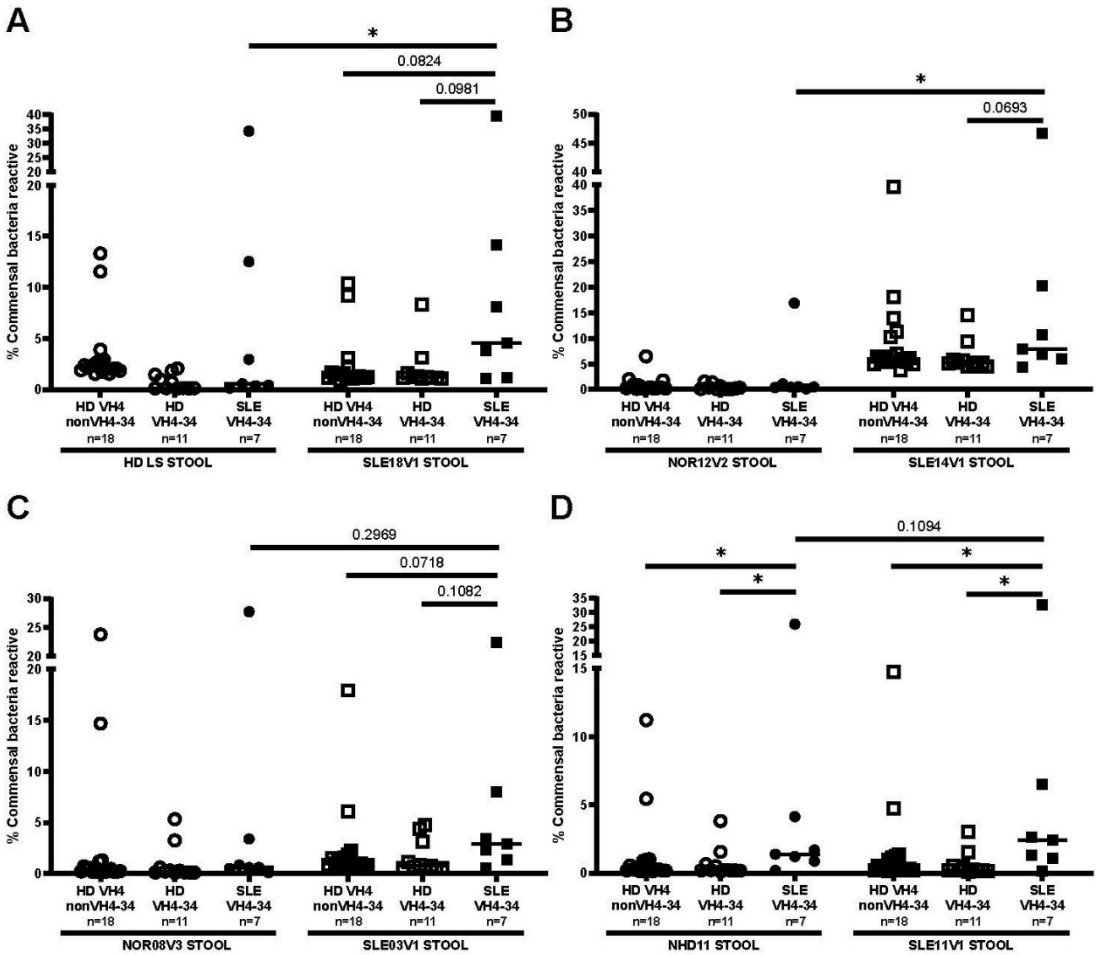


Figure 4.3: The reactivity of SLE VH4-34+IgG antibodies towards gut bacteria is seen to be higher in SLE feces compared to healthy donor stools. A. The percentages of gut bacteria reactivity for healthy donor HD LS and SLE stool SLE18 are illustrated. **B.** The reactivity percentages of gut bacteria in healthy donor NOR12V2 and SLE stool SLE12 are shown. **C.** The reactivity proportions of gut bacteria in healthy donor HD NOR08V3 and SLE stool SLE03 are represented. **D.** The reactivity percentages of gut bacteria in the stool samples of healthy donor HD NHD11 and SLE stool SLE11 are displayed. Each circle represents the results that were tested on a healthy donor stool, whereas each square symbol shows the data obtained from a SLE patient stool sample, and horizontal bars denote medians. In addition, symbols

denoting an unfilled gap indicate the HD IgG antibodies, whereas symbols filled with black represent the SLE VH4-34+IgG antibodies in Figures **C** and **D**. [* p-value <0.05]

4.4. 9G4$^+$(VH4-34) CD27$^+$IgG$^+$ B cell clones with unmutated AVY and NHS motifs are associated with an increased level of anti-commensal reactivity

In order to evaluate if VH4-34-expressing IgG clones of SLE patients exhibit recognition of commensal bacteria, we conducted an analysis of the frequencies and reactivity of 9G4+CD27+IgG+ B lymphocytes expressing VH4-34-encoded antibodies obtained from one active and seven quiescent SLE patients that displayed autoantibodies characteristic of the disease (Table 4.1). The 9G4 monoclonal antibody specifically identifies an epitope encoded by the FWR1 region of the VH4-34 gene, thereby facilitating the detection and separation of VH4-34+IgG+ B lymphocytes.(193) Consistent with our sequencing findings, we observed elevated frequencies of 9G4+ B lymphocytes in the IgG+ pool of SLE patients; on the other hand, 9G4+IgG+ B lymphocytes were few in healthy donors, as formerly shown (Figure 4.4A and B).(5, 7)

The analysis of somatic hypermutation in immunoglobulin genes expressed by 9G4+IgG+ B lymphocytes obtained from three SLE patients (one active with a SLEDAI of 8 and two quiescent with SLEDAI <4) revealed a notable reduction in the overall number of mutations seen in VH and Vκ immunoglobulin genes, confirming decreased SHM frequencies identified in total IgG$^+$ B lymphocytes from these patients (p-value < 0.0001 for V_H, p-value <0.01 for Vκ, Figure 4.4C).(6)

Nevertheless, the majority of 9G4$^+$IgG$^+$ B cell clones derived from both healthy donors and SLE patients possessed an unmutated AVY motif, which was distinct from VH4-34 sequences acquired from the entire population of IgG+ B cells Figure 4.4D). The presence of bias in this context is probably due to the 9G4 identification of the germline encoded FWR1 epitope, which is located near or overlaps with the AVY sequence. This bias has hindered further evaluations of the effects of the AVY mutation on the detection of commensal bacteria. Notably, owing to the aforementioned bias, we had already checked the CD27+ IgG+ B cells of SLE patients and healthy donors without using the 9G4 sorting strategy, and their results were also comparable with the ones obtained from 9G4$^+$(VH4-34) CD27$^+$IgG$^+$ B cell

clones. It is important to acknowledge that due to the limited presence of VH4-34+IgG+ B cells in healthy donors (HDs), we employed the 9G4 sorting technique to get a greater quantity of 9G4+(VH4-34) CD27+IgG+ B cells from both healthy and SLE subjects for our study. Nevertheless, a notable difference in the mutational state of the NHS motif was observed when comparing healthy donor and SLE patient VH4-34 sequences. In fact, the majority of VH4-34 sequences derived from SLE 9G4+IgG+B lymphocytes had an unmutated NHS motif in their CDR2, as seen in total IgG+ B cells in Figure 1. Conversely, VH4-34 sequences from healthy donors mostly exhibited altered NHS motifs (Figure 4.4D), therefore suggesting that the elimination of glycosylation in VH4-34 CDR2 is a prevalent characteristic linked to immunological responses in healthy controls.

We additionally examined the 9G4+IgG+ B cell reactivity by assessing their antibodies' ability to bind commensal bacteria. Lysates of bacteria contained commensal bacteria Bacteroides fragilis, Enterobacter cloacae, Enterococcus faecalis, and Clostridium difficile. The reactivity investigations demonstrated the presence of antibodies produced by 9G4+IgG+ B lymphocytes with an unmutated AVY motif, which were linked with anti-commensal bacteria.(7) We observed an enriched anti-commensal bacteria activity in antibodies produced by 9G4+IgG+ B lymphocytes in patients with SLE. (Figure 4.4E) In fact, the anti-commensal 9G4+IgG+ B lymphocyte frequencies in both healthy donors and SLE patients were averaged at 36.8 and 54.8%, respectively. (Figure 4.4E) In contrast, this reactivity was only seen in around 20% of non-VH4-34 IgG+ B lymphocytes in healthy donors, as stated previously.(7) The observed elevation in reactivity towards anti-commensal bacteria in HD 9G4+IgG+ B cells could possibly be attributed to the isolation of rare IgG clones containing unmutated AVY motifs in these individuals owing to the sorting technique involving the use of the 9G4 anti-VH4-34 antibody. It is worth noting that findings from our previous work indicated that VH4-34-encoded antibodies derived from unbiased total circulating IgG+ B cells in HDs did not exhibit reactivity towards anti-commensal bacteria.(7)

Furthermore, there was an increased presence of anti-commensal reactivity seen in clones expressing VH4-34 with an unmutated NHS motif in healthy donors

(Figure 4.4F). It may be inferred that the AVY motif, in its unmutated state, which imparts self-reactivity, along with the presence of CDR2 glycosylation coming from the intact NHS sequence, promotes a preference for the reactivity of VH4-34-encoded antibodies towards anti-commensal bacteria.

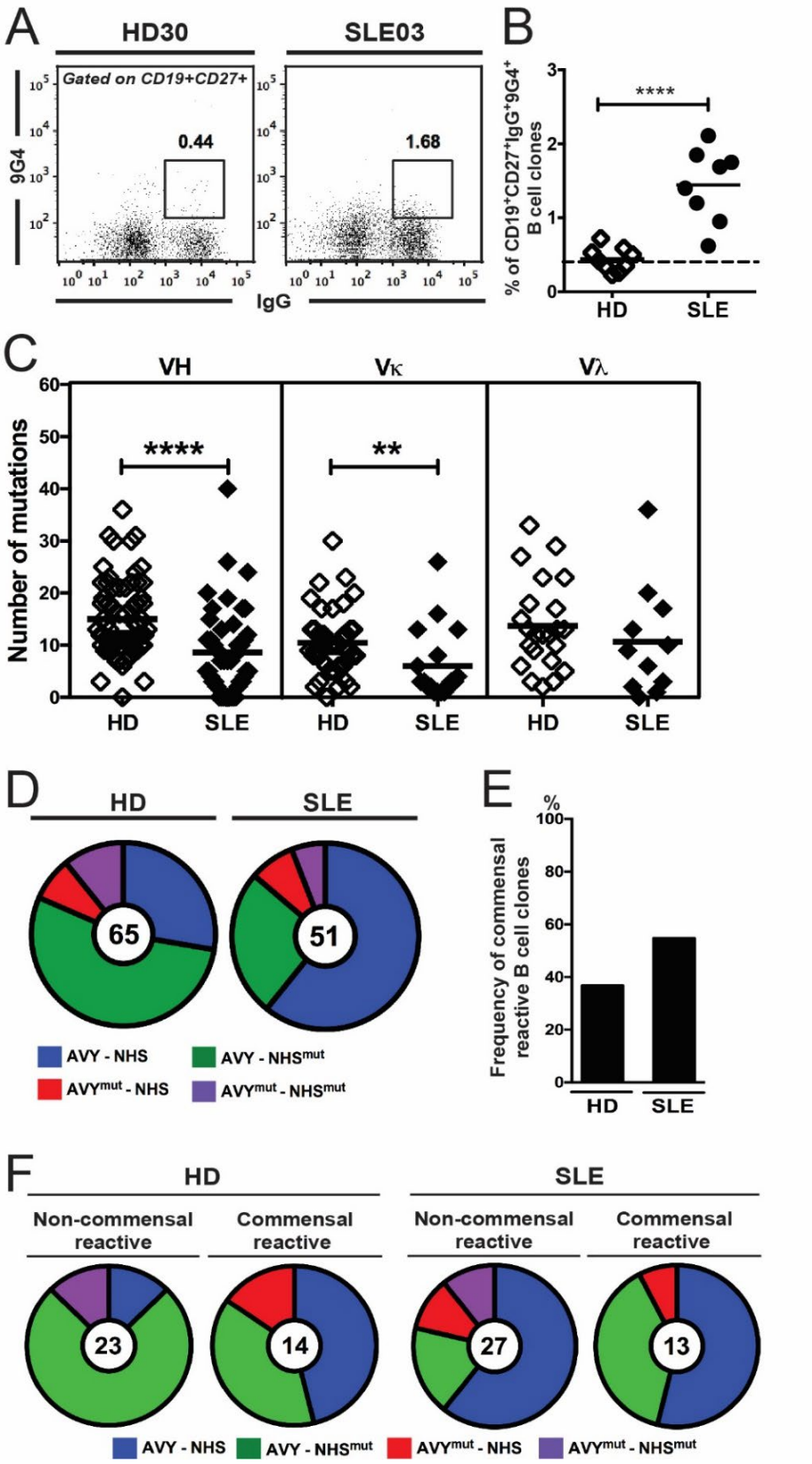


Figure 4.4: The VH4-34-expressing antibodies produced by 9G4+IgG+ B lymphocytes are increased in SLE patients and preferentially bind commensal bacteria antigens. **A.** Dot plots showing the staining results for 9G4, which

recognizes VH4-34-encoding heavy chains, and CD19 expression on gated CD19+CD27+IgG+ memory B lymphocytes from both a representative healthy donor and an SLE patient. **B** shows the frequencies of CD19+CD27+IgG+9G4+ B cell clones in a cohort of 11 healthy controls and 8 SLE patients. In Figure **C,** the number of mutations seen in the VH, Vκ, and Vλ genes of antibodies derived from memory B cells expressing the 9G4+IgG+ phenotype was assessed in a cohort of five healthy donors and three SLE patients. The pie charts in Figure **D** illustrate the ratios of 9G4+IgG+ memory B lymphocyte clones that have undergone mutations in the AVY and/or NHS sequences in healthy donors and SLE patients. For Figure **E**, the frequencies of commensal reactive $9G4^{+}IgG^{+}$ B cells are depicted. Pie charts in Figure **F** were used to visually show the percentage of clones that had mutations in the AVY and NHS sequences within noncommensal and commensal-reactive 9G4+IgG+ B lymphocytes derived from healthy donors as well as SLE patients.

4.5. Stool bacteria recognition by expanded 9G4+IgG+ B cells in SLE patients

The identification of a distinct subset of autoreactive B lymphocytes, characterized by the use of the immunoglobulin variable heavy chain gene VH4-34 and recognized by the rat monoclonal antibody 9G4, was also ascertained. Our primary focus lies in examining the responsiveness of 9G4+ memory B cells towards commensal gut bacteria in individuals with SLE in comparison to those without the disease. The primary objective of these experiments is to find out if there are observable differences in fecal gut bacteria reactivity between patients with SLE and healthy donors, specifically in relation to 9G4+ (VH4-34) memory B cells. Therefore, we tested the binding of VH4-34 antibodies of 9G4+CD27+IgG+ B cells towards commensal bacteria using bacterial flow cytometry.

We detected substantially greater binding of 9G4+ VH4-34-encoded antibodies generated from IgG+ memory B cells of healthy donors to commensal bacteria derived from SLE stool than from HD stool (the average on HD stool samples at 9.1 versus the average on SLE stool samples at 19.85, p-value < 0.0001, Figure 4.5D). In a similar fashion, 9G4+ VH4-34-encoded recombinant antibodies obtained from SLE patients demonstrated a considerable rise in commensal bacteria binding in SLE stool relative to the fecal samples of healthy donors (the average on HD stool samples at 7.53 versus the average on SLE stool samples at 19.17, p-value < 0.0001, Figure 4.5D). There was no statistically significant difference seen in the reactivity levels between SLE patients and healthy donor 9G4+IgG+ B cell clones when exposed to bacteria generated from healthy donor stool samples. Likewise, no statistically

significant distinction was found in the reactivity levels between SLE patients and HD 9G4+IgG+ B cell clones when exposed to bacteria obtained from stool samples of SLE patients. Together, these data reflect a significant factor influencing the variations in bacterial adherence to VH4-34 clones between healthy donors and those with SLE is most likely attributed to differences in the bacterial species present in each of these settings.

Collectively, the findings presented indicate that 9G4+ VH4-34 IgG antibodies derived from both healthy individuals and SLE patients displayed increased reactivity against commensal gut bacteria found in the fecal sample derived from SLE patients.

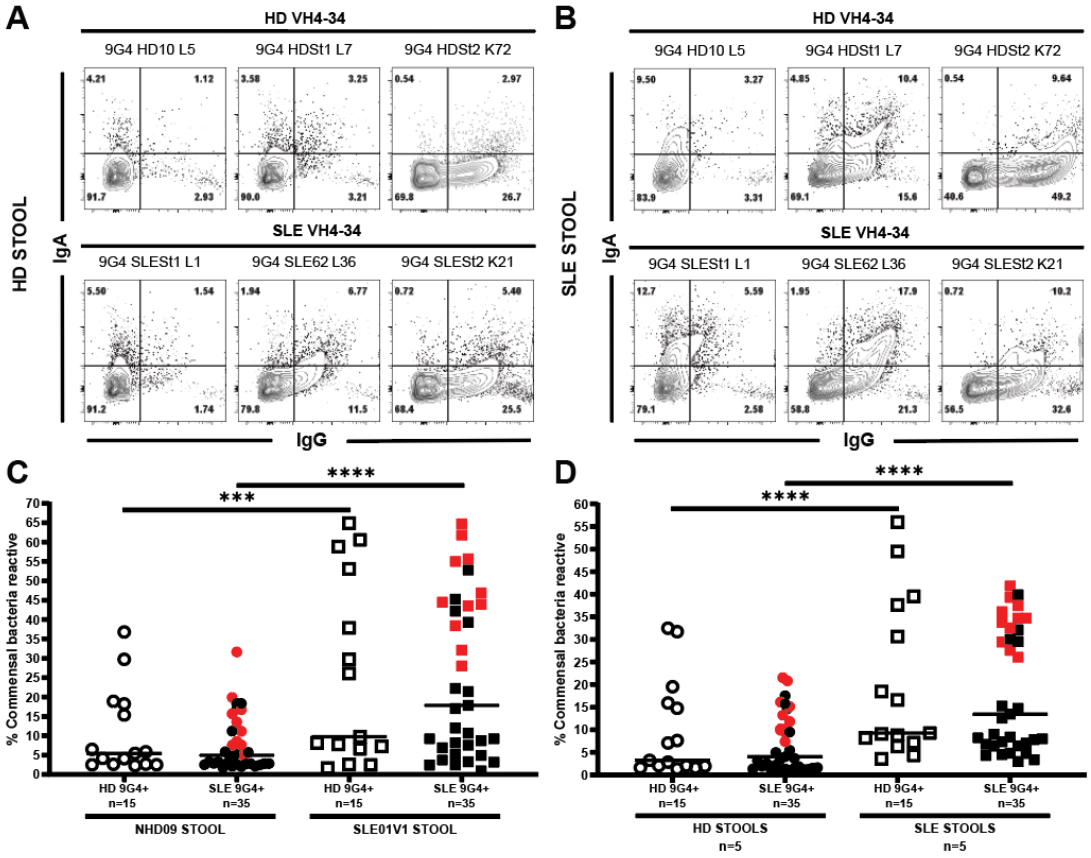


Figure 4.5: Anti-gut bacteria 9G4^{+}IgG^{+} B cells are expanded in SLE patients. Representative gating strategy and flow cytometry plots of IgG versus IgA on bacteria that were tested for 9G4+CD27+IgG+ B cell binding to gut bacteria are depicted in Figures **A** and **B**. Flow cytometry plots in Figure **A** show the binding of VH4-34 antibodies from 9G4^{+}IgG^{+} B cells on a healthy donor stool. In contrast, Figure **B** depicts the binding of VH4-34-encoded antibodies expressed by 9G4^{+}IgG^{+} B cells to gut bacteria on an SLE stool. Figure **C** depicts the reactivity percentages of gut bacteria

for a representative healthy donor and SLE stool pair. Figure **D** illustrates the frequencies of gut bacteria reactivity for the average values of HD and SLE 9G4+IgG+ recombinant antibodies on HD and SLE stools. Each circle represents the results that were tested on a healthy donor stool, whereas each square symbol shows the data obtained from a SLE patient stool sample, and horizontal bars denote medians. In addition, symbols denoting an unfilled gap indicate the HD IgG antibodies, whereas symbols filled with black represent the SLE VH4-34+IgG antibodies in Figures **C** and **D.** Also, symbols labeled in red for SLE 9G4+IgG+ recombinant antibodies illustrate the 9G4 B cells that show clonal expansion in the SLESt2 patient (see in the following section).

The primary objective of our study was to assess the reactivity of recombinant IgG VH4-34 antibodies derived from both healthy individuals and patients with systemic lupus erythematosus (SLE) towards commensal bacteria obtained from both healthy individuals and SLE patients. Consequently, our study incorporates a stool cohort of five healthy donors and five individuals with SLE in order to assess the gut bacteria reactivity of VH4-34 IgG+ B cells. The reactivity of VH4-34-encoded recombinant antibodies, which were cloned from 9G4-enriched IgG+ memory B cells obtained from either healthy participants or SLE patients, was also represented on a separate set of healthy donor and SLE stool pairs in Figure 4.6. Concerning other stool comparisons, we also noticed a significant rise in the bacterial binding of the total IgG+ fraction when $9G4^{+}IgG^{+}$ recombinant antibodies derived from healthy individuals and SLE patients were tested against SLE stool as compared to healthy donor stool.

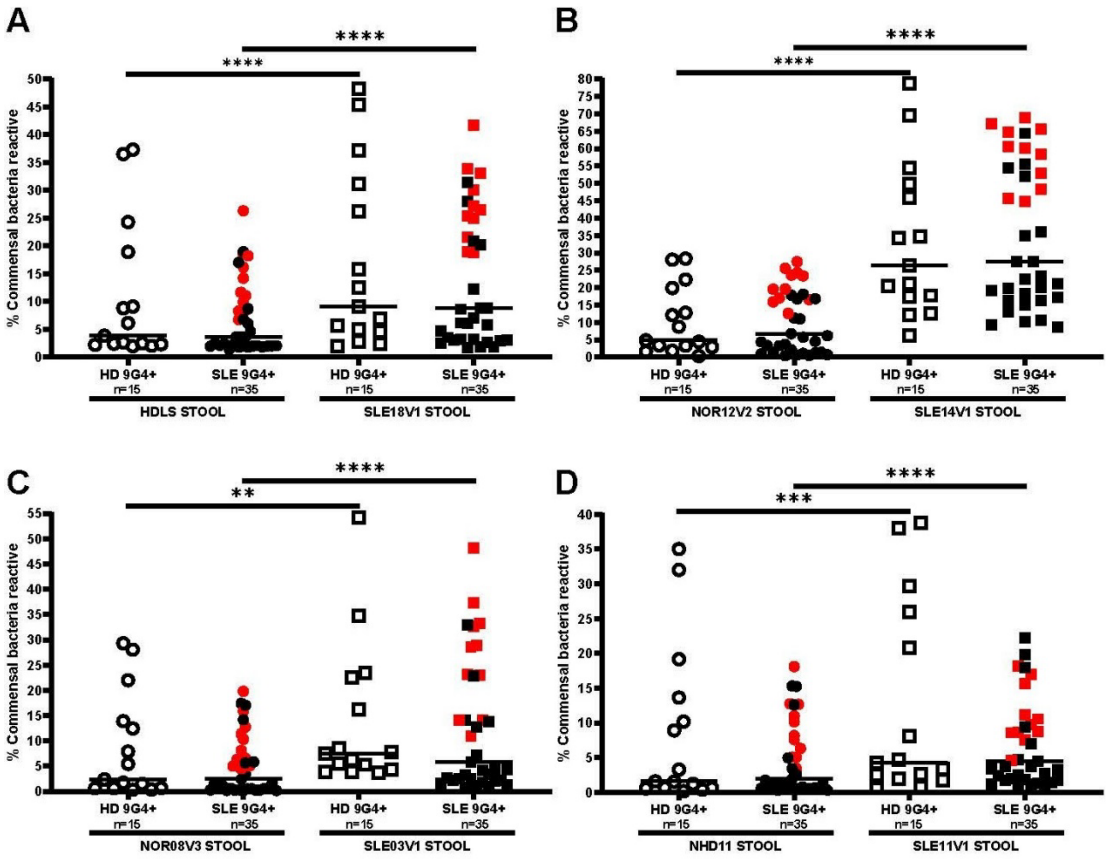


Figure 4.6: **Recognition of fecal bacteria by B lymphocytes expressing the 9G4+IgG+ phenotype. A.** The percentages of commensal bacteria reactivity for healthy donor HD LS and SLE stool SLE18 are illustrated. **B.** The reactivity percentages of gut bacteria in healthy donor NOR12V2 and SLE stool SLE12 are shown. **C.** The reactivity proportions of gut bacteria in healthy donor HD NOR08V3 and SLE stool SLE03 are represented. **D.** The reactivity percentages of gut bacteria in the stool samples of healthy donor HD NHD11 and SLE stool SLE11 are displayed. Each circle represents the results that were tested on a healthy donor stool, whereas each square symbol shows the data obtained from a SLE patient stool sample, and horizontal bars denote medians. In addition, symbols denoting an unfilled gap indicate the HD IgG antibodies, whereas symbols filled with black represent the SLE VH4-34+IgG antibodies in Figures **A, B, C** and **D.** Also, symbols labeled in red for SLE 9G4+IgG+ recombinant antibodies illustrate the 9G4 B cells that show clonal expansion in the SLESt2 patient (see in the following section). [**** p-value < 0.0001, *** p-value <0.001, ** p-value <0.01]

After our investigation, which evaluated the reactivity of recombinant 9G4+IgG+ antibodies obtained from both healthy persons and SLE patients towards fecal gut bacteria acquired from both healthy individuals and SLE patients, we want to find out how many SLE and HD 9G4+IgG+ B clones, which can be categorized into

non-commensal and commensal-reactivity groups for the stools of both healthy people and SLE patients, have mutations in the AVY and NHS VH4-34 motifs.

Pie charts in Figure 4.7 were used to illustrate the proportion of clones exhibiting mutations in the AVY and NHS sequences within non-commensal and commensal-reactive 9G4+IgG+ B lymphocytes isolated from healthy donors as well as SLE patients when tested on the stools of healthy participants and SLE patients.

All the high-positive SLE 9G4+ clones on the SLE stools are also highly commensal reactive on HD stools, meaning that they match, giving the same mutational status of VH4-34 AVY and NHS motifs. This fact is also consistent with highly reactive HD 9G4+ clones.

9G4+IgG+ expressing antibodies possessing unmutated AVY motifs exhibit greater binding towards commensal gut bacteria present in the stool samples of both healthy individuals and lupus patients. Conversely, these antibodies with mutated AVY and NHS demonstrated minimal binding to commensal gut bacteria found in control stool samples as well as SLE stools.

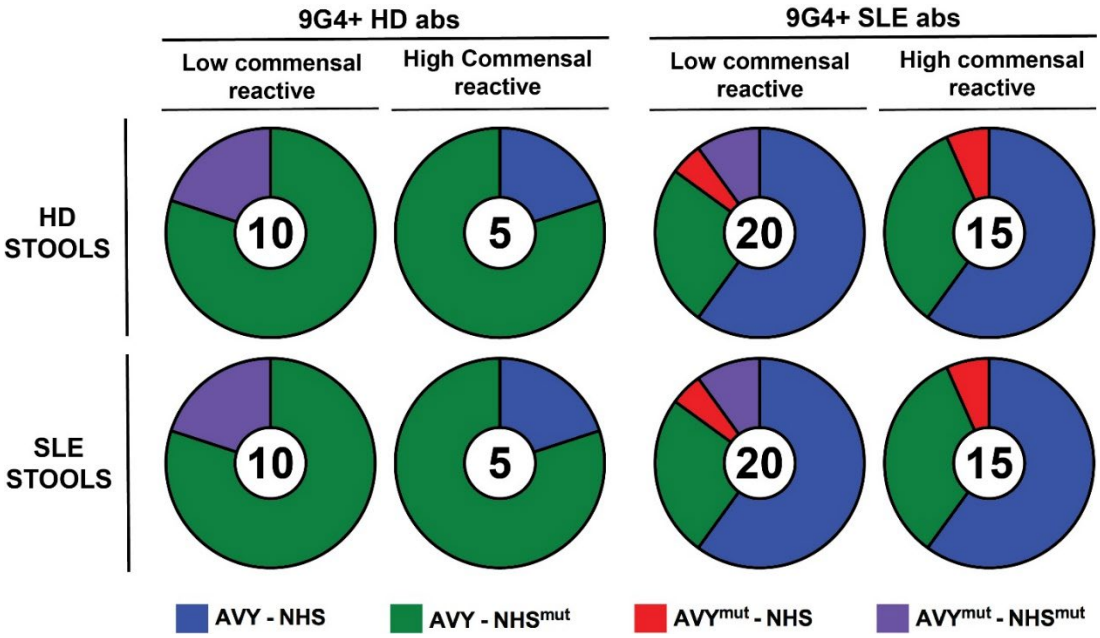


Figure 4.7: The 9G4+IgG+ B cell clones with an unmutated AVY motif showed higher anti-commensal reactivity.

The following tables show the repertoire and reactivity of the antibodies derived from IgG+ memory B cells, of which cloned antibodies were tested for flow experiments and for other analysis.

Table 4.4: Antibody repertoire and reactivity of HD10 9G4+IgG+ memory B lymphocytes

Ig	HEAVY																LIGHT																REACTIVITY							
								FWR1		CDR1		FWR2		CDR2		FWR3						FWR1		CDR1		FWR2		CDR2		FWR3		CDR3								
	VH	D	RF	JH	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vκ	Jκ	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Stalning	Flagellin	E.coli	S.aureus	E. faeca	E.cloaca
HD10 G8	4-34	3-3	1	6	GRGGNFLDIYHYFMDV	16	16	1	2	4	1	0	0	2	0	4	2	3-11	5	QQRSDWPPRIT	11	7	0	0	1	1	2	1	0	0	1	0	1	0	-	ND	ND	-	-	-	+	+
HD10 G14#	4-34	1-24	2	6	AYSVVARNQRYYFYYYMDV	19	13	1	1	2	0	1	1	2	0	2	3	1-9	4	QQLNSYPLT	9	3	0	0	2	0	0	0	0	0	0	1	0	0								
HD10 G24#	4-34	3-3	3	4	GGSHLTSTFGVVNVGAYFDS	20	31	0	0	3	0	4	3	10	2	5	4	4-1	1	QQYYRAPWT	9	9	0	0	0	0	3	0	0	1	1	2	2	0								
								FWR1		CDR1		FWR2		CDR2		FWR3						FWR1		CDR1		FWR2		CDR2		FWR3		CDR3								
	VH	D	RF	JH	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vλ	Jλ	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Stalning	Flagellin	E.coli	S.aureus	E. faeca	E.cloaca
HD10 G3	4-34	2-2	2	4	GHPYSSKSSSWYYFAL	16	30	3	0	5	0	0	2	7	3	6	4	1-40	1	QSHDSLLSGYI	11	33	1	2	3	2	4	0	4	1	7	3	3	3	+	ND	ND	+	-	-	-	-
HD10 G4#	4-34	5-12	3	4	GKKLNIVATNRMGRYYFES	19	24	2	1	3	1	0	1	4	3	6	3	2-11	3	CSYAGTSAV	9	13	2	2	1	1	3	2	1	0	0	1	0	0								
HD10 G5	4-34	/	/	6	QPTFYGYFQYYYMGV	15	11	1	0	2	0	0	0	1	3	1	3	1-40	3	QSYDISLGGRRV	12	15	2	1	2	0	2	2	1	1	0	2	2	0	-	ND	ND	-	-	-	-	-
HD10 G13	4-34	1-7	2	4	SPRWNQRSSYYFDY	14	19	1	1	2	0	0	1	4	1	6	3	2-23	5	CSYSRSRI	8	12	1	1	1	1	1	1	0	0	1	1	4	0	+	ND	ND	+	+	+	+	+
HD10 G15	4-34	4-17	1	6	GRLRTENMDV	10	19	1	0	1	0	2	2	4	1	5	3	1-51	3	GTWDSSLSGVV	11	12	0	2	2	1	0	2	1	1	0	0	1	2	-	ND	ND	-	-	-	-	-
HD10 G19#	4-34	2-2	2	6	RYCSSVSCTGRTLHHYMDV	19	31	4	0	0	2	2	3	6	1	7	6	2-11	3	CSYSDTSTRWV	11	23	2	2	4	1	4	0	1	1	1	1	4	2								
HD10 G28	4-34	2-2	3	4	GPHNEDIVVVPGAMVLFDY	19	7	0	1	3	0	0	0	2	0	1	0	2-8	1	SSYAGSKTLYV	11	3	0	0	0	0	0	0	0	1	2	0	0	-	ND	ND	-	+	+	-	-
HD10 G33	4-34	5-5	2	4	GAYGDDVLDF	10	25	1	1	2	1	1	0	5	3	8	3	1-47	3	AAWDDSLTGPV	11	9	0	0	1	0	0	1	1	1	3	0	1	1	-	ND	ND	-	-	-	+	+
HD10 G36	4-34	/	/	4	GWTNFLRQKYLDY	13	9	0	0	0	0	1	0	4	0	3	1	2-14	3	SSYTGSSTWV	10	7	3	1	0	0	0	1	1	0	0	0	1	0	-	ND	ND	-	-	-	-	-
HD10 G37	4-34	2-15	2	4	VRGYGSAYSRARLDS	15	18	1	1	1	0	1	0	4	1	6	3	2-14	3	SSYTSSSTWV	10	3	1	0	0	0	0	1	0	0	1	0	0	0	-	ND	ND	-	-	-	-	-
HD10 G46	4-34	3-9	2	4	GLGDYDYLDF	10	10	0	1	0	0	0	0	3	0	6	0	2-14	3	SSYTGSSTWV	10	6	1	3	0	0	0	0	1	0	0	0	1	0	-	ND	ND	-	-	-	-	-
HD10 G47#	4-34	/	/	4	GSLRARFDY	9	8	0	1	1	0	0	0	3	0	0	3	2-18	3	RSYNTTSTSLISA	13	18	1	1	2	1	1	1	2	0	3	1	4	1								
								FWR1		CDR1		FWR2		CDR2		FWR3																								
	VH	D	RF	JH	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S																								
HD10 G2	4-34	5-5	1	5	GSKPSIKLWPARKVHNWFDP	20	8	0	0	1	1	0	1	2	0	2	1																								
HD10 G18	4-34	3-22	3	6	GVMKINMVVVVMSSYSYYMDV	21	12	0	0	2	0	1	2	2	0	3	2																								
HD10 G20	4-34	4-23	3	4	GHRVVMVTANFDY	13	23	1	3	3	1	0	2	5	3	1	4																								
HD10 G34	4-34	6-6	3	4	GSQGFHSARRPRRAFDY	17	17	2	1	2	0	1	1	3	1	4	2																								
HD10 G40	4-34	1-7	1	4	LDGSENNNWVDP	12	36	2	4	2	0	2	1	8	1	8	8																								
HD10 G43	4-34	6-13	3	5	RLGSSRTSPTLGARGT	16	11	1	1	0	1	1	0	3	0	3	1																								

RF, reading frame: # antibody failed to be expressed- non reactive; +, reactive

Table 4.5: Antibody repertoire and reactivity of HDSt1 9G4+IgG+ memory B lymphocytes

Ig	HEAVY																LIGHT														REACTIVITY						
							FWR1		CDR1		FWR2		CDR2		FWR3						FWR1		CDR1		FWR2		CDR2		FWR3		CDR3							
	VH	D	RF	JH	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Vκ	Jκ	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Staining	Flagellin	E.coli	S.aureus	E.faecal	E.cloaca
HDSt1 G08	4-34	/	/	6	PAAYYHHYRGLDV	13	11	1	1	0	1	1	2	0	1	4	0	3-20	1	QQYGNSRRT	9	18	2	2	2	4	0	0	2	0	1	3	1	1	-	ND	ND	+	+	+	+	+
HDSt1 G15	4-34	3-10	3	6	GQGRAFGELLVPYNGMDV	18	9	0	0	2	0	0	2	1	0	1	3	1-8	4	QQYYSYPLS	9	8	3	0	3	0	1	0	0	0	1	0	0	0	-	ND	ND	+	+	-	+	+
HDSt1 G16	4-34	2-2	2	6	VKINCISSTSCSFFFYYYGMDV	22	12	2	2	1	0	0	1	3	1	2	0	1-9	4	QQLNSYPLT	9	12	3	0	1	0	0	0	1	0	5	0	0	2	+	ND	ND	+	+	+	+	+
HDSt1 G18	4-34	/	/	5	GSDPYKTRL	9	22	1	2	4	1	2	1	4	1	3	3	4-1	2	QQYYDSPRT	9	13	2	0	2	0	1	1	0	0	3	1	3	0	-	ND	ND	-	-	-	-	-
HDSt1 G20	4-34	3-3	2	6	VFYDFWSGYGTDV	13	14	0	0	1	1	2	1	1	1	3	4	3-20	2	HQYGSSPPST	10	13	3	2	3	3	0	0	0	0	0	0	1	1	-	ND	ND	+	+	+	+	-
HDSt1 G23	4-34	/	/	4	GGGWSPLDY	9	12	0	0	1	2	0	0	4	0	5	0	3-20	4	QQYDTSPHT	9	6	0	1	2	1	0	0	0	0	0	0	2	0	-	ND	ND	-	-	-	-	-
HDSt1 G24	4-34	4-23	1	4	GRRGLRRQRRGSHVDY	16	14	3	0	2	0	1	0	3	2	2	1	1-39	4	QQSYSTPLT	9	2	1	1	0	0	0	0	0	0	0	0	0	0	+	ND	ND	+	+	+	+	+
HDSt1 G25	4-34	2-2	3	6	VRVRPAAIRRLYYYYGMDV	19	16	0	0	2	1	1	2	5	1	3	1	1-9	3	QQLNAYPFT	9	13	2	0	3	0	2	1	0	0	1	2	2	0	-	ND	ND	+	+	-	+	-
HDSt1 G27	4-34	4-17	3	4	GGEIGMVTTMTYYFDH	16	11	1	0	2	0	1	1	1	0	3	2	1-39	2	QQSDDTPYT	9	17	1	2	3	1	1	1	0	0	3	2	3	0	-	ND	ND	+	-	-	+	+
HDSt1 G30	4-34	3-9	2	6	FPFYIQYSDGYYSNGMDV	18	13	0	2	1	1	0	0	4	2	1	2	1-9	3	QQLTSYPFT	9	10	3	0	1	0	1	1	0	0	1	2	1	0	-	ND	ND	-	-	-	-	-
HDSt1 G33	4-34	4-17	2	4	AQGDSYEDF	9	11	0	0	0	0	0	1	2	2	2	4	3-20	2	QQYAGSPQT	9	6	0	1	1	0	0	0	0	0	1	1	2	0	-	ND	ND	-	-	-	-	-
HDSt1 G37#	4-34	3-3	3	6	VRSRDYGVVIIPVDGMDV	18	6	0	0	0	0	0	0	3	2	1	0	1-5	5	QQYYSYSIT	9	11	0	1	4	0	0	1	2	1	0	0	1	1								
HDSt1 G38	4-34	6-13	2	6	GGRDSSNLFRPYYYYGMDI	19	14	3	1	1	0	1	0	3	1	1	3	1-39	1	QQRYSSPPT	9	12	0	2	0	1	1	1	1	1	1	2	2	0	+	ND	ND	+	+	+	+	+
HDSt1 G42#	4-34	3-22	2	6	DFEIDSSGYHRYYYDMDV	18	9	0	0	2	0	0	0	1	0	3	3	2-30	4	MQGTHWPVT	9	8	1	0	0	0	1	0	1	0	2	3	0	0								
HDSt1 G45	4-34	/	/	5	GAARSDSTGWFD	12	18	1	1	2	1	0	4	4	0	3	2	3-20	2	QQYGSSPPEYT	11	12	0	2	3	1	0	1	4	0	1	0	0	0	-	ND	ND	-	-	-	-	-
HDSt1 G46	4-34	/	/	5	TVLQRYFDL	9	22	2	1	1	2	0	1	4	1	6	4	1-5	1	QQYKSPSWT	9	20	0	0	2	2	2	2	1	1	2	4	3	1	-	ND	ND	-	-	-	-	-
HDSt1 G47	4-34	3-16	3	2	RRFQTFGQKYWYFDL	15	11	1	0	1	1	1	1	3	1	2	0	3-20	2	QQYGSSPFT	9	8	2	1	0	0	2	2	0	0	0	1	0	0	+	ND	ND	+	+	+	+	+
HDSt1 G48	4-34	2-21	2	4	VPTLSRVCGDGLCRRGMFDQ	20	11	0	1	0	0	0	0	5	0	4	1	3-20	1	QQYDNSRWT	9	9	0	1	0	1	0	0	2	1	1	0	2	1	+	ND	ND	+	+	+	+	+

							FWR1		CDR1		FWR2		CDR2		FWR3						FWR1		CDR1		FWR2		CDR2		FWR3		CDR3							
	VH	D	RF	JH	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Vλ	Jλ	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Staining	Flagellin	E.coli	S.aureus	E.faecal	E.cloaca
HDSt1 G07	4-34	6-13	1	4	GLVRSQQTFDF	11	12	2	1	2	0	0	0	1	0	4	2	3-1	3	QVWDSNTVV	9	13	1	2	1	1	0	1	1	0	3	1	2	0	-	ND	ND	+	-	+	+	+
HDSt1 G40	4-34	6-19	2	6	GPGSSGYMRFVKYGMDV	11	25	1	2	2	2	1	1	9	2	4	1	2-11	3	CSYAGNYLLV	11	27	1	0	6	1	3	2	2	0	4	4	3	1	-	ND	ND	-	-	-	+	+

							FWR1		CDR1		FWR2		CDR2		FWR3	
	VH	D	RF	JH	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S
HDSt1 G12	4-34	2-21	3	4	GVKGVVVVTTIYYFDS	16	15	0	2	0	1	0	1	2	0	6	3
HDSt1 G36	4-34	3-10	3	6	GVRGVIITYYYNAMDV	16	12	1	0	1	0	0	0	2	0	4	4

RF, reading frame: # antibody failed to be expressed- non reactive; +, reactive

Table 4.6 Antibody repertoire and reactivity of HDSt2 9G4+IgG+ memory B lymphocytes

Ig	HEAVY							FWR1		CDR1		FWR2		CDR2		FWR3		LIGHT					FWR1		CDR1		FWR2		CDR2		FWR3		CDR3		REACTIVITY							
	VH	D	RF	JH	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	VΚ	JΚ	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Staining	Flagellin	E.coli	S.aureus	E. faeca	E.cloaca
HDSt2 G51	4-34	3-16	3	5	GEGLQGVVITS	11	30	2	2	2	1	0	1	6	2	9	5	3-11	4	QQRYNGRPLT	10	17	0	2	2	2	0	0	3	0	1	2	4	1	-	ND	ND	-	-	-	-	-
HDSt2 G63	4-34	4-17	2	4	GTSDEYGDYQRYFDY	15	8	1	0	0	0	0	0	5	0	2	0	1-33	2	QQYDALPFT	9	6	3	0	0	0	0	1	0	0	0	0	2	0								
HDSt2 G64	4-34	2-15	2	2	QFCLSGTCYSLFPDAYWFFDF	21	21	0	0	0	0	0	0	6	2	4	9	3-15	2	QQYNNWPPYN	10	22	2	2	2	2	1	1	1	2	6	2	0	1								
HDSt2 G66	4-34	3-10	2	4	VSAALPPGGTYFMFFDY	17	17	1	1	0	0	3	1	3	0	4	4	1-39	4	QQSFVTPRT	9	30	1	3	5	2	3	4	2	0	3	3	3	1	-	ND	ND	+	+	+	+	+
HDSt2 G67	4-34	5-24	2	6	GPLITRYNYLYYNMDV	16	9	1	0	0	0	0	0	2	0	2	4	3-20	2	QQYSISPPRYT	11	10	0	0	3	0	1	1	0	1	0	2	2	0	+	ND	ND	+	+	+	+	+
HDSt2 G68	4-34	/	/	4	VSATLSPTNTYFMFFDS	17	22	1	0	1	1	1	1	5	2	4	6	1-39	4	QQSYTSPRT	9	23	0	0	0	0	4	6	1	1	5	3	2	1		ND	ND					
HDSt2 G72	4-34	6-6	3	6	VTVGRTRITPRPKPDYYMDV	20	22	1	1	2	1	0	0	2	2	7	6	1-39	1	QQSYSAPRT	9	19	0	1	4	0	2	0	3	0	4	3	1	1	+	ND	ND	+	+	+	+	+
								FWR1		CDR1		FWR2		CDR2		FWR3							FWR1		CDR1		FWR2		CDR2		FWR3		CDR3									
	VH	D	RF	JH	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	VΚ	JΚ	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Staining	Flagellin	E.coli	S.aureus	E. faeca	E.cloaca
HDSt2 G57	4-34	/	/	2	GDSAGPFDV	9	21	2	0	1	1	0	1	3	1	6	6	1-40	3	QSYDTSLGGSGV	12	23	1	1	3	1	2	2	4	0	3	3	2	1								
HDSt2 G60	4-34	5-12	2	4	RRGYDQFYYFDN	12	13	0	0	1	0	0	1	1	0	6	4	2-23	3	CSYAGSVI	8	5	0	0	1	0	1	0	0	0	2	0	0	1	-	ND	ND	-	-	-	+	-
HDSt2 G69	4-34	6-19	2	4	RPGWFQYYFDS	11	9	1	0	1	0	0	0	2	1	4	0	2-14	3	SSHISSSIHMV	11	10	2	1	0	0	0	0	2	0	0	1	2	2	-	ND	ND	+	+	+	+	+

RF, reading frame: # antibody failed to be expressed- non reactive; +, reactive

Table 4.7: Antibody repertoire and reactivity of HD IY01 9G4+IgG+ memory B lymphocytes

Ig	HEAVY																LIGHT																REACTIVITY							
								FWR1		CDR1		FWR2		CDR2		FWR3							FWR1		CDR1		FWR2		CDR2		FWR3		CDR3							
	VH	D	RF	JH	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vκ	Jκ	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Staining	Flagellin	E.coli	S.aureus	E. faecal	E.cloaca
HD IY01 G52#	4-34	4-17	2	6	GRDEYGDSVDYYYYKMDV	18	3	1	0	0	0	0	0	2	0	0	0	2-24	1	MQATQFPWT	9	8	1	0	1	0	2	0	2	0	1	1	0	0								
HD IY01 G55	4-34	6-6	3	5	VPHIAARRQGSRYFDL	16	10	0	1	2	0	0	1	1	1	2	2	1-39	1	QQSYSTPPT	9	0	0	0	0	0	0	0	0	0	0	0	0	0	+	ND	ND	+	+	+	+	+
HD IY01 G58	4-34	3-22	2	4	RRAWYDKFVYYFDN	14	11	1	0	0	1	0	1	2	2	3	1	3-15	1	QQYHNWSPAT	10	11	1	0	4	1	0	0	2	0	0	1	2	0	+	ND	ND	+	+	-	+	+
								FWR1		CDR1		FWR2		CDR2		FWR3							FWR1		CDR1		FWR2		CDR2		FWR3		CDR3							
	VH	D	RF	JH	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vλ	Jλ	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Staining	Flagellin	E.coli	S.aureus	E. faecal	E.cloaca
HD IY01 G50	4-34	2-2	3	4	GPAYIVVVPAAVLNYYFDY	19	7			0	0	0	0	1	0	2	4	2-14	1	SSYTSSNTYV	10	12	2	0	3	0	0	1	2	0	2	0	1	1	-	ND	ND	-	-	+	+	+
HD IY01 G54	4-34	/	/	5	GGIKVHRARGSRRKWFDP	18	18	1	2	1	1	0	3	3	1	4	2	1-40	1	QSYDSSLSAFV	11	13	1	1	2	2	0	1	3	0	2	0	1	0	-	ND	ND	-	-	-	+	+

RF, reading frame: # antibody failed to be expressed- non reactive; +, reactive

Table 4.8: Antibody repertoire and reactivity of HD MK27 9G4+IgG+ memory B lymphocytes

Ig	HEAVY															LIGHT														
								FWR1		CDR1		FWR2		CDR2		FWR3							FWR1		CDR1		FWR2		CDR2		FWR3		CDR3	
	VH	D	RF	JH	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vκ	Jκ	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S
HD MK27 G67	4-34	2-2	2	5	PTYCSSTNCAGPLAF	15	15	3	1	0	0	0	2	3	1	4	1	3-15	2	QQYNNWPPYT	10	9	1	0	4	0	0	0	1	0	3	0	0	0
HD MK27 G69	4-34	6-13	2	3	GRSWGWAFDY	10	21	6	2	0	0	0	1	0	3	7	2	1-5	2	QQYNRYSAYT	10	10	0	1	1	1	1	1	1	0	3	0	1	0
								FWR1		CDR1		FWR2		CDR2		FWR3							FWR1		CDR1		FWR2		CDR2		FWR3		CDR3	
	VH	D	RF	JH	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vλ	Jλ	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S
HD MK27 G60	4-34	1-26	2		FNSKDAFDL	9	11	3	0	0	0	0	0	3	1	2	2	2-23	1	CSRRESGTRYV	11	29	1	2	4	2	2	1	2	0	4	3	6	2
HD MK27 G91	4-34	3-22	2	6	WPDDSSGYYVPGGDGPYYGMDV	22	11	0	0	0	0	0	1	2	0	3	5	1-44	3	AAWDDSMNGVV	11	10	0	0	2	1	1	0	1	1	1	1	1	1
								FWR1		CDR1		FWR2		CDR2		FWR3																	
	VH	D	RF	JH	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S																
HD MK27 G61	4-34	6-19	2	4	GRSSGWYPAPNYFDS	15	18	1	1	1	0	0	1	5	0	7	2																
HD MK27 G64	4-34	3-10	2	3	EGEDYSGSGSNGIRALDI	18	18	5	3	2	0	1	1	1	0	3	2																
HD MK27 G76	4-34	2-2	3	5	RPRHRRVPAAIGV	13	0	0	0	0	0	0	0	0	0	0	0																
HD MK27 G77	4-34	4-17	2	6	TGDYYYYGMDV	11	11	5	1	1	0	1	2	0	0	1	0																
HD MK27 G79	4-34	3-22	2	4	TTGDYYLDN	9	10	1	0	0	0	0	2	2	0	5	0																
HD MK27 G52	4-34	3-10	2	6	SIGGSGNYNNRGYYGMDV	18	22	3	1	0	2	3	2	4	0	5	2																
HD MK27 G57	4-34	3-3	2	5	VNVWSGYYT	9	7	1	3	0	0	0	0	0	0	2	1																
HD MK27 G72	4-34	3-16	3	4	LGVPEYGDFQYYFDY	15	3	1	1	0	0	0	0	0	0	1	0																
HD MK27 G73	4-34	1-26	3	4	VSPPHTVGVQSTYYHGLDF	19	18	1	2	1	0	0	0	3	1	6	4																

RF, reading frame

Table 4.9: Antibody repertoire and reactivity of SLE St1 9G4+IgG+ memory B lymphocytes

Ig	HEAVY							FWR1		CDR1		FWR2		CDR2		FWR3		LIGHT					FWR1		CDR1		FWR2		CDR2		FWR3		CDR3		REACTIVITY							
	VH	D	RF	JH	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vκ	Jκ	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Staining	Flagellin	E.coli	S.aureus	E.faecalis	E.cloaca
SLE St1 G05#	4-34	6-19	3	6	SALYPSAGIYGIDYGMDV	18	17	2	2	0	2	1	1	1	0	6	2	3-20	1	QQSVTSPWT	9	16	0	2	1	1	1	2	0	1	2	3	3	0								
SLE St1 G10#	4-34	3-10	3	6	GKMIRGVRITTHYSYSDGVDV	21	26	0	2	3	2	2	2	3	1	4	7	1-17	1	LQHQTYPRT	9	13	0	1	3	0	3	0	1	0	1	1	3	0								
SLE St1 G19	4-34	6-19	2	5	GLGYSSSYDWFDP	13	3	0	0	0	0	0	1	0	1	1	0	3-11	5	QHRSNWPPEIT	11	4	0	1	1	0	0	0	0	0	0	0	2	0	-	ND	ND	-	-	-	-	-
SLE St1 G24#	4-34	2-21	2	2	DENGDSDWYFDL	12	12	0	0	0	0	2	3	4	2	1	0	1-5	1	QQYNSYWT	8	1	0	1	0	0	0	0	0	0	0	0	0	0								
SLE St1 G26	4-34	3-10	3	4	ASHRLSMVFDY	11	2	0	0	0	0	0	0	0	1	0	1	1-39	1	QQSYSTPRT	9	3	0	0	1	0	1	1	0	0	0	0	0	0	+	ND	ND	+	+	+	+	+
SLE St1 G29	4-34	5-24	1	4	GKRWLQPEAFFDY	13	3	0	0	0	1	1	1	0	0	0	0	1-39	2	QQSYTKRS	8	8	1	1	1	0	0	0	1	0	1	0	3	0	+	ND	ND	+	+	+	+	+
SLE St1 G32	4-34	4-11	2	6	GGRDYSKYSSKKFYAMDV	18	11	1	1	2	0	0	1	3	0	2	1	3-20	1	QQYGSSPT	8	1	1	0	0	0	0	0	0	0	0	0	0	0	-	ND	ND	-	-	-	-	-
SLE St1 G40#	4-34	3-3	3	6	GFTPLDHFGVVVYYYYGMDV	20	0	0	0	0	0	0	0	0	0	0	0	2-28	1	MQALQTPWT	9	1	0	1	0	0	0	0	0	0	0	0	0	0								
SLE St1 G41	4-34	4-17	2	6	GPGYGDYPAILYYYGMDV	18	0	0	0	0	0	0	0	0	0	0	0	1-27	1	QKYNSAPRT	9	3	2	0	0	0	0	0	0	1	0	0	0	0	-	ND	ND	-	-	-	-	-
SLE St1 G42	4-34	2-2	2	5	GLNVVVPAALNWFDP	15	0	0	0	0	0	0	0	0	0	0	0	1-5	1	QQYNSYPVT	9	5	1	1	0	0	0	0	0	0	0	0	3	0	-	ND	ND	-	-	-	-	-
SLE St1 G15																		3-20	1	QQYGSSPWT	9	2	1	1	0	0	0	0	0	0	0	0	0	0								
								FWR1		CDR1		FWR2		CDR2		FWR3							FWR1		CDR1		FWR2		CDR2		FWR3		CDR3								
	VH	D	RF	JH	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vλ	Jλ	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Staining	Flagellin	E.coli	S.aureus	E.faecalis	E.cloaca
SLE St1 G01	4-34	5-5	2	4	EGGRGYSYGYFSLTYYFDY	19	0	0	0	0	0	0	0	0	0	0	0	1-40	3	QSYDSSLSGRV	11	2	0	1	0	0	0	0	0	0	0	1	0	+	ND	ND	+	+	+	+	+
SLE St1 G03	4-34	3-3	2	6	LSVAGTEYYGMDV	13	40	0	3	3	0	4	5	5	1	11	8	2-14	3	SSYTRGSIWV	10	38	1	1	5	4	5	3	3	2	8	3	0	3	-	ND	ND	-	-	-	-	-
SLE St1 G27	4-34	4-17	2	2	DDNGDSDWYFDL	12	15	0	0	0	1	2	2	3	0	3	4	1-40	2	QSYDNTLSGFVL	12	17	1	2	2	0	2	0	3	0	2	3	2	0	-	ND	ND	-	-	-	-	-
								FWR1		CDR1		FWR2		CDR2		FWR3																									
	VH	D	RF	JH	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S																									
SLE St1 G37	4-34	6-6	3	5	GRRTTVAARPRFAWPWFDP	19	11	1	0	1	0	0	1	1	0	7	0																									

RF, reading frame: # antibody failed to be expressed- non reactive; +, reactive

Table 4.10: Antibody repertoire and reactivity of SLE St2 9G4+IgG+ memory B lymphocytes

Ig	HEAVY															LIGHT														REACTIVITY						
								FWR1		CDR1		FWR2		CDR2		FWR3						FWR1		CDR1		FWR2		CDR2		FWR3		CDR3							
	VH	D	RF	JH	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vκ	Jκ	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Staining	Flagellin	E.coli	S.aureus	E.faecali	E.cloaca
SLE St2 G02	4-34	2-2	3	2	GGVVFVPTAKRRWYFDL	17	5	0	0	1	1	0	1	0	0	1	1	1-5	2	QQYNSFPYT	9	7	2	0	1	0	1	1	0	0	0	0	1	1	-	ND	ND	-	-	-	+	-
SLE St2 G04	4-34	2-2	3	2	GGVVVVPAAIRRWYFDL	17	7	1	0	2	0	0	0	1	0	1	2	1-5	2	QQYNNFPYT	9	10	0	0	0	0	1	0	2	1	2	1	2	1	-	ND	ND	+	-	-	-	+
SLE St2 G05	4-34	2-2	3	2	GGTAVIPTALRRWYFDL	17	10	1	1	1	0	0	0	2	2	2	1	1-5	2	QQYNSFPYT	9	8	2	1	0	0	2	0	0	0	1	1	1	0	+	ND	ND	+	+	+	+	+
SLE St2 G06	4-34	2-2	3	2	GGVAVVPAALRRYYFDL	17	10	1	0	1	0	0	1	2	0	4	1	1-5	2	QQYNSFPYT	9	5	0	1	0	0	0	1	2	0	0	0	1	0								
SLE St2 G07	4-34	2-2	3	2	GGIVVKATDLRRWYFDL	17	7	0	1	1	1	0	0	1	0	2	1	1-5	2	QQYNGFPYT	9	9	0	3	1	1	1	0	0	0	1	0	2	0	-	ND	ND	+	+	+	+	+
SLE St2 G09#	4-34	2-2	3	2	GGVAVVPAALRRWYFDL	17	7	0	0	0	0	0	0	3	0	4	0	1-5	2	QQYNSFPYT	9	4	0	0	1	0	1	0	0	0	0	0	2	0								
SLE St2 G11	4-34	2-2	3	2	GGVVVVPADKRRWYFDL	17	8	1	0	1	0	2	1	2	0	1	0	1-5	2	QYNSFPYT	8	0	0	3	1	0	1	1	0	0	1	1	1	0	-	ND	ND	+	+	+	+	+
SLE St2 G17	4-34	2-2	3	4	GGGDIVVVPAANMGLDY	17	0	0	0	0	0	0	0	0	0	0	0	3-20	1	QQYGSSPWT	9	1	0	1	0	0	0	0	0	0	0	0	0	0	-	ND	ND	-	-	-	-	-
SLE St2 G18	4-34	4-17	3	2	VRLMTAVSYWNFDL	14	7	0	0	0	0	0	0	4	0	1	2	2-28	5	MQGRQTPIT	9	2	0	0	0	0	0	0	0	0	0	0	2	0	+	ND	ND	+	+	+	+	+
SLE St2 G19	4-34	1-26	2	4	GYGSAFDH	8	4	1	0	1	0	0	1	0	0	1	0	3-15	4	QQYNNWPL	8	2	1	1	0	0	0	0	0	0	0	0	0	0	-	ND	ND	-	-	-	-	-
SLE St2 G21	4-34	2-2	3	2	GGIVVIPTALRRWYFDL	17	4	0	1	1	0	0	0	0	0	1	1	1-5	2	QQYNSFPYT	9	5	0	1	1	0	2	0	0	0	0	0	1	0	+	ND	ND	+	+	+	+	+
SLE St2 G22	4-34	2-2	3	2	GGIVVIPTALRRWYFDL	17	7	0	1	1	0	0	0	2	0	1	2	1-5	2	QQYNTFPYT	9	14	0	2	1	1	1	1	0	2	3	0	2	1	-	ND	ND	+	+	+	+	+
SLE St2 G25	4-34	5-24	2	4	LLGRDGYNLFDY	12	3	0	0	2	0	0	0	1	0	0	0	1-39	2	QQSYSTPPYT	10	4	0	1	1	0	1	1	0	0	0	0	0	0	-	ND	ND	+	-	-	-	+
SLE St2 G26	4-34	2-2	3	2	GGIVVIPTALRRWYFDL	17	3	0	0	1	0	0	0	0	0	2	0	1-5	2	QQYNTFPYS	9	8	0	1	1	0	2	2	0	0	0	0	2	0	+	ND	ND	+	+	+	+	+
SLE St2 G27	4-34	2-2	3	2	GGIVVKPTDLRRWYFDL	17	10	1	1	1	0	0	0	3	1	3	0	1-5	2	QQYNAFPYT	9	10	0	2	1	0	2	1	1	0	2	0	1	0	+	ND	ND	+	+	+	+	+
SLE St2 G28	4-34	6-19	2	5	DSSGWYRRFDP	11	0	0	0	0	0	0	0	0	0	0	0	3-20	4	QQYGSSPPVLT	11	1	0	1	0	0	0	0	0	0	0	0	0	0	+	ND	ND	-	-	-	-	-
SLE St2 G29	4-34	2-2	2	5	PYETVPAARGYNWFDP	16	13	1	3	1	0	1	2	0	1	3	1	1-39	4	QQSFITPLT	9	18	3	1	5	1	1	0	1	1	2	1	2	0	-	ND	ND	+	+	-	-	+
SLE St2 G30	4-34	3-22	2	6	ERYDSSGYYSSYYYYGMDV	19	0	0	0	0	0	0	0	0	0	0	0	3-11	4	QQRSTWLLT	9	2	0	1	0	0	0	0	0	0	0	1	0	-	ND	ND	+	-	-	+	+
SLE St2 G34	4-34	3-10	1	4	LKGFGELLY	9	0	0	0	0	0	0	0	0	0	0	0	1-33	4	QQYNNCPPSF	10	4	1	2	0	0	0	0	0	0	1	0	0	0	-	ND	ND	-	-	-	-	-
SLE St2 G38	4-34	2-2	3	2	GGIVVIPTALRRWCFDL	17	2	0	0	1	0	0	0	1	0	0	0	1-5	2	QQFNTFPYT	9	12	3	1	1	0	2	1	1	0	0	0	3	0	+	ND	ND	+	+	+	+	+
SLE St2 G39	4-34	2-2	3	2	GGVVVVPAAIRRWYFDL	17	10	1	0	2	1	0	0	1	0	2	3	1-5	2	QQYNNFPYT	9	13	0	2	0	0	1	0	2	1	2	2	2	1	-	ND	ND	+	+	-	+	+
SLE St2 G46	4-34	3-10	1	2	SFRFGSKRWYFDL	13	5	0	0	1	1	0	0	1	0	1	1	1-5	2	QQYNSFPYT	9	5	0	0	0	0	0	0	0	0	0	1	3	1	+	ND	ND	-	-	-	-	-
SLE St2 G47#	4-34	2-2	3	2	GGIVVIPTALRRWYFDL	17	2	0	0	0	0	0	0	0	0	2	0	1-5	2	QQYNTFPYT	9	6	0	1	1	0	2	0	0	0	0	0	2	0								
								FWR1		CDR1		FWR2		CDR2		FWR3						FWR1		CDR1		FWR2		CDR2		FWR3		CDR3							
	VH	D	RF	JH	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vλ	Jλ	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Staining	Flagellin	E.coli	S.aureus	E.faecali	E.cloaca
SLE St2 G06#	4-34	2-2	3	2	GGVAVVPAALRRYYFDL	17	10	1	0	1	0	0	1	2	0	4	1	2-8	2	SSYASSNNLV	10	4	3	1	0	0	0	0	0	0	0	0	0	0								
SLE St2 G10	4-34	/	/	2	LYFYATGIYGSPLL	14	0	0	0	0	0	0	0	0	0	0	0	2-23	1	CSYADSSVYV	10	13	2	0	1	1	1	0	3	0	3	1	1	0	+	ND	ND	+	+	+	+	+
SLE St2 G31#	4-34	2-2	3	2	GGVAVVPAALRRYYFDL	17	9	0	0	0	0	0	0	3	1	4	1	2-14	3	SSYTSSSFWV	10	2	0	0	0	0	1	0	1	0	0	0	0	0								
								FWR1		CDR1		FWR2		CDR2		FWR3																							
	VH	D	RF	JH	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S																								
SLE St2 G37	4-34	2-2	3	2	GGVVVLPAAIRRWYFDL	17	4	0	0	0	0	0	0	0	0	2	2																								
SLE St2 G40	4-34	6-19	3	6	DRIAVAGTHYGMDV	14	1	0	0	0	0	0	0	0	0	0	1																								
SLE St2 G43	4-34	2-2	3	2	GGIVVIPTALRRWYFDL	17	2	0	0	0	0	0	0	0	0	2	0																								

RF, reading frame: # antibody failed to be expressed- non reactive; +, reactive

Table 4.11: Antibody repertoire and reactivity of SLE62 9G4+IgG+ memory B lymphocytes

Ig	HEAVY															LIGHT														REACTIVITY							
								FWR1		CDR1		FWR2		CDR2		FWR3																					
	VH	D	RF	JH	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vk	Jk	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Staining	Flagellin	E.coli	S.aureus	E.faecal	E.cloaca
SLE62 G1	4-34	1-26	2	4	QSGIHGGNSLI	11	11	0	0	0	1	0	0	5	1	3	1	2-28	4	MQALQTPLT	9	3	0	0	1	0	0	0	0	0	2	0	0	+	ND	ND	+	-	-	+	+
SLE62 G3	4-34	3-22	2	4	ERGGEYYDSSGLDY	14	0	0	0	0	0	0	0	0	0	0	0	2-28	2	MQALQTPYT	9	1	0	0	0	0	0	0	0	0	0	0	1	-	ND	ND	-	-	-	-	-
SLE62 G6	4-34	2-15	3	4	GHRVVMVTANFDY	11	24	1	3	3	1	0	2	6	3	1	4	1-27	1	QKYNSAPWT	9	13	0	0	2	2	1	0	1	1	3	3	0	0	-	ND	ND	-	-	-	-	-
SLE62 G7	4-34	2-2	2	4	GPYCSSTSCHMFRSDY	16	14	1	0	1	0	1	2	2	1	4	2	3-20	1	QQYRNSPRT	9	8	0	0	1	0	0	0	2	0	2	1	2	0	+	ND	ND	+	+	+	+	+
SLE62 G8	4-34	/	/	4	DPGIPHYFDY	11	5	2	1	0	0	1	0	1	0	0	0	3-20	4	QQYGSSPLT	9	2	0	0	1	0	0	0	0	0	1	0	0	0	+	ND	ND	-	-	-	-	-
SLE62 G13	4-34	/	/	3	GGQGDAFDI	9	9	0	0	3	0	0	2	0	1	2	1	1-5	1	QQYNAYSWT	11	4	0	0	0	0	1	0	1	0	0	0	2	0	+	ND	ND	+	+	+	+	+
								FWR1		CDR1		FWR2		CDR2		FWR3																					
	VH	D	RF	JH	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vλ	Jλ	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Staining	Flagellin	E.coli	S.aureus	E.faecal	E.cloaca
SLE62 G9	4-34	5-5	3	5	GVDPAMAKFDL	11	19	1	0	0	0	0	2	5	3	5	3	1-44	2	AAWDDYLNAL	10	20	2	1	5	2	1	0	2	1	2	1	2	1	+	ND	ND	+	+	+	-	-
SLE62 G18	4-34	3-3	1	4	RFSEWFAFDC	10	20	2	1	1	0	0	2	5	0	5	4	1-40	3	QSYGSSLSLYVV	12	9	0	1	2	0	1	0	1	0	2	0	1	1	+	ND	ND	-	-	-	+	+
SLE62 G36	4-34	3-22	3	4	KVVGIDYFEY	10	17	3	0	3	1	0	0	5	0	4	1	1-47	3	AAWDDSLSGRL	11	6	0	0	1	1	0	2	0	0	2	0	0	0	-	ND	ND	-	-	-	-	-
SLE62 G41	4-34	6-19	3	6	GGGHSAYSSTRYYFYMEV	18	17	1	2	1	1	0	2	3	2	2	3	1-33	4	QQYDNLPLT	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	ND	ND	-	-	-	-	-
SLE62 G42	4-34	3-3	2	4	GWDFWRLLDY	10	5	0	1	0	0	1	0	0	0	2	1	3-1	1	QAWDSSTAGVV	11	3	0	0	1	0	2	0	0	0	0	0	0	0	-	ND	ND	-	-	-	-	-

RF, reading frame: # antibody failed to be expressed- non reactive; +, reactive

Table 4.12: Antibody repertoire and reactivity of IgG+ memory B lymphocytes for the HD VH4 non-VH4-34 group

Ig	HEAVY							FWR1		CDR1		FWR2		CDR2		FWR3		LIGHT				FWR1		CDR1		FWR2		CDR2		FWR3		CDR3		REACTIVITY		
	VH	D	RF	JH	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vκ	Jκ	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Staining
HD10 G22	4-61	2-15	2	4	DSVDCSGGNCYRRDDF	16	9	1	0	0	2	0	0	3	0	2	1	1-39	1	QQSYSTPQT	9	11	1	0	2	0	3	1	2	1	0	1	0	0	-	-	-
HD10 G131	4-59	3-16	2	5	LHYDYDGPEFDP	12	24	5	0	3	1	5	1	2	3	1	3	3-20	1	QQHGRSPRT	9	8	0	1	4	0	0	0	1	0	0	0	2	0	-	-	-
								FWR1		CDR1		FWR2		CDR2		FWR3							FWR1		CDR1		FWR2		CDR2		FWR3		CDR3				
	VH	D	RF	JH	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vλ	Jλ	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Staining
HD10 G162	4-38	3-3	2	4	DHDFWGGIFDY	11	18	2	1	2	0	3	0	5	0	4	1	2-8	3	SSYADSNSVV	10	8	0	0	2	0	1	1	1	0	2	0	1	0	-	-	-

Ig	HEAVY							FWR1		CDR1		FWR2		CDR2		FWR3		LIGHT				FWR1		CDR1		FWR2		CDR2		FWR3		CDR3		REACTIVITY		
	VH	D	RF	JH	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vκ	Jκ	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Staining
HD11 G115	4-39	3-10	3	5	MVRGVIARWFDP	12	16	2	1	4	0	1	2	3	0	2	1	3-20	5	QQYGGSSSIT	10	15	2	1	5	0	0	0	2	1	0	0	2	2	-	-	-
								FWR1		CDR1		FWR2		CDR2		FWR3							FWR1		CDR1		FWR2		CDR2		FWR3		CDR3				
	VH	D	RF	JH	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vλ	Jλ	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Staining
HD11 G103	4-39	3-22	1	6	DRWLLLPYYHYTMDV	15	12	0	0	1	1	1	0	1	2	4	2	2-8	1	SSYAGSNNYV	10	3	0	0	0	1	0	0	1	0	1	0	0	0	+	+	-

Ig	HEAVY							FWR1		CDR1		FWR2		CDR2		FWR3		LIGHT				FWR1		CDR1		FWR2		CDR2		FWR3		CDR3		REACTIVITY		
	VH	D	RF	JH	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vκ	Jκ	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Staining
HD29 G256	4-31	2-2	3	6	GGLPAASSRYYYYAMDV	17	36	7	2	7	3	1	1	0	3	8	4	3-20	3	QQYDTSPPLFT	11	24	1	3	4	0	1	2	2	0	2	4	3	2	-	+	-
HD29 G293	4-39	3-10	1	4	GVRLWYAVQ	9	22	1	1	3	3	1	2	7	0	2	2	2-28	2	MQALQTPQT	9	10	0	1	3	1	0	1	0	0	2	0	1	1	+	+	-
								FWR1		CDR1		FWR2		CDR2		FWR3							FWR1		CDR1		FWR2		CDR2		FWR3		CDR3				
	VH	D	RF	JH	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vλ	Jλ	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Staining
HD29 G09	4-31	/	/	4	ETWPDTSYFDY	11	29	7	2	2	1	3	0	4	1	1	8	3-21	2	QVWASRGDCPVV	12	29	5	1	4	1	1	0	3	0	2	7	5	0	-	-	-

Ig	HEAVY							FWR1		CDR1		FWR2		CDR2		FWR3		LIGHT				FWR1		CDR1		FWR2		CDR2		FWR3		CDR3		REACTIVITY		
	VH	D	RF	JH	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vκ	Jκ	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	poly	HEp-2	Staining
HD15 G19	4-b	3-22	2	6	MWEKHGFYYYGMDV	14	36	3	2	5	1	2	2	5	6	5	5	1-39	1	QQSYSTPWT	9	27	1	1	10	0	2	1	2	0	3	7	0	0	-	+	-
HD15 G31	4-39	6-6	3	2	RAARRRGYWYFDL	13	30	1	0	4	0	2	1	2	4	11	5	1-39	2	QQSHSRPYI	9	24	0	1	4	1	2	3	3	1	3	2	3	1	+	+	c
HD15 G83	4-61	3-3	3	3	THSFAGHPILSAFDL	15	29	0	0	4	0	4	2	5	3	8	3	3-20	3	QQYGGSPTYT	10	16	3	0	5	1	1	1	0	0	2	1	2	0	-	-	-

Table 4.12: Antibody repertoire and reactivity of IgG+ memory B lymphocytes for the HD VH4 non-VH4-34 group (continued)

Ig	HEAVY																LIGHT														REACTIVITY		
								FWR1		CDR1		FWR2		CDR2		FWR3						FWR1		CDR1		FWR2		CDR2		FWR3		CDR3				
	VH	D	RF	JH	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vκ	Jκ	CDR3(aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	poly	HEp-2	Staining
HDP7 G02	4-31	3-9	2	5	KGEERDILTGEYSSNWFDP	19	14	1	0	0	0	0	0	5	3	3	2	4-1	2	HQYYSLPDT	9	10	2	0	1	1	1	1	1	0	0	0	3	0	-	-	-
HDP7 G05	4-4	3-10	3	3	VTVVSARVIVESGASAFDI	19	14	3	0	2	0	0	0	1	1	5	2	3-15	3	HSGT	4	15	3	2	1	1	1	1	0	0	5	1	0	0	-	-	c
HDP7 G124	4-31	2-21	2	3	ACGDCLWNAAFDI	13	17	4	0	2	1	0	0	2	3	2	3	3-11	1	QQRSNWWT	8	4	0	0	2	0	1	0	0	0	1	0	0	0	-	-	-

Ig	HEAVY																LIGHT														REACTIVITY		
								FWR1		CDR1		FWR2		CDR2		FWR3						FWR1		CDR1		FWR2		CDR2		FWR3		CDR3				
	VH	D	RF	JH	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vκ	Jκ	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Staining
HD09 G26	4-59	2-21	3	4	GRYDDLLTASYNGGYYYFDS	20	30	3	0	1	0	1	3	6	0	8	8	1-8	2	QQYFTYPS	8	9	3	0	1	0	0	0	0	0	1	0	2	2	-	-	-

Ig	HEAVY																LIGHT														REACTIVITY		
								FWR1		CDR1		FWR2		CDR2		FWR3						FWR1		CDR1		FWR2		CDR2		FWR3		CDR3				
	VH	D	RF	JH	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vκ	Jκ	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	poly	HEp-2	Staining
HD12 G162	4-39	5-5	2	5	HRAGYKYEEGFDP	13	20	1	0	2	0	2	1	3	1	6	4	1-33	4	QQYDNLFAVN	10	27	0	1	4	2	0	6	2	1	4	1	4	2	-	-	A

Ig	HEAVY																LIGHT														REACTIVITY		
								FWR1		CDR1		FWR2		CDR2		FWR3						FWR1		CDR1		FWR2		CDR2		FWR3		CDR3				
	VH	D	RF	JH	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	Vκ	Jκ	CDR3 (aa)	Length	Mutations	R	S	R	S	R	S	R	S	R	S	R	S	Poly	HEp-2	Staining
HDbuf2 G46	4-31	3-3	2	5	MGYYDLPWFDP	11	23	3	1	4	1	1	1	3	0	4	5	3-11	4	QQRSNWIT	8	5	0	0	1	0	0	1	1	0	2	0	0	0	+	-	c

RF, reading frame

- non reactive; +, reactive

A, actin staining

C, diffuse cytoplasmic staining

4.6. The expansion of gut bacteria-reactive 9G4+IgG+ B lymphocytes has been observed in SLE patients

We examined the clonality for identification of clonal selection in order to determine if there is any fecal gut bacteria-reactive 9G4+IgG+ memory B cell clone that is specifically expended in SLE patients. The sequences of each clone were examined to see whether they exhibited shared rearrangements of diversity heavy-chain (D) and joining heavy-chain (J) gene segments, as well as similarities in the CDR3. Particularly, the VH4-34 region genes were analyzed and compared to the germline VH4-34*01 sequence to determine the presence and arrangement of somatic mutations in order to investigate the impact of antigen-mediated clonal selection. We found that a clonal expansion of 9G4+ memory B cells characterized by a non-mutant AVY motif was observed in SLESt2 patient. SLESt2 9G4+ VH4-34 clones pertaining to VH4-34/D2-2/RF3/JH2 and CDR3 length of 17 amino acid arrangement are overrepresented. This disproportionate representation in the VH4-34 B cell compartment has not been described to date. In this study, we found that one of three SLE patients, whom we sorted with the 9G4 strategy and tested for fecal bacteria staining, displayed a clonal expansion in their VH4-34 memory B cell compartment. In fact, we have observed not only the circulating 9G4+ (VH4-34) memory B cell clonal expansion evidence in SLE patients but also established a connection between this expansion and the gut microbiota. This finding may be attributed to the preferred rearrangement of the VH4-34 gene.

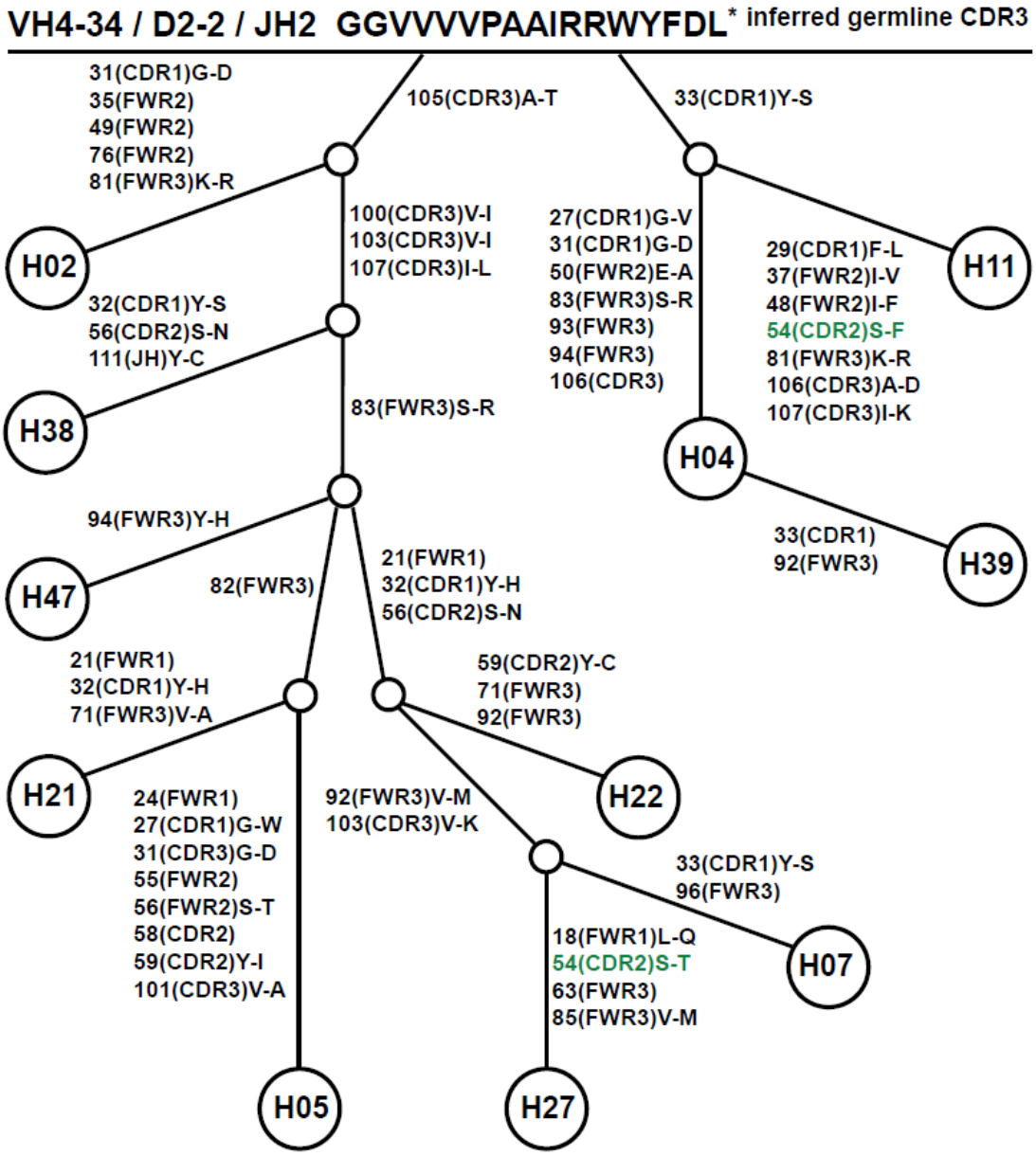


Figure 4.8: A clonal tree of the SLESt2 patient's 9G4 B cells with respect to clonal expansion. The clonal tree of the clonal expansion detected in the 9G4-sorted SLESt2 patient is depicted. Mutations and their codon positions are indicated on the branch sides, with the affected regions enclosed in parentheses. The presence of alterations in the amino acid composition is denoted.

4.7. Reversion of HD VH4-34+IgG+ memory B cells with mutated AVY motifs to their germline unmutated AVY form

After testing the VH4-34-encoded IgG antibodies directly cloned from IgG+ memory B cells from SLE and HD to avoid the bias from sorting B cells with the 9G4 anti-VH4-34, we found that indeed, most of the HD clones had the mutated AVY motif and no longer bound commensal gut bacteria. Consequently, we wanted to check if this commensal bacteria reactivity ability of VH4-34-expressing IgG antibodies comes from the germline unmutated VH4-34 AVY motif. Therefore, we reverted 8 HD VH4-34^{+} CD27^{+}IgG^{+} memory B cells bearing mutated AVY motifs into germline AVY form and tested those possessing an unmutated VH4-34 AVY motif with fecal bacteria staining for our flow analysis.

The VH4-34 sequences with unmutated AVY motifs seem to be required for engagement with the gut bacteria, which can lead to the expansion of the autoreactive 9G4 B cell subset and subsequently cause the deterioration of the SLE patient's condition.

Additionally, SLE may lead to the development of anti-gut bacteria-reactive VH4-34+IgG+ memory B lymphocytes with unmutated AVY motifs, which could point to the presence of systemic immune responses targeting the gut microbiota that are unable to be contained in individuals with SLE.(204)

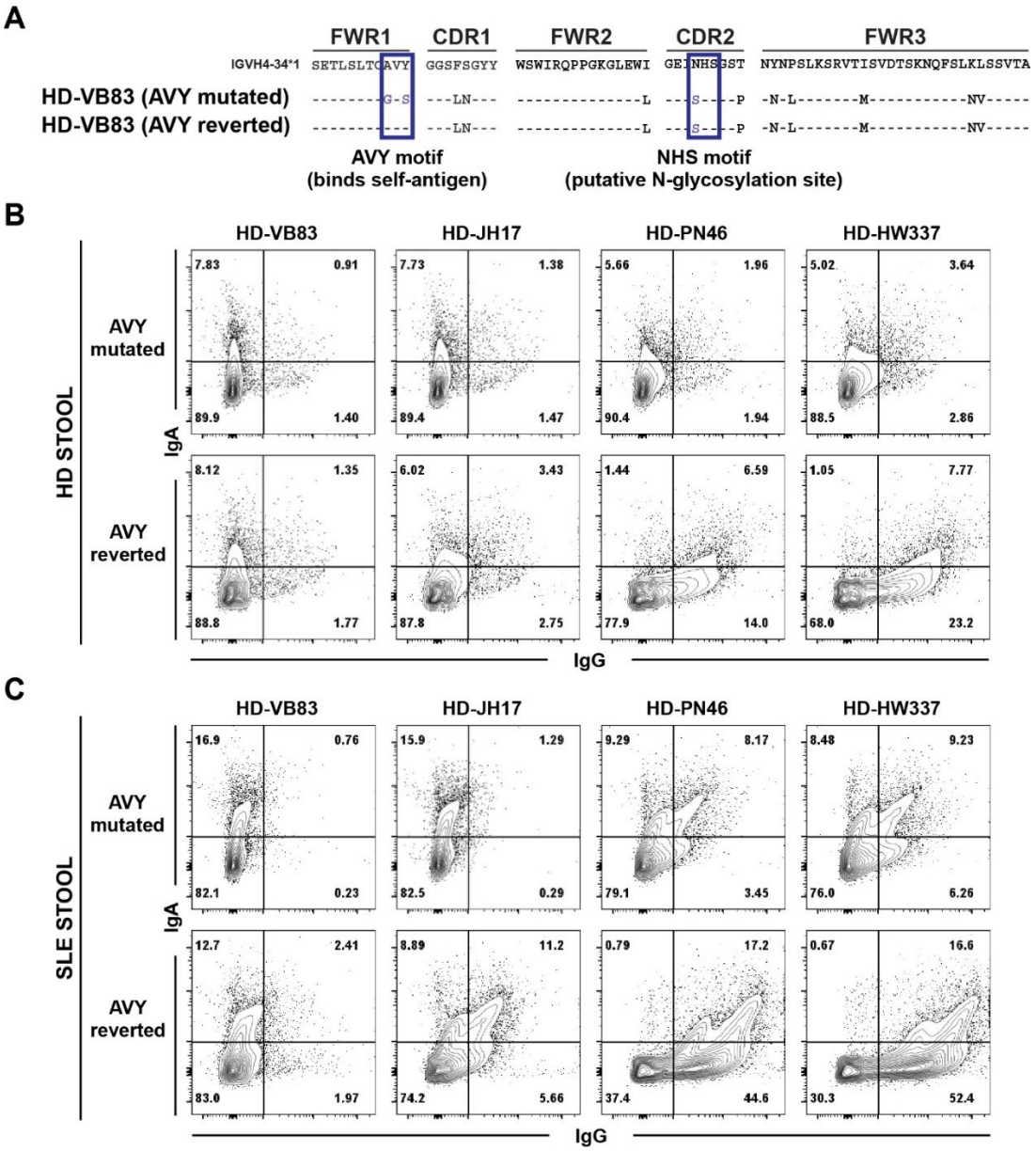


Figure 4.9: The presence of VH4-34 sequences containing unmutated AVY motifs can be an essential factor in an interaction with gut bacteria.

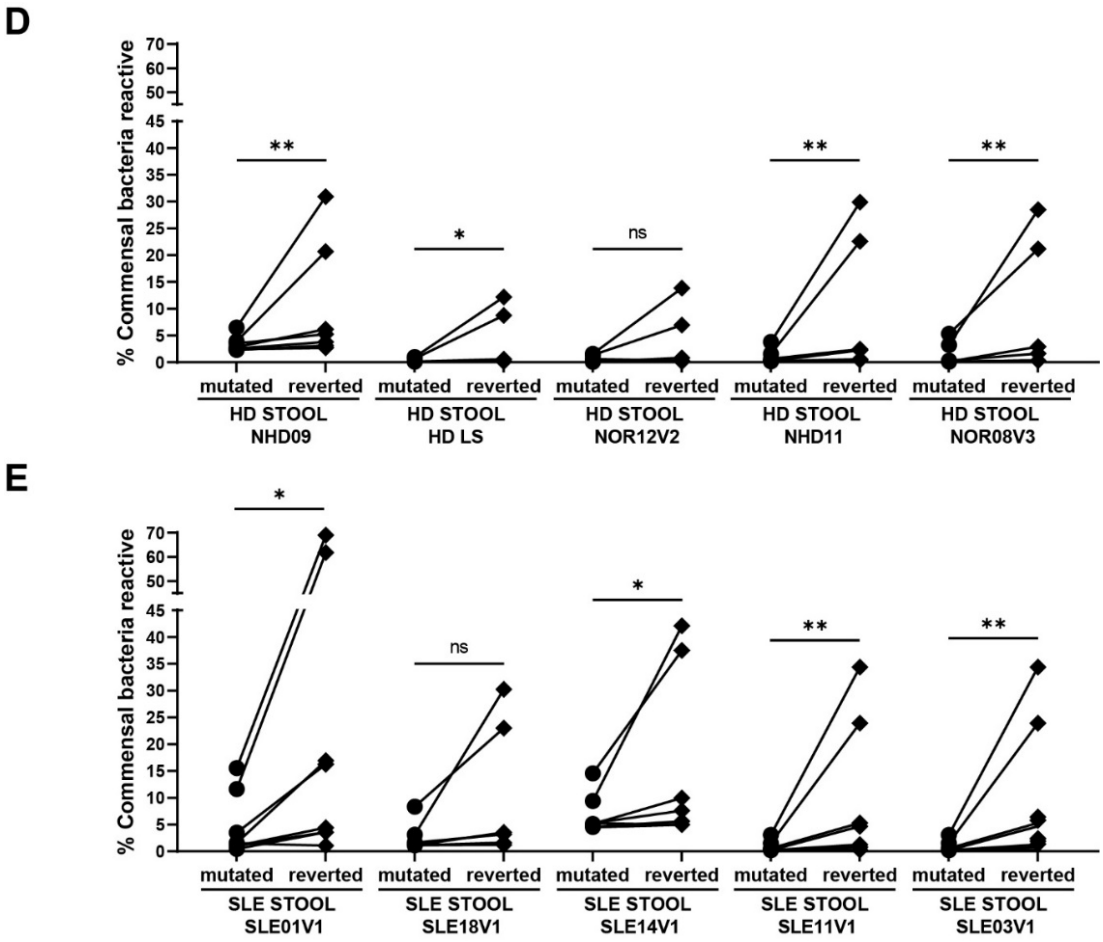


Figure 4.9: The presence of VH4-34 sequences containing unmutated AVY motifs can be an essential factor in an interaction with gut bacteria (continued). In figure **A,** the amino acid sequence alignment of VH4-34 for IgG+ memory B cell derived from HD VB83 harboring mutated AVY and NHS motifs is shown. Under the sequence of the HD VB83 clone, a reverted AVY motif expressing HD VB83 clone is also presented. The amino acid sequence of the germline IGHV4-34*01 is shown in the uppermost section. The identification of germline identity is shown by a dash, whereas replaced residues are represented in black. The germline AVY and NHS sequences in FWR1 and CDR2 are shown in blue. Mutations occurring in clones that are mutated for both AVY and NHS are depicted in purple. Representative flow cytometry plots of IgG versus IgA on bacteria that were tested for the gut bacteria binding of VH4-34 IgG+ B cell clones and their corresponding AVY unmutated versions are depicted in Figures **B** and **C**. Flow cytometry plots in Figure **B** show the binding of VH4-34 antibodies on a healthy donor stool, whereas Figure **C** shows the binding of indicated antibodies on a SLE stool. The percentages of gut bacteria reactivity for 8 HD VH4-34^{+} CD27^{+}IgG^{+} memory B cells with mutated AVY motifs (represented in circles) and their corresponding AVY reverted versions (denoted as diamonds) are shown for HD stools in figure **C** and for SLE stools in figure **D**.

In Table 4.13, each total IgG+ percentage value for all tested IgG+ antibody reactivity against fecal gut bacteria is shown.

Table 4.13: Total IgG+ Percentage for Each Stool Sample in Flow Analysis

		STOOL SAMPLES									
Type of Rabs	Name of Rabs	NHD09	SLE01V1	NOR12v2	SLE14V1	HDLS	SLE18V1	NOR08V3	SLE03V1	NHD11	SLE11V1
9G4+rabs	HD10 K14	5.85	29.7	12.09	34.6	8.79	15.86	5.43	7.47	3.27	4.68
	HD10 K24	18.23	53.1	19.9	54.5	18.91	31.19	12.45	23.50	10.17	25.98
	HD10 L13	36.8	60.6	28.32	78.7	37.3	48.3	28.06	54.20	32	38.01
	HD10 L15	2.51	2.57	0.32	26.31	2.3	2.44	2.38	3.78	0.37	0.72
	HD10 L3	2.51	2.48	1.82	12.17	1.95	2	0.34	4.30	0.168	0.59
	HD10 L5	4.06	6.58	2.87	12.68	2.03	4.64	0.67	5.30	0.63	2.46
	HD IV01 L50	5.44	9.73	4.81	17.71	2.64	12.47	1.74	3.80	1.53	1.72
	HD IV01 L54	18.9	37.87	12.77	45.8	9.08	26.21	13.88	22.52	19.16	20.79
	HDSt1 K23	2.3	8.16	4.39	20.38	2.19	4.94	0.36	8.54	0.533	1.94
	HDSt1 K48	15.35	64.9	22.31	49.9	24.28	37.14	22	16.17	13.64	29.63
	HDSt1 L40	2.67	1.66	1.8	6.22	2.48	2.8	0.67	3.66	0.66	3.38
	HDSt1 L7	6.47	26.1	8.72	34.3	6.12	9.08	7.88	5.47	8.9	8.11
	HDSt2 K51	4.15	7.25	3.25	17.41	3.84	5.71	1.49	6.35	1.59	4.24
	HDSt2 K66	2.62	7.78	3.38	21.19	2.39	6.88	0.62	7.83	1.19	2.64
	HDSt2 K72	29.71	58.85	28.02	69.6	36.5	45.4	29.3	34.8	35	38.77
	SLESt1 K19	2.6	9.24	1.23	19.72	1.93	4.75	0.61	2.12	0.835	3.29
	SLESt1 K29	6.05	17.03	11.21	36.1	4.65	8.81	3.39	7.21	1.94	4.68
	SLESt1 K32	2.79	9.23	3.65	17.21	2.01	3.23	0.55	2.68	0.65	1.68
	SLESt1 K37	16.83	42.17	17.86	54.4	6.04	27.99	17.4	14.09	15.31	22.23
	SLESt1 K41	2.47	3.18	1.1	16.33	1.83	1.83	0.233	1.37	0.361	1.05
	SLESt1 K42	2.82	3.27	3.33	22.34	1.94	2.49	0.51	2.15	0.59	1.92
	SLESt1 L1	3.29	8.19	4.54	18.19	2.45	5.85	1.14	4.11	1.28	3.68
	SLESt1 L27	1.76	6.9	10.95	27.48	1.92	6.08	0.59	2.13	0.71	2.37
	SLESt1 L3	2.06	0.94	0.63	10.13	1.77	1.86	0.18	1.03	0.321	0.87
	SLESt2 K11	8.51	43.5	19.53	44.9	9.87	18.84	8.09	23.00	5.04	7.59
	SLESt2 K17	4.97	5.2	5.72	19.99	3.66	3.55	1.57	3.24	1.72	3.41
	SLESt2 K19	2.89	8.69	4.35	21.2	2.32	6.13	0.55	3.42	0.799	2.5
	SLESt2 K2	4.87	64.7	16.97	52.9	6.74	29.98	4.99	28.60	3.42	4.58
	SLESt2 K21	31.61	43.96	23.58	58.4	18.53	41.7	15.87	48.10	18.1	16.99
	SLESt2 K22	19.89	46.9	25.57	67.2	26.3	27.15	19.81	14.15	12.66	18.2
	SLESt2 K25	2.95	10.68	0.71	19.16	2.1	6.98	0.53	2.50	1.2	1.82
	SLESt2 K27	7.55	28	15.91	45.6	11.06	25	6.36	23.19	6.32	8.7
	SLESt2 K28	2.73	4.92	2.09	12.99	2.35	3.09	0.29	1.72	0.55	1.44
	SLESt2 K29	4.76	11.96	6.14	35	3.66	8.62	2.43	13.77	1.35	7
	SLESt2 K30	3.26	3.72	0.46	8.6	2.04	3.18	0.32	4.30	0.57	1.75
	SLESt2 K38	15.64	44.5	19.64	65.6	14.21	21.52	6.56	33.20	10.17	8.75
	SLESt2 K39	13.58	32.09	16.52	64.7	8.29	19.01	5.02	37.30	7.58	9.89
	SLESt2 K4	7.64	61.8	24.24	60.5	8.05	33.03	4.49	32.60	5.09	8.58
	SLESt2 K46	18.35	52.8	18.09	64.4	18.96	31.42	17.05	33.00	15.24	17.95
	SLESt2 K47	16.74	55	23.35	68.8	18.2	33.86	11.38	14.14	11.01	15.6
	SLESt2 K5	11.16	55.7	27.42	48.3	16.14	25.34	12.74	28.90	8.15	10.55
	SLESt2 K7	11.91	38.4	12.57	60.1	11.64	26.48	10.33	10.97	12.75	11.12
	SLESt2 L10	11.19	45.26	16.77	52	8.68	20.62	5.84	22.82	4.96	9.4
	SLE62 K1	2.52	2.39	0.9	9.32	1.91	3.01	0.27	0.87	0.58	1.168
	SLE62 K13	2.13	21.4	0.39	10.61	2.02	1.71	0.155	1.68	0.302	1.22
	SLE62 K3	2.14	2.47	1.46	14.88	1.42	2.67	0.173	1.17	0.295	1.34
	SLE62 K7	5.84	22.22	3.25	23.42	6.8	12.27	5.65	5.76	3.43	3.71
	SLE62 L36	18.31	38.3	16.75	55.6	17.02	20.2	14.2	12.79	12.57	19.84
	SLE62 L42	5.7	17.84	6.71	27.56	3.64	8.8	1.62	4.34	2.6	4.42
	SLE62 L9	2.79	7.57	1.39	16.2	1.77	2.96	0.262	2.47	0.574	2.72

Table 4.13: Total IgG+ Percentage for Each Stool Sample in Flow Analysis (continued)

		STOOL SAMPLES									
Type of Rabs	Name of Rabs	NHD09	SLE01V1	NOR12v2	SLE14V1	HDLS	SLE18V1	NOR08V3	SLE03V1	NHD11	SLE11V1
VH4-34 rabs	HD29 K49	3.27	2.76	0.3	5.21	2.07	1.18	0.59	4.77	0.51	0.491
	HD29 K54	2.34	0.85	0.38	5.74	1.85	1.23	0.31	0.78	0.18	0.276
	HD29 K91	2.48	1.94	0.172	5.75	1.45	1.05	0.209	0.82	0.117	0.199
	HD HW 337	6.5	15.52	1.565	9.39	0.966	3.11	3.25	3.16	3.82	3.05
	HD JH 17	2.85	1.57	0.098	5.12	0.126	1.13	0.121	0.7	0.216	0.18
	HD PN 116	2.42	1.04	0.113	5.25	0.134	1.29	0.109	0.52	0.195	0.147
	HD PN 148	2.55	1.5	0.089	4.55	0.11	1.24	0.12	0.43	0.195	0.095
	HD PN 33	3.52	3.49	0.62	5.24	0.135	1.62	0.292	1.11	0.65	0.54
	HD PN 46	3.9	11.63	1.35	14.55	0.706	8.33	5.32	4.4	1.56	1.5
	HD PN 57	2.41	0.43	0.075	4.48	0.146	1.13	0.119	0.54	0.229	0.121
	HD VB 83	2.31	1	0.084	4.49	0.094	1.11	0.085	0.53	0.19	0.198
	SLE174 117	7.2	10.87	1.06	20.34	0.372	1.18	0.502	1.37	1.69	1.32
	SLE174 154	3.81	10.05	0.37	6.81	12.51	14.12	0.85	2.88	1.21	2.63
	SLE174 84	3.96	7.58	0.435	6	0.415	4.52	0.59	3.42	1.39	2.44
	SLE175 116	2.45	0.8	0.094	4.38	0.137	1.05	0.108	0.52	0.199	0.141
	SLE175 80	4.21	7	0.511	7.87	0.568	3.83	0.601	2.32	0.87	1.11
	SLE175 95	34.95	63.9	16.88	46.7	34.2	39.5	27.7	22.4	25.9	32.54
	SLE176 270	8.88	15.77	0.466	10.65	2.95	8.07	3.39	8.01	4.14	6.53
HD VH4 nonVH4-34 rabs	HDbuf2 G46	3.76	2.1	0.79	7	2.67	1.71	1.3	2.23	0.97	1.27
	HD12 G162	2.53	0.9	0.42	5.64	2.25	1.15	0.205	0.87	0.194	0.378
	HD09 G26	2.31	0.52	0.31	4.89	1.8	1.62	0.25	0.82	0.34	0.565
	HD10 G22	3.18	2.23	1.77	14.01	3.89	2.28	0.57	1.52	0.56	1.13
	HD10 G131	2.13	0.77	0.28	5.3	2.05	1.44	0.246	0.93	0.25	0.281
	HD10 G162	1.9	0.89	0.139	5.95	2.13	0.92	0.144	1.39	0.132	0.186
	HD11 K115	1.73	0.42	0.163	5.8	1.53	1.17	0.189	0.56	0.181	0.238
	HD11 G103	2.56	0.97	0.42	3.71	2.33	1.33	0.72	1.38	0.9	1.34
	HD11 L115	1.91	0.39	0.196	6.04	1.55	1.07	0.167	0.73	0.26	0.224
	HD29 G09	2.29	1.34	0.183	10.25	2.74	1.23	0.555	1.85	0.242	0.401
	HD29 G256	2.33	0.55	0.21	4.88	2	1.17	0.23	0.82	0.181	0.186
	HD29 G293	2.45	0.36	0.186	5.45	1.83	1.06	0.174	0.64	0.135	0.19
	HD15 G19	2.22	0.47	0.185	5.58	1.77	1.14	0.187	0.83	0.13	0.141
	HD15 G31	6.09	22.15	1.98	18.03	13.32	9.2	14.68	6.11	5.45	4.75
	HD15 G83	2.77	1.9	0.42	6.36	2.38	1.58	0.45	0.97	0.27	0.538
	HDP7 G124	2.05	0.59	0.44	6.48	2.01	1.25	0.48	1.02	0.35	0.339
	HDP7 G02	3.28	1.33	0.96	11.26	2.91	3.11	1.18	1.60	1.01	1.005
	HDP7 G05	12.03	25.73	6.48	39.6	11.55	10.39	23.75	17.92	11.22	14.77
Reverted VH4-34 rabs	HD HW 337 Reverted	30.93	69	13.85	42.1	12.18	30.25	28.5	24.9	29.8	34.39
	HD JH 17 Reverted	6.17	16.93	0.82	9.96	0.57	3.53	2.93	3.62	2.26	4.66
	HD PN 116 Reverted	2.79	3.55	0.124	4.98	0.12	1.18	0.318	0.89	0.42	0.76
	HD PN 148 Reverted	3.8	1.05	0.104	5.62	0.12	1.17	0.235	0.49	0.299	0.234
	HD PN 33 Reverted	5.24	16.26	0.31	7.6	0.263	3.15	1.63	3.83	2.47	5.29
	HD PN 46 Reverted	20.68	61.8	6.96	37.5	8.77	23	21.19	19.51	22.6	23.92
	HD PN 57 Reverted	2.71	3.57	0.104	5.02	0.154	1.21	0.34	0.53	0.38	0.71
	HD VB 83 Reverted	3.12	4.39	0.118	4.94	0.153	1.61	0.37	0.97	0.59	1.27
	SLE174 117 Reverted	4.5	24.44	0.305	5.7	0.217	1.7	0.97	3.12	2.5	3.8
	SLE175 116 Reverted	3.86	5.6	0.203	5.85	0.24	1.5	0.6	1.53	0.65	1.24

4.8. Identification of bacterial taxa bound to VH4-34-encoded antibodies

While investigating the anti-commensal bacteria reactivity of VH4-34-encoded IgG antibodies in SLE patients, we evaluated the composition of bacterial populations in the feces of healthy controls and SLE patients that exhibit binding to the 9G4+ VH4-34 IgG+ antibodies from both healthy donors and SLE patients. Therefore, our final step involved the sorting of IgG+ fecal gut bacteria, followed by their submission for sequencing to determine which strains in SLE stools are recognized by VH4-34 clones.

The construction of the amplicon sequence variant (ASV) abundance heatmap by ZYMO involved the utilization of the abundance of unique amplicon sequences that are derived from the raw sequencing data. ASV heatmaps with sample clustering for all stool pairs tested based on 9G4+ VH4-34 IgG+ antibody binding are shown below. The application of heatmaps in conjunction with sample clustering serves as an efficient method for discerning patterns of microbial distribution across various samples. Heatmaps illustrate the presence and abundance of up to fifty taxa that are most prevalent at a specific taxonomic level. The abundance for each taxon can be seen in each row, with the taxonomy ID provided on the right. The abundance of each VH4-34 IgG antibody sample is represented by each column, with the sample ID displayed at the bottom. The grouping of information is denoted by the presence of a colored bar positioned at the top of each column. The application of hierarchical clustering was carried out on the samples, employing the Bray-Curtis dissimilarity measure by ZYMO. In addition, a hierarchical clustering analysis was conducted on the taxa in order to group together those taxa that have similar distributions.

With sorting, we divided the bacteria into 9G4 antibody-bound (positive) and 9G4-unbound bacteria (negative) categories. While looking at specific taxonomic groups found in the stool of SLE patients that exhibit a higher degree of binding affinity towards VH4-34 IgG recombinant antibodies, we observed that Intestinibacter;s_bartlettii is the most frequently bound bacteria by 9G4+ antibodies cloned from B cells from healthy donors and SLE patients based on SLE stool analysis. Intestinibacter;s_bartlettii, classified under the Eubacteriales order (Clostridium bartlettii sp. nov., isolated from human faeces, (205, 206)), yielded a higher signal of binding of 9G4+ antibodies in three out of five SLE stool samples, in particular SLE11,

SLE03, and SLE14, referring that patients with SLE have immune responses that are directed against microorganisms present in the gastrointestinal tract. Moreover Christensenellaceae;g_NA;s_sp30434, which also belongs to Eubacteriales (207), is the common bacteria species bound by 9G4+ antibodies on SLE01. On the other hand, healthy donor stool analysis shows that 9G4+ antibodies from healthy donors and SLE patients most often bind to the bacteria Anaerostipes;s_hadrus, which is also categorized under the Eubacteriales order.(208) The fact that IgG+ B lymphocytes with VH4-34 antibodies that react to both self- and anti-gut bacteria were found in SLE patients suggests that their gut microbiota are not being kept in check properly. This discovery provides evidence in favor of our theory that VH4-34-encoded memory B cells are linked to leaky gut, where gut bacteria can travel into blood circulation, thereby leading to the expansion of intrinsically autoreactive 9G4 B cells.

The fecal bacterial populations associated with VH4-34 IgG clones from healthy controls and SLE patients on all stool samples are demonstrated in the following figures.

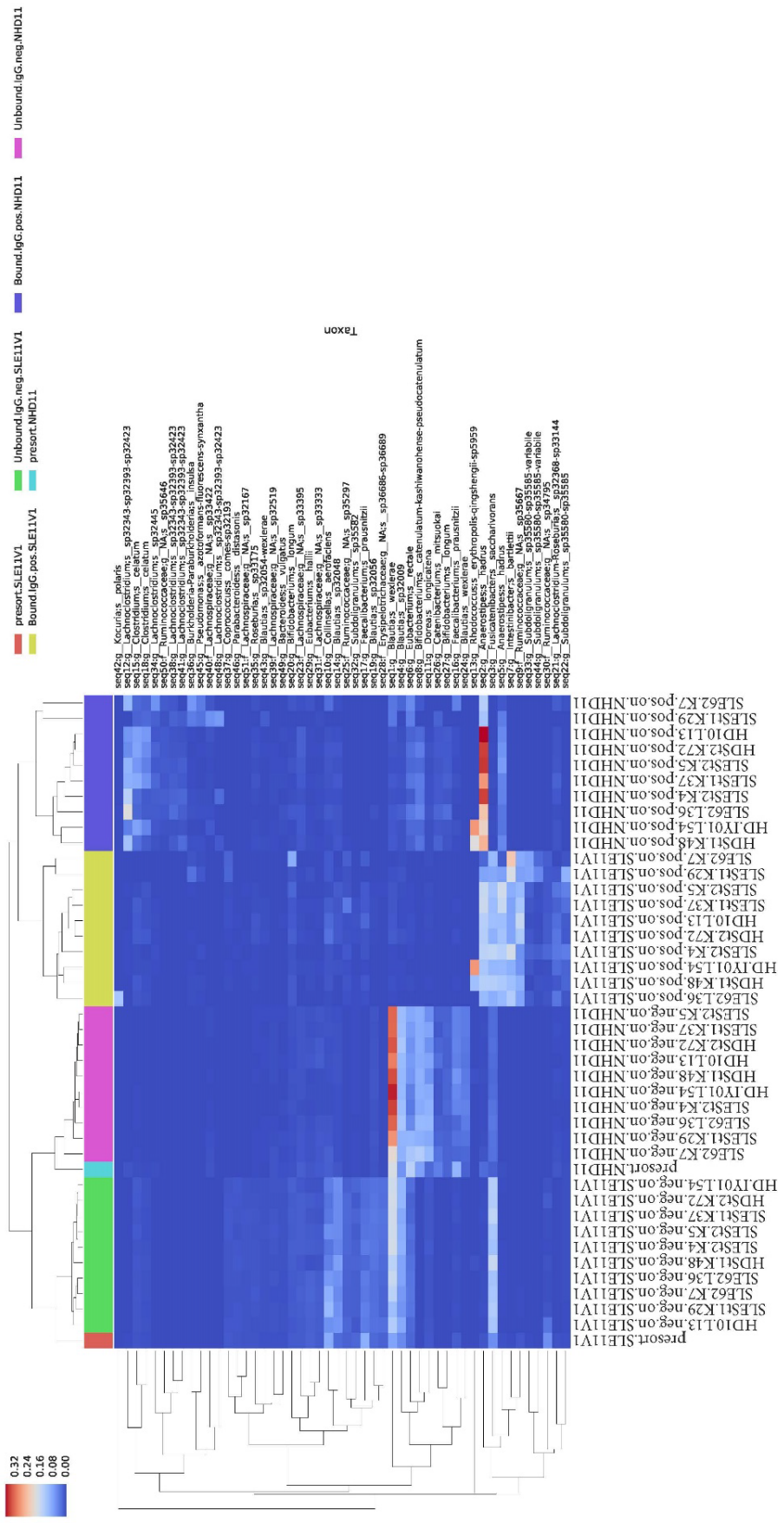
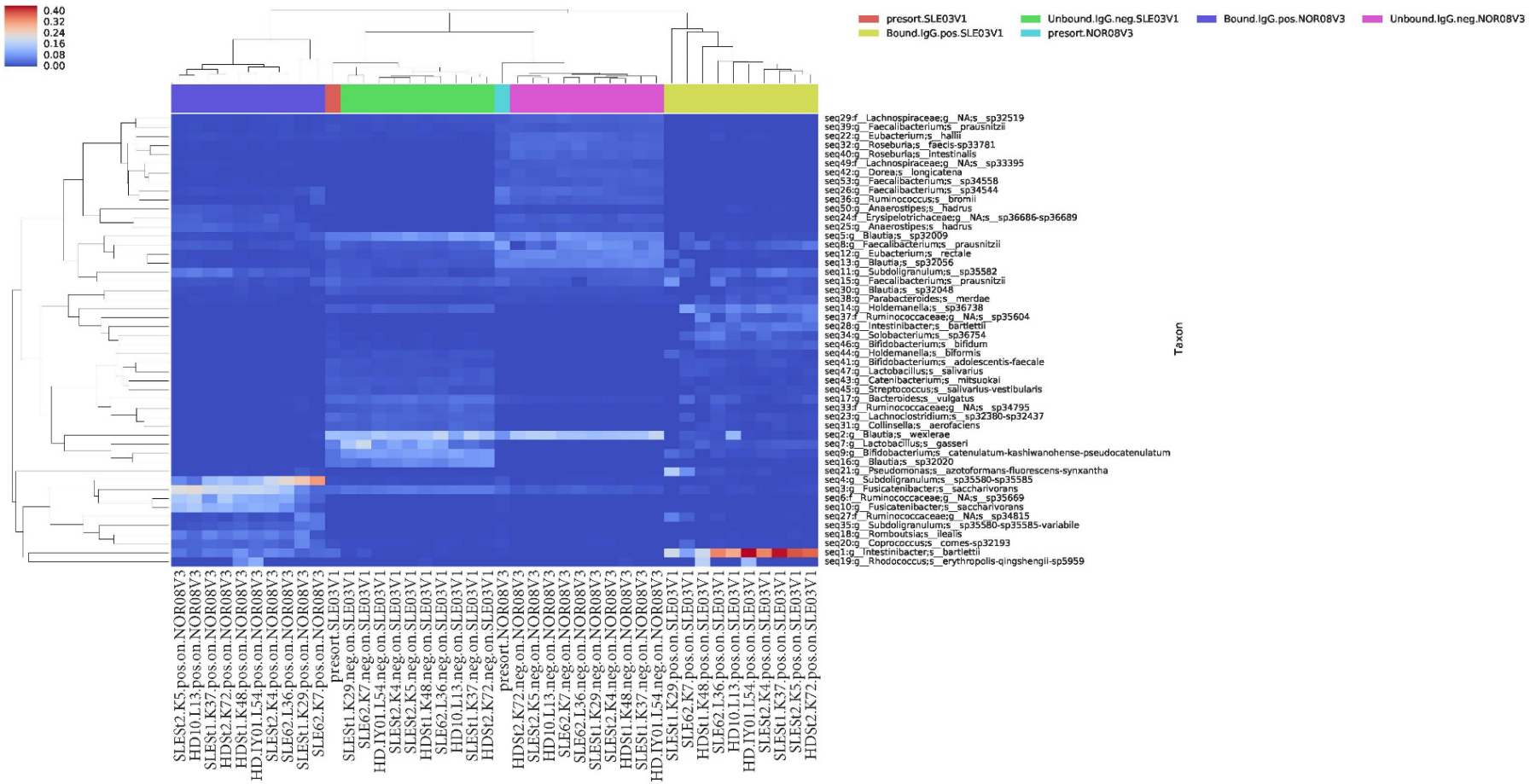


Figure 4.10: The bacterial populations associated with VH4-34 IgG clones for SLE11 and NHD11 stools.

Figure 4.11: The bacterial populations associated with VH4-34 IgG clones for SLE03 and NOR08 stools.



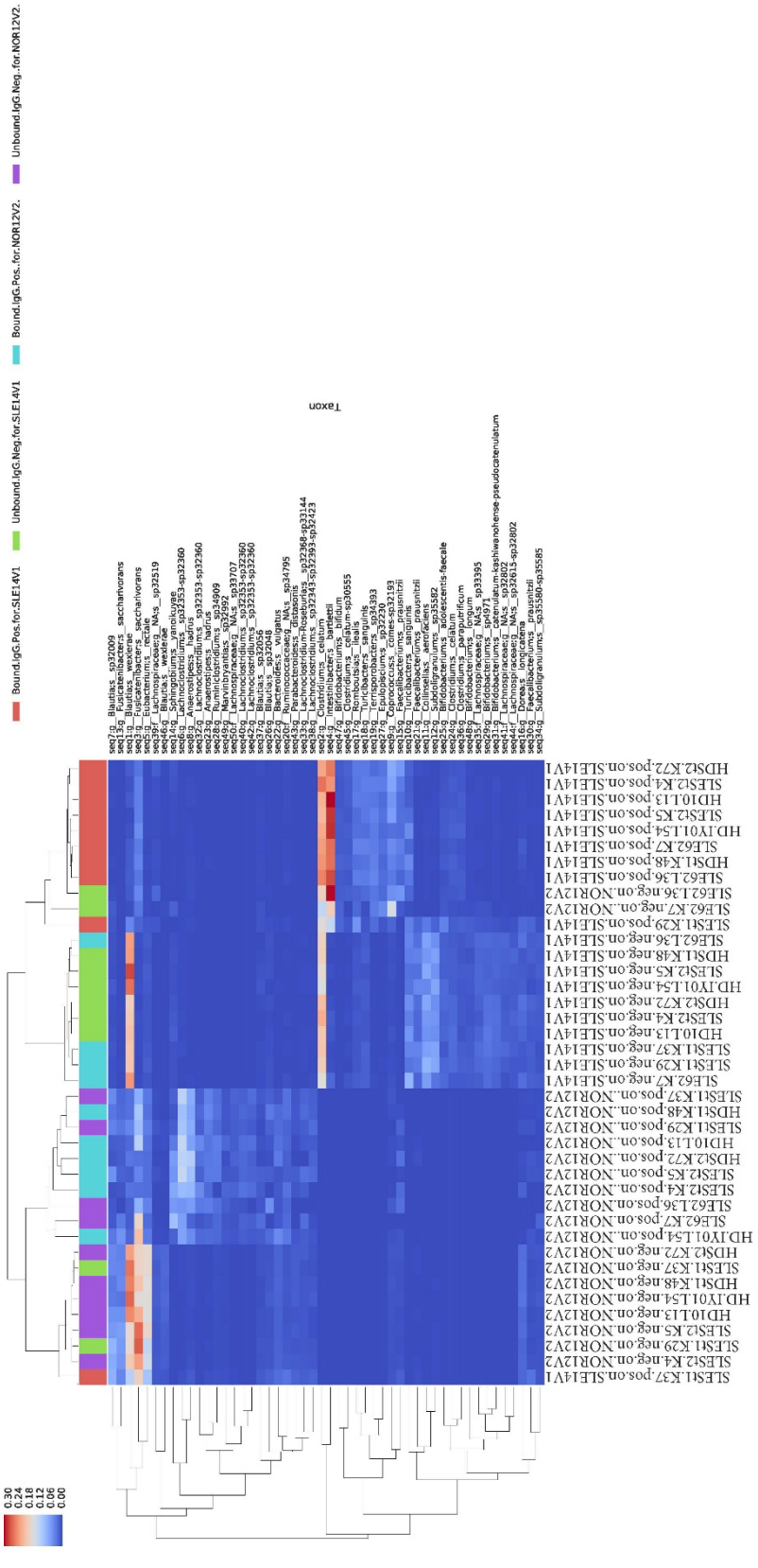
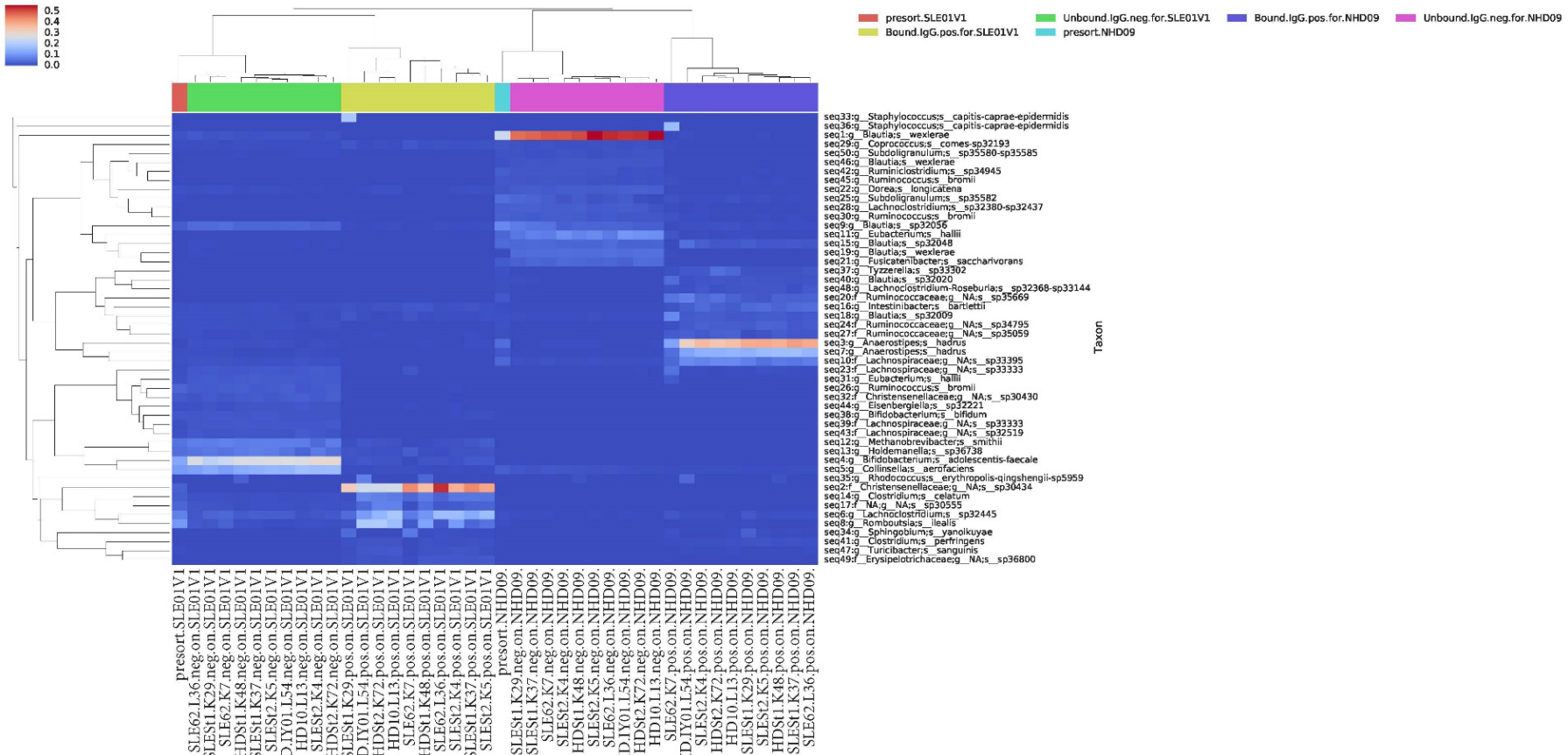


Figure 4.12: The bacterial populations associated with VH4-34 IgG clones for SLE14 and NOR12 stools.

Figure 4.13: The bacterial populations associated with VH4-34 IgG clones for SLE01 and NHD09 stools.



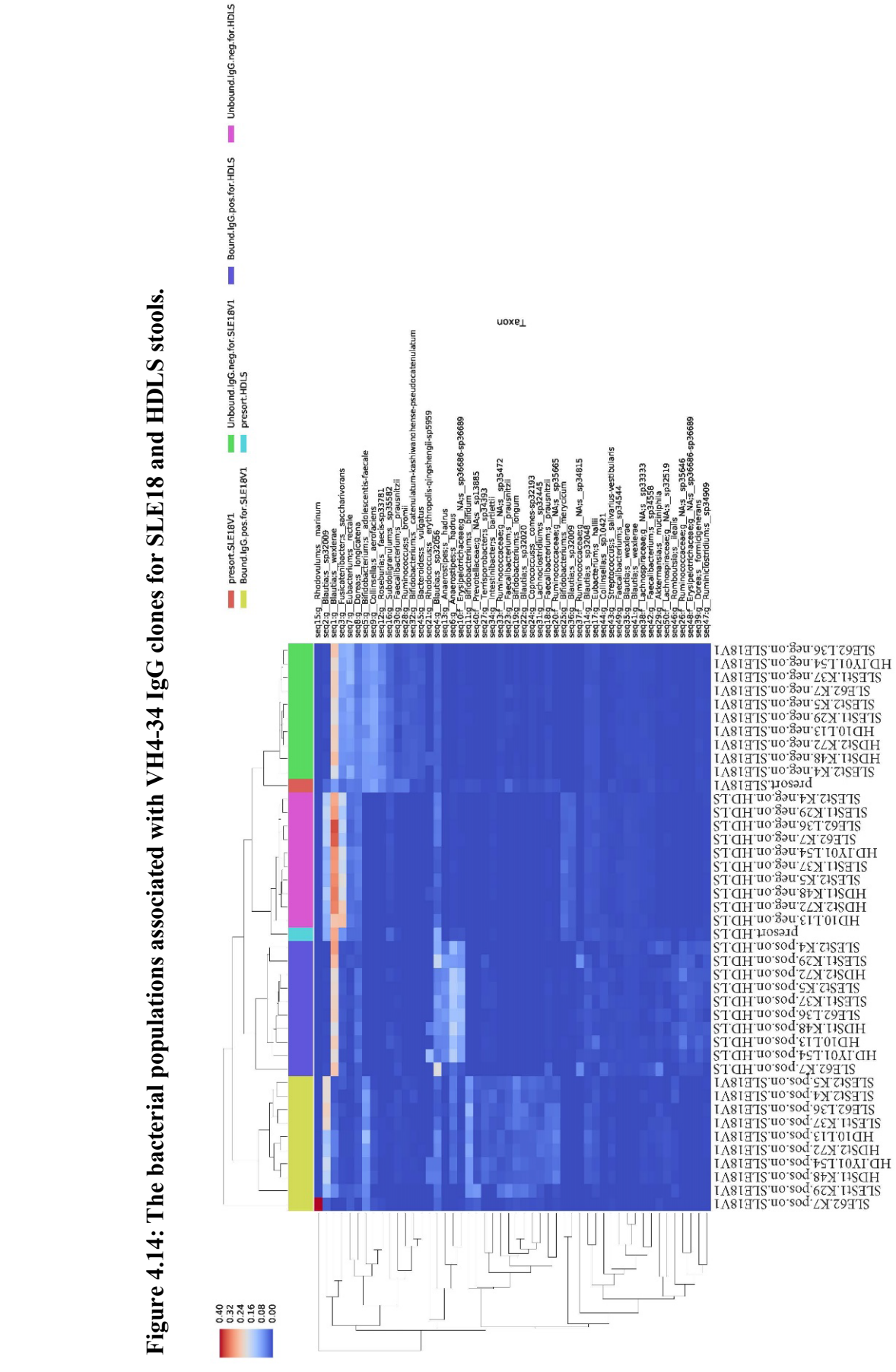


Figure 4.14: The bacterial populations associated with VH4-34 IgG clones for SLE18 and HDLS stools.

5. DISCUSSSION

To date, the association between circulating 9G4 memory B cells isolated from individuals with SLE, and the gut microbiota has not been investigated clearly. In this study, we have identified aberrant VH4-34 gene use, reduced frequencies of somatic hypermutation (SHM), a propensity for reactivity towards commensal microorganisms, and 9G4 clonal expansion that has great reactivity to commensal bacteria, and observed unique species of gut bacteria that bind circulating 9G4 antibodies. Therefore, it is possible that the germline-encoded autoreactive VH4-34 antibodies, which can detect I/i carbohydrates found on RBCs and B lymphocytes, might cross-react with antigens produced by commensal bacteria that enter the bloodstream due to a failure to contain the gut microbiota. The aforementioned concept has been previously shown in the research conducted by Schickel et al. in 2017 (7), specifically in relation to individuals with deficiencies in IRAK4 and MYD88, and our findings align with these earlier observations when SLE patients are included in the study. In SLE, there are anti-gut bacteria-reactive VH4-34+IgG+ memory B lymphocytes, which could point to the presence of systemic immune responses targeting the leaked gut microbiota in SLE patients.(204) The presence of IgG+ B lymphocytes that encode VH4-34 antibodies with unmutated AVY motif and react to both self- and anti-commensal bacteria in individuals with SLE can indicate a compromised ability to control the gut microbiota.

Normally, the V4-34 gene expression in the peripheral blood is selectively repressed; however, in SLE, this censorship is bypassed, and IgG-9G4 antibodies are significantly increased in around 75% of individuals diagnosed with active SLE. In general, there is evidence to support a favorable correlation between serum 9G4 antibodies and both overall disease activity as well as the occurrence of lupus nephritis and central nervous system involvement.(1, 171, 189, 191) Although the direct pathogenic impact of these antibodies in SLE has yet to be demonstrated, there are various pieces of data that support this claim in addition to the high quantity of these antibodies and their relationship with the illness.(1) Firstly, several IgM and IgG anti-DNA antibodies are encoded by the V4-34 gene in SLE patients (195), and 9G4 antibodies could have a role in the immune response to Smith antigens in SLE.(1, 209)

Secondly, it has been shown that 9G4 antibodies have the ability to attach to a significant proportion of naive B lymphocytes through the identification of a specific glycoform of CD45/B220.(171) One may argue that this characteristic has the potential to play a role in the development of naïve lymphopenia often seen in active SLE, as it may lead to the elimination of naive lymphocytes and/or facilitate their transformation into class-switched B lymphocytes.(1, 175, 210) On top of that, the capacity of 9G4 antibodies to establish binding with B220 might potentially explain the observation that these antibodies exhibit recognition of apoptotic cells.(1, 171, 211) Hence, it is reasonable to suggest that the presence of 9G4 antibodies could possibly serve as an important factor in the ability of lupus sera to stimulate an ongoing release of an immunostimulatory cytokine, interferon-alpha (IFN-α), a cytokine that is presently recognized as a key factor in the development of this illness. (1, 212, 213) Furthermore, via their interaction with CD45, 9G4 antibodies have the potential to impact the disruption of B cell tolerance by altering the activation threshold of B lymphocytes.(1, 214, 215) Thirdly, the presence of 9G4 antibodies could possibly be involved a role in the development of neuropsychiatric systemic lupus erythematosus (SLE) by virtue of their capacity to specifically bind to gangliosides, which are very prevalent in neuronal cells.(1, 216) The apparent link between central nervous system manifestation in SLE and anti-lymphocyte antibodies may be explained by the antigenic reactivity mentioned.(1, 217) Finally, we herein disclose our discovery of a pathogenetic connection between gut bacteria present in SLE patients and circulating 9G4 antibodies exhibiting an unmutated AVY motif. The preservation of VH4-34 germline-encoded AVY and NHS sequences in SLE patients raises the possibility that the AVY motif may have a significant impact on systemic responses that target gut bacteria.

It is suggested that VH4-34+ ASC clonal expansion and a high prevalence of VH4-34+ activated naive cells in SLE flares indicate the involvement of an autoantigen-driven mechanism. However, there is still a lack of comprehensive understanding of the specific autoantigens that are the focus of ASC oligoclonal expansion during SLE exacerbations.(2) A recent study indicated a correlation between the occurrence of flares in patients with SLE and the existence of VH4-34 antibodies that are either unmutated or weakly mutated. However, the specific antigens

that are recognized by these antibody clones have yet to be identified.(2) Given the fact that VH4-34-expressing antibodies exhibit inherent self-reactivity and detect I/i carbohydrates present on hematopoietic cells (8, 9), our lab has previously found that these clones can bind to commensal gut bacteria as well, implying that SLE flares may arise due to disruptions in the gut microbiota, which could then potentially trigger the generation of VH4-34+IgG+ B lymphocytes from naïve B lymphocytes.(7) We revealed that VH4-34-encoding antibodies possessing unmutated AVY motifs (because they were sorted with the 9G4 antibody) from both healthy donors and SLE patients bind very well to commensal bacteria in stools from SLE compared to healthy donor stools. This seems specific to VH4-34-encoded antibodies because other VH4-non-VH4-34 antibodies do not bind commensal bacteria in SLE stool. We suggest that SLE patients have more 9G4+ CD27+IgG+ memory B cells encoding VH4-34 antibodies, which frequently have unmutated AVY and NHS sites and can recognize commensal gut bacteria.

Previous studies have demonstrated an elevated occurrence of VH4-34+IgG+B lymphocytes with reduced SHMs in the memory pool among SLE patients.(218) In fact, our SHM analysis of Ig genes cloned from 9G4+IgG+ B lymphocytes obtained from three SLE patients revealed a notable reduction in the total number of mutations in VH and Vκ genes. In parallel, our research revealed that a majority of IgG+ B cells expressing VH4-34 had an unmutated AVY site, frequently in conjunction with the unmutated NHS in SLE patients.

The findings from mutagenesis research provide compelling evidence that the unmutated germline AVY motif of VH4-34 monoclonal antibodies originating from lupus patients' blood has a notable influence on the activity of these SLE 9G4 B cell clones towards fecal gut bacteria.

Furthermore, we demonstrated clonal expansion in one out of three SLE patients whose PBMC were sorted using a 9G4 monoclonal antibody strategy. The VH4-34 region genes were analyzed to determine the presence and arrangement of somatic mutations in order to evaluate the impact of antigen-mediated clonal selection. We saw repeated arrangement of VH4-34/ D2-2 / RF3 / JH2 and CDR3 length of 17 amino acids in the cloned 9G4+ B cell compartment of a SLESt2 patient potentially

as a sign of clonal expansion and somatic diversity. Our data indicate that a group of anti-commensal class-switched V4-34-encoded IgG antibodies of memory 9G4 B lymphocytes from a SLESt2 patient went through clonal expansion associated with somatic hypermutation. It is noteworthy to observe that this clonal proliferation exhibits significant responsiveness towards commensal microorganisms. The phenomenon of SLE VH4-34 clonal expansion has been seen to be correlated with clinical exacerbation. It has been determined that this clonal expansion exhibits a high degree of specificity towards commensal microorganisms. The result of SLE patients displaying a clonal expansion of 9G4 antibodies with a non-mutant AVY motif can imply a limited use of the VH4-34 gene, hence suggesting a non-random biological selection process.

The intriguing scientific inquiries arise from the observed cross-reactivity between a bacterial antigen and an autoantigen. There is a growing acceptance of molecular resemblances between gut microorganisms and autoantigens in various autoimmune disorders, which has led to the proposition that translocation of gut bacteria into blood known as leaky gut may play an important role in the initiation or worsening of these autoimmune illnesses.(219) The concept of leaky gut in SLE was further supported by elevated concentrations of endotoxins; however, it is important to note that this endotoxin phenomenon may not be directly related to VH4-34 reactivity in SLE patients. Nevertheless, such recognition of VH4-34+ memory B lymphocyte clones to commensal bacteria antigen suggests that there may be systemic immune reactions directed towards foreign antigens that might potentially exhibit cross-reactivity with the I/i antigen identified by VH4-34 antibodies. Hence, it is plausible that the VH4-34 autoreactive region has undergone selection within the genome of humans due to its inherent ability to generate antibodies, which have the potential to counteract uncontrolled gut flora.(7) Given the fact that, in SLE patients, the gut equilibrium would be shifted in the direction of the autoimmunity state, either due to leaky gut, molecular mimicry, epitope spreading, bystander activation, gender bias, or biofilms, molecular mimicry may be an important factor for VH4-34 antibodies, facilitating their ability to bind to commensal gut bacteria.

In certain individuals with autoimmune disorders, there may be a disruption in anti-bacterial responses, particularly in the generation of IgA antibodies that specifically target commensal bacteria, leading to an inability to adequately preserve the balance of the gut microbiota. (57, 220) Moreover, it has been previously reported that CD27+IgM+ memory B lymphocytes are believed to be responsible for mediating immune responses to carbohydrates and providing defense against gram-positive encapsulated bacteria.(221) Consistent with this concept, individuals with SLE have self-reactive VH4-34-encoding IgG+ memory B lymphocytes, which could develop as a result of systemic anti-commensal bacteria responses and then undergo expansion during disease flares.(2, 7, 57) Furthermore, a recent study revealed that the escape of commensal bacteria, particularly Enterococcus gallinarum, from the intestines to the liver contributes to the progression of SLE, further referring to the fact that commensal microorganisms from the gut of SLE patients are not contained properly.(57, 143, 222) Additionally, the investigation of the monocyte transcriptome in individuals with SLE demonstrated the presence of a distinctive pattern associated with prolonged exposure to endotoxins, which might point to a potential disruption of the intestinal barrier, leading to the release of commensal bacteria antigens into other body sites.(220) In relation to this matter, the first study documenting the presence of intestinal dysbiosis among SLE patients showed an elevated prevalence of the gut Bacteroides phylum.(139) Strikingly, VH4-34-expressing IgG+ B lymphocytes exhibited recognition of B. fragilis, a member of the Bacteroides phylum. This finding provides more evidence that dysbiosis of the microbiota may contribute to the emergence and proliferation of these 9G4+IgG+ B lymphocytes in SLE patients.(7) Moreover, the formation of autoimmunity has been linked to the presence of modified gut flora.(57, 139, 223-225) Whether poor B-cell tolerance checkpoints or alterations in the B-cell repertoire, in particular intrinsically autoreactive VH4-34+IgG+ B cell expansion in lupus is linked to gut dysbiosis in SLE patients and thereby favor SLE pathophysiology, was undetermined.(57) To fill this knowledge gap, we investigated the anti-commensal bacteria reactivity of VH4-34-encoded IgG antibodies from healthy donors and SLE patients. We found that the involvement of VH4-34 sequences with unmutated AVY motifs appears to be necessary for interaction with gut bacteria,

resulting in the proliferation of the autoreactive 9G4 B cell subgroup and consequent exacerbation of the SLE patient's symptoms.

There are several possible explanations that account for the preferential selection of a certain VH4-34 gene segment. One possible reason can be functional selection according to the recognition of specific antigenic determinants. These determinants provide advantages either in expanding the B cell repertoire or in defending against prevalent pathogens.(180) In this study, we showed not only that 9G4+IgG+ B cells bind the fecal bacteria in flow experiments but also found the composition of bacterial populations in the feces of a small group of healthy donors and SLE patients that exhibit binding to the 9G4+ VH4-34 IgG+ antibodies from both healthy donors and SLE patients. Our observations indicate that Intestinibacter;s_bartlettii were found to be the most common bacteria among three out of five 9G4-bound fractions from different SLE patients. Intestinibacter;s_bartlettii, a common 9G4 antibody-bound bacteria among SLE patients, was classified under the class Clostridia, which is known to be important in SLE pathogenesis.(120, 167)The taxonomic classification of the genus Intestinibacter was established in a relatively recent timeframe. This genus encompasses the previously identified human strain known as Clostridium bartletti, which has subsequently been reclassified as Intestinibacter bartlettii. Limited knowledge exists on the involvement of this entity within the colon ecology and its potential impact on human health.(226) Importantly, I. bartlettii is believed to possess resistance to oxidative stress and be capable of breaking fucose, implicating an indirect role in the breakdown of mucus. (226, 227) Based on the fact that this phenomenon may serve as an additional component within the leaky gut idea pertaining to the SLE exacerbation for those patients, it is tempting to speculate that the systemic 9G4 antibody response in SLE patients could be attributed to the translocation of Intestinibacter bartlettii in some SLE patients. This observation offers supportive evidence for the notion that VH4-34-encoded memory B cells are associated with increased intestinal permeability, allowing gut bacteria to enter the bloodstream, consequently resulting in the inherently autoreactive 9G4 B cell expansion. Also, although the presence of germline-encoded VH4-34 antibodies has been shown to exhibit cross-reactivity with bacterial lipopolysaccharide (LPS), indicating a potential involvement in the defensive mechanisms of the host organism,

9G4 antibody is responsible for encoding all pathogenic anti-I/i cold-agglutinin antibodies, which suggests that these natural antibodies may really have detrimental consequences.(179, 228) Consequently, knowledge of identifying 9G4-reactive species from SLE stool unique to that patient might be an advantageous approach to diving into more tailored treatments based on personalized medicine, like giving antibiotics targeting the translocated 9G4-bound bacteria.

Furthermore, commensal reactivity was also one of the features of fetal B cells, which is likely attributed to the germline encoded VH3 gene regions. However, stool-reactive fetal innate-like B lymphocyte antibodies exhibited reactivity against the bacterial species Blautia and Dorea, unlike VH4-34-expressing antibodies.(229) On the other hand, our study was conducted to reveal the interaction between the microbiota and VH4-34–expressing IgG B cells in patients with SLE. Results from the pairs having SLE11, SLE03, and SLE14 patient stools refer to the importance of Intestinibacter;s_bartlettii regarding 9G4 activity on SLE stools. A comprehensive analysis of the SLE 9G4 B cells in relation to gut bacteria like Intestinibacter;s_bartlettii has the potential to shed significant light on the underlying mechanisms driving disease progression, including leaky gut. Consequently, this research could offer valuable guidance for the identification of novel therapeutic strategies.

Despite making significant advances in the field, the precise molecule with which the 9G4 antibody recognizes the gut bacteria is yet unclear. A prominent candidate consists of carbohydrate molecules present in the bacterial wall, which may resemble I/i antigens, although more research is needed to be conducted to confirm this. Still, it is plausible that these antigens might potentially originate from the same antigens that are detected by 9G4 antibodies, such as N-acetyllactosamine-containing glycosphingolipids, gangliosides, and bacterial glycolipids. (1, 181, 216, 230) It is crucial to acknowledge that N-acetyllactosamine is present in a diverse range of glycoproteins, such as CD45 and other differentiation antigens, gastrointestinal mucins, gangliosides, glycolipids, and apoptotic cells. (1, 231-236) As a result, self-antigens containing N-acetyllactosamine are abundantly present and capable of interacting with 9G4 B cells, whether in a soluble state or on the plasma membrane of

various cell types such as erythrocytes, lymphocytes, epithelial cells, apoptotic cells, and tumor cells. Indeed, previous studies have demonstrated that 9G4 antibodies exhibit reactivity towards a variety of antigens or cells, as mentioned earlier. (1, 173, 182, 186, 194, 210, 216, 228, 237-240) It is possible that 9G4 antibodies detect an epitope found in bacterial carbohydrates like N-acetyllactosamine, suggesting a potential cross-reactivity between self and microbial glycolipid antigens. (241) The precise characteristics of the antigens/epitopes on gut bacteria recognized by 9G4 B cells will be investigated in our laboratory as a continuation of this project.

In light of the possibility that lupus may consist of a group of interconnected pathologies rather than a uniform illness, our research was insufficient in size to prove our hypothesis for all the patients, like previous studies. The observed findings might benefit from additional elucidation by conducting tests on a broader range of antibodies and stool samples from patients. However, we can conclude that a particular group of SLE patients do possess gut bacteria that are recognized by VH4-34 antibodies harboring an unmutated AVY motif. Moreover, given the gut complexity and different factors influencing the gut microbiota and lupus itself, it is hard to draw solid conclusions for all SLE patients. Rather, it is more realistic to find unique bacterial taxa like Intestinibacter;s_bartlettii bound to 9G4 recombinant antibodies for a group of SLE patients. Moreover, perhaps other SLE patients may have different gut bacteria, which have bacterial entities in common with Intestinibacter;s_bartlettii, causing the expansion of 9G4 B cell clones. It is of utmost importance to emphasize that targeting these bacteria could be beneficial in terms of reducing the risk of triggering 9G4 clonal expansion.

Our inquiry was centered around the etiology of VH4-34-encoding CD27+IgG+B lymphocyte expansion in individuals diagnosed with SLE and our studies offer valuable insights into the involvement of the gut microbiota in the pathophysiological mechanisms underlying lupus flares. More specifically, the findings shown in this research point toward a specific bacterial community, which is recognized by VH4-34-encoded IgG antibodies, inside the gut microbiota of a group of SLE patients potentially contributing to SLE flare.

6. CONCLUSIONS AND PERSPECTIVES

1. The VH4-34 autoreactive motif is conserved in IgG+ B lymphocytes of SLE patients.
2. Our study showed evidence of the interaction between VH4-34-expressing IgG antibodies and the microbiota in lupus patients.
3. The process of antigenic selection in immune responses can involve the use of VH4-34 memory B clones in SLE patients, which sustains unmutated AVY and NHS sequences that are linked to reactivity against commensal bacteria.
4. SLE VH4-34 IgG+ B cell antibodies show a higher level of anti-fecal gut bacteria reactivity.
5. VH4-34-encoded antibodies of both healthy individuals and SLE patients bearing unmutated AVY motifs, which were selected using the 9G4 antibody, exhibit greater binding towards commensal gut bacteria present in the SLE stools samples as opposed to control stools. This phenomenon appears to be exclusive to VH4-34-encoded antibodies since other VH4 antibodies that are not VH4-34 do not exhibit binding capabilities to commensal bacteria found in the feces of individuals with SLE.
6. Unmutated or weakly mutated VH4-34-expressing antibodies recognize gut bacteria, which can give a hint that SLE flares in fact might be due to a breach in the gut microbiota, leading to a contribution to the expansion of VH4-34+IgG+ B cells in SLE patients.
7. SLESt2 9G4+ VH4-34 clones pertaining to VH4-34/D2-2/RF3/JH2 and CDR3 lengths of 17 amino acid arrangements are overrepresented. This disproportionate representation in the VH4-34 B cell compartment for SLE patients has not been described to date.
8. Since SLE VH4-34 clonal expansion is associated with disease flare, we must highlight our discovery of the SLE VH4-34 clonal expansion of gut bacteria-reactive circulating 9G4+IgG+ B cells in one SLE patient, suggesting that commensal bacteria translocating from the gut to the blood are therefore responsible for the VH4-34 expansion in SLE.

9. The commensal bacteria reactivity ability of VH4-34-expressing IgG antibodies comes from the germline unmutated VH4-34 AVY motif based on mutagenesis experiments in which we reverted HD VH4-34+IgG+ memory B cells with mutated AVY motifs to their germline unmutated AVY form.
10. The presence of IgG+ B lymphocytes that encode VH4-34 antibodies and exhibit reactivity against both self and anti-commensal bacteria in individuals with SLE indicates a compromised containment of the intestinal microbiota.
11. Certain taxonomic groups were found in the stools of SLE patients that bind to 9G4+ VH4-34 IgG recombinant antibodies with a greater affinity. Similarly, particular taxa present in healthy controls' fecal samples bind to VH4-34 IgG recombinant antibodies from $9G4^{+}IgG^{+}$ B cells more strongly.
12. Intestinibacter;s_bartlettii yielded a higher signal of binding of 9G4+ antibodies in three out of five SLE stool samples, in particular SLE11, SLE03, and SLE14, referring that patients with SLE have immune responses that are directed against microorganisms present in the gastrointestinal tract. Moreover, Christensenellaceae;g_NA;s_sp30434, is the common bacteria species bound by 9G4+ antibodies on SLE01. On the other hand, healthy donor stool analysis shows that 9G4+ antibodies from healthy donors and SLE patients most often bind to the bacteria Anaerostipes;s_hadrus. To the best of our knowledge, those bacterial taxa, which are all classified under the Eubacteriales order, have not been associated with VH4-34 B cells previously.
13. Our findings suggest that SLE flare may correlate with the presence of Intestinibacter bartettii in the blood due to the dysbiosis of the intestinal flora and leaky gut. Given the fact that Intestinibacter bartettii bound to 9G4 in the feces of SLE patients was important in disease pathogeneses, potential therapeutic modalities for gut microbiota management should be strengthened to target this possible bug, which may speed up the worsening of SLE flares by causing the growth of VH4-34 memory B cells that are responsible for autoreactive antibodies attacking multiple tissues.
14. This work enhances our understanding of the microbiome's involvement in the development of SLE and to discover specific bacterial communities that could be

targeted to inhibit VH4-34 antibody production and reduce disease exacerbations in SLE patients.

15. We confirmed the importance of leaky gut mechanisms in SLE pathogenesis, which was initially suggested with the identification of Ro60 commensal bacteria as a trigger for autoimmunity in lupus.(143)
16. VH4-34 immunoglobulins cloned from 9G4 memory B cell lymphocytes could provide a potent tool to examine and characterize SLE patient fecal microbial profiles, which are instrumental in uncovering the pathogenesis of SLE.
17. SLE patients' dysbiosis may improve to some extent after possible prophylaxis or potential therapy against Intestinibacter bartlettii or other potential gut bacteria that are present in SLE patients and bind with high affinity to 9G4 antibodies.
18. Unanswered questions remaining in the details of gut bacteria unique to SLE patients include the following: Is it possible to use gut bacteria screening from SLE patient stools as a means of predicting illness prognosis, outcome, or treatment approach? Since the PhyloChip (16S rRNA gene DNA microarray) is an efficient and practical device for studying intestinal microbiota (242), in the future, perhaps patients with SLE may want to use the PhyloChip to check whether Intestinibacter bartlettii, known as a target for 9G4 memory B cells in SLE, is present in their body. In this case, SLE patients may benefit from a tailored antibiotic regimen that will help eliminate Intestinibacter bartlettii in their gut, thereby decreasing the expansion of VH4-34 IgG B cell clones and reducing the SLE exacerbation.
19. Similar autoimmune diseases should be tested to see if self-reactive 9G4 B cells could play a role in binding commensal gut bacteria entities. Accordingly, the appropriate personalized treatment against the possible 9G4-bound bacteria antigen might be considered, like future candidates for SLE management mentioned above.
20. More studies are needed to investigate what molecule is responsible for the binding of 9G4 monoclonal antibodies to gut bacteria, leading to the exacerbation of SLE flares. Therefore, our lab intends to further explore the intricate chemical mechanism behind this recognition as a prospective avenue for our project,

although we have not yet identified the particular molecule that accounts for the binding of 9G4 antibodies to gut bacteria.

21. In conclusion, a better understanding of how the VH4-34 clones with unmutated AVY motifs contribute to the pathogenesis of SLE by recognizing the gut bacteria, which in turn causes an increase in the number of self-reactive autoantibodies, will expedite the integration of new diagnostic, preventative, and therapeutic strategies.

7. REFERENCES

1. Milner EC, Anolik J, Cappione A, Sanz I. Human innate B cells: a link between host defense and autoimmunity? Springer Semin Immunopathol. 2005;26(4):433-52.

2. Tipton CM, Fucile CF, Darce J, Chida A, Ichikawa T, Gregoretti I, et al. Diversity, cellular origin and autoreactivity of antibody-secreting cell population expansions in acute systemic lupus erythematosus. Nat Immunol. 2015;16(7):755-65.

3. Cappione A, 3rd, Anolik JH, Pugh-Bernard A, Barnard J, Dutcher P, Silverman G, et al. Germinal center exclusion of autoreactive B cells is defective in human systemic lupus erythematosus. J Clin Invest. 2005;115(11):3205-16.

4. Yurasov S, Wardemann H, Hammersen J, Tsuiji M, Meffre E, Pascual V, et al. Defective B cell tolerance checkpoints in systemic lupus erythematosus. Journal of Experimental Medicine. 2005;201(5):703-11.

5. Pugh-Bernard AE, Silverman GJ, Cappione AJ, Villano ME, Ryan DH, Insel RA, et al. Regulation of inherently autoreactive VH4-34 B cells in the maintenance of human B cell tolerance. J Clin Invest. 2001;108(7):1061-70.

6. Mietzner B, Tsuiji M, Scheid J, Velinzon K, Tiller T, Abraham K, et al. Autoreactive IgG memory antibodies in patients with systemic lupus erythematosus arise from nonreactive and polyreactive precursors. Proc Natl Acad Sci U S A. 2008;105(28):9727-32.

7. Schickel JN, Glauzy S, Ng YS, Chamberlain N, Massad C, Isnardi I, et al. Self-reactive VH4-34-expressing IgG B cells recognize commensal bacteria. J Exp Med. 2017;214(7):1991-2003.

8. Potter KN, Hobby P, Klijn S, Stevenson FK, Sutton BJ. Evidence for involvement of a hydrophobic patch in framework region 1 of human V4-34-encoded Igs in recognition of the red blood cell I antigen. J Immunol. 2002;169(7):3777-82.

9. Reed JH, Jackson J, Christ D, Goodnow CC. Clonal redemption of autoantibodies by somatic hypermutation away from self-reactivity during human immunization. J Exp Med. 2016;213(7):1255-65.

10. Murphy KM, Weaver C. Janeway's Immunobiology: Garland Science/Taylor & Francis Group, LLC; 2017.

11. Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. J Exp Med. 1991;173(5):1213-25.

12. Schatz DG, Oettinger MA, Baltimore D. The V(D)J recombination activating gene, RAG-1. Cell. 1989;59(6):1035-48.

13. Oettinger MA, Schatz DG, Gorka C, Baltimore D. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. Science. 1990;248(4962):1517-23.

14. Li YS, Hayakawa K, Hardy RR. The regulated expression of B lineage associated genes during B cell differentiation in bone marrow and fetal liver. J Exp Med. 1993;178(3):951-60.

15. Meffre E, Casellas R, Nussenzweig MC. Antibody regulation of B cell development. Nature Immunology. 2000;1(5):379-85.

16. O'Riordan M, Grosschedl R. Transcriptional regulation of early B-lymphocyte differentiation. Immunol Rev. 2000;175:94-103.

17. Nagata K, Nakamura T, Kitamura F, Kuramochi S, Taki S, Campbell KS, et al. The Ig alpha/Igbeta heterodimer on mu-negative proB cells is competent for transducing signals to induce early B cell differentiation. Immunity. 1997;7(4):559-70.

18. ten Boekel E, Melchers F, Rolink A. The status of Ig loci rearrangements in single cells from different stages of B cell development. Int Immunol. 1995;7(6):1013-9.

19. Ehlich A, Martin V, Müller W, Rajewsky K. Analysis of the B-cell progenitor compartment at the level of single cells. Curr Biol. 1994;4(7):573-83.

20. Alt FW, Yancopoulos GD, Blackwell TK, Wood C, Thomas E, Boss M, et al. Ordered rearrangement of immunoglobulin heavy chain variable region segments. EMBO J. 1984;3(6):1209-19.

21. Tsubata T, Reth M. The products of pre-B cell-specific genes (lambda 5 and VpreB) and the immunoglobulin mu chain form a complex that is transported onto the cell surface. J Exp Med. 1990;172(3):973-6.

22. Kerr WG, Cooper MD, Feng L, Burrows PD, Hendershot LM. Mu heavy chains can associate with a pseudo-light chain complex (psi L) in human pre-B cell lines. Int Immunol. 1989;1(4):355-61.

23. Mårtensson IL, Keenan RA, Licence S. The pre-B-cell receptor. Curr Opin Immunol. 2007;19(2):137-42.

24. Reth M, Petrac E, Wiese P, Lobel L, Alt FW. Activation of V kappa gene rearrangement in pre-B cells follows the expression of membrane-bound immunoglobulin heavy chains. EMBO J. 1987;6(11):3299-305.

25. Schlissel MS, Baltimore D. Activation of immunoglobulin kappa gene rearrangement correlates with induction of germline kappa gene transcription. Cell. 1989;58(5):1001-7.

26. Maki K, Nagata K, Kitamura F, Takemori T, Karasuyama H. Immunoglobulin beta signaling regulates locus accessibility for ordered immunoglobulin gene rearrangements. J Exp Med. 2000;191(8):1333-40.

27. Ritchie KA, Brinster RL, Storb U. Allelic exclusion and control of endogenous immunoglobulin gene rearrangement in kappa transgenic mice. Nature. 1984;312(5994):517-20.

28. Rusconi S, Köhler G. Transmission and expression of a specific pair of rearranged immunoglobulin mu and kappa genes in a transgenic mouse line. Nature. 1985;314(6009):330-4.

29. Yamagami T, ten Boekel E, Andersson J, Rolink A, Melchers F. Frequencies of multiple IgL chain gene rearrangements in single normal or kappaL chain-deficient B lineage cells. Immunity. 1999;11(3):317-27.

30. Tiegs SL, Russell DM, Nemazee D. Receptor editing in self-reactive bone marrow B cells. J Exp Med. 1993;177(4):1009-20.

31. Yamagami T, ten Boekel E, Schaniel C, Andersson J, Rolink A, Melchers F. Four of five RAG-expressing JCkappa-/- small pre-BII cells have no L chain gene rearrangements: detection by high-efficiency single cell PCR. Immunity. 1999;11(3):309-16.

32. Gay D, Saunders T, Camper S, Weigert M. Receptor editing: an approach by autoreactive B cells to escape tolerance. J Exp Med. 1993;177(4):999-1008.

33. Radic MZ, Erikson J, Litwin S, Weigert M. B lymphocytes may escape tolerance by revising their antigen receptors. J Exp Med. 1993;177(4):1165-73.

34. Rajewsky K. Clonal selection and learning in the antibody system. Nature. 1996;381(6585):751-8.

35. Sandel PC, Monroe JG. Negative selection of immature B cells by receptor editing or deletion is determined by site of antigen encounter. Immunity. 1999;10(3):289-99.

36. Hartley SB, Crosbie J, Brink R, Kantor AB, Basten A, Goodnow CC. Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens. Nature. 1991;353(6346):765-9.

37. Pike BL, Boyd AW, Nossal GJ. Clonal anergy: the universally anergic B lymphocyte. Proc Natl Acad Sci U S A. 1982;79(6):2013-7.

38. Fulcher DA, Basten A. Reduced life span of anergic self-reactive B cells in a double-transgenic model. J Exp Med. 1994;179(1):125-34.

39. Cyster JG, Hartley SB, Goodnow CC. Competition for follicular niches excludes self-reactive cells from the recirculating B-cell repertoire. Nature. 1994;371(6496):389-95.

40. Bell SE, Goodnow CC. A selective defect in IgM antigen receptor synthesis and transport causes loss of cell surface IgM expression on tolerant B lymphocytes. EMBO J. 1994;13(4):816-26.

41. Hibbs ML, Tarlinton DM, Armes J, Grail D, Hodgson G, Maglitto R, et al. Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease. Cell. 1995;83(2):301-11.

42. Khan WN, Alt FW, Gerstein RM, Malynn BA, Larsson I, Rathbun G, et al. Defective B cell development and function in Btk-deficient mice. Immunity. 1995;3(3):283-99.

43. Engel P, Zhou LJ, Ord DC, Sato S, Koller B, Tedder TF. Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. Immunity. 1995;3(1):39-50.

44. Rickert RC, Rajewsky K, Roes J. Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. Nature. 1995;376(6538):352-5.

45. Sato S, Miller AS, Inaoki M, Bock CB, Jansen PJ, Tang ML, et al. CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: altered signaling in CD22-deficient mice. Immunity. 1996;5(6):551-62.

46. O'Keefe TL, Williams GT, Davies SL, Neuberger MS. Hyperresponsive B cells in CD22-deficient mice. Science. 1996;274(5288):798-801.

47. Kishihara K, Penninger J, Wallace VA, Kündig TM, Kawai K, Wakeham A, et al. Normal B lymphocyte development but impaired T cell maturation in CD45-exon6 protein tyrosine phosphatase-deficient mice. Cell. 1993;74(1):143-56.

48. Rolink AG, Andersson J, Melchers F. Characterization of immature B cells by a novel monoclonal antibody, by turnover and by mitogen reactivity. Eur J Immunol. 1998;28(11):3738-48.

49. Yu W, Nagaoka H, Jankovic M, Misulovin Z, Suh H, Rolink A, et al. Continued RAG expression in late stages of B cell development and no apparent re-induction after immunization. Nature. 1999;400(6745):682-7.

50. Meffre E, Davis E, Schiff C, Cunningham-Rundles C, Ivashkiv LB, Staudt LM, et al. Circulating human B cells that express surrogate light chains and edited receptors. Nature Immunology. 2000;1(3):207-13.

51. Osmond DG. Proliferation kinetics and the lifespan of B cells in central and peripheral lymphoid organs. Curr Opin Immunol. 1991;3(2):179-85.

52. Förster I, Rajewsky K. The bulk of the peripheral B-cell pool in mice is stable and not rapidly renewed from the bone marrow. Proc Natl Acad Sci U S A. 1990;87(12):4781-4.

53. Lam KP, Kühn R, Rajewsky K. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. Cell. 1997;90(6):1073-83.

54. Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC. Predominant autoantibody production by early human B cell precursors. Science. 2003;301(5638):1374-7.

55. Abbas A, Lichtman A, Pillai S. Basic Immunology: Basic Immunology E-Book: Elsevier Health Sciences; 2019.

56. Kuraoka M, Meffre E, Kelsoe G. Chapter Two - The First B-Cell Tolerance Checkpoint in Mice and Humans: Control by AID. In: Alt F, editor. Advances in Immunology. 139: Academic Press; 2018. p. 51-92.

57. Meffre E, O'Connor KC. Impaired B-cell tolerance checkpoints promote the development of autoimmune diseases and pathogenic autoantibodies. Immunol Rev. 2019;292(1):90-101.

58. Nemazee D. Mechanisms of central tolerance for B cells. Nat Rev Immunol. 2017;17(5):281-94.

59. Allman DM, Ferguson SE, Lentz VM, Cancro MP. Peripheral B cell maturation. II. Heat-stable antigen(hi) splenic B cells are an immature developmental intermediate in the production of long-lived marrow-derived B cells. J Immunol. 1993;151(9):4431-44.

60. Sng J, Ayoglu B, Chen JW, Schickel JN, Ferre EMN, Glauzy S, et al. AIRE expression controls the peripheral selection of autoreactive B cells. Sci Immunol. 2019;4(34).

61. Chen JW, Schickel JN, Tsakiris N, Sng J, Arbogast F, Bouis D, et al. Positive and negative selection shape the human naive B cell repertoire. J Clin Invest. 2022;132(2).

62. Viant C, Weymar GHJ, Escolano A, Chen S, Hartweger H, Cipolla M, et al. Antibody Affinity Shapes the Choice between Memory and Germinal Center B Cell Fates. Cell. 2020;183(5):1298-311 e11.

63. Domeier PP, Schell SL, Rahman ZS. Spontaneous germinal centers and autoimmunity. Autoimmunity. 2017;50(1):4-18.

64. Reynaud C-A, Weill J-C. Memory B Cells. In: Ratcliffe MJH, editor. Encyclopedia of Immunobiology. Oxford: Academic Press; 2016. p. 195-9.

65. Manz RA, Löhning M, Cassese G, Thiel A, Radbruch A. Survival of long-lived plasma cells is independent of antigen. Int Immunol. 1998;10(11):1703-11.

66. Allen CD, Okada T, Tang HL, Cyster JG. Imaging of germinal center selection events during affinity maturation. Science. 2007;315(5811):528-31.

67. Bende RJ, van Maldegem F, Triesscheijn M, Wormhoudt TA, Guijt R, van Noesel CJ. Germinal centers in human lymph nodes contain reactivated memory B cells. J Exp Med. 2007;204(11):2655-65.

68. Mesin L, Schiepers A, Ersching J, Barbulescu A, Cavazzoni CB, Angelini A, et al. Restricted Clonality and Limited Germinal Center Reentry Characterize Memory B Cell Reactivation by Boosting. Cell. 2020;180(1):92-106 e11.

69. Robinson WH. Sequencing the functional antibody repertoire--diagnostic and therapeutic discovery. Nat Rev Rheumatol. 2015;11(3):171-82.

70. Yu YH, Lin KI. Chapter Two - Factors That Regulate the Generation of Antibody-Secreting Plasma Cells. In: Alt FW, editor. Advances in Immunology. 131: Academic Press; 2016. p. 61-99.

71. Klein U, Rajewsky K, Küppers R. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. J Exp Med. 1998;188(9):1679-89.

72. Zinöcker S, Schindler CE, Skinner J, Rogosch T, Waisberg M, Schickel JN, et al. The V gene repertoires of classical and atypical memory B cells in malaria-susceptible West African children. J Immunol. 2015;194(3):929-39.

73. Anderson SM, Tomayko MM, Ahuja A, Haberman AM, Shlomchik MJ. New markers for murine memory B cells that define mutated and unmutated subsets. Journal of Experimental Medicine. 2007;204(9):2103-14.

74. Giesecke C, Frölich D, Reiter K, Mei HE, Wirries I, Kuhly R, et al. Tissue Distribution and Dependence of Responsiveness of Human Antigen-Specific Memory B Cells. The Journal of Immunology. 2014;192(7):3091-100.

75. MacLennan ICM. Germinal Centers. Annual Review of Immunology. 1994;12(1):117-39.

76. Tiller T, Tsuiji M, Yurasov S, Velinzon K, Nussenzweig MC, Wardemann H. Autoreactivity in human IgG+ memory B cells. Immunity. 2007;26(2):205-13.

77. Taylor JJ, Pape KA, Jenkins MK. A germinal center–independent pathway generates unswitched memory B cells early in the primary response. Journal of Experimental Medicine. 2012;209(3):597-606.

78. Fagarasan S, Muramatsu M, Suzuki K, Nagaoka H, Hiai H, Honjo T. Critical roles of activation-induced cytidine deaminase in the homeostasis of gut flora. Science. 2002;298(5597):1424-7.

79. Boullier Sv, Tanguy M, Kadaoui KA, Caubet Cc, Sansonetti P, Corthésy B, et al. Secretory IgA-Mediated Neutralization of Shigella flexneri Prevents Intestinal Tissue Destruction by Down-Regulating Inflammatory Circuits1. The Journal of Immunology. 2009;183(9):5879-85.

80. Kiriakidou M, Ching CL. Systemic Lupus Erythematosus. Ann Intern Med. 2020;172(11):ITC81-ITC96.

81. Durcan L, O'Dwyer T, Petri M. Management strategies and future directions for systemic lupus erythematosus in adults. Lancet. 2019;393(10188):2332-43.

82. Agmon-Levin N, Mosca M, Petri M, Shoenfeld Y. Systemic lupus erythematosus one disease or many? Autoimmunity Reviews. 2012;11(8):593-5.

83. Kaul A, Gordon C, Crow MK, Touma Z, Urowitz MB, van Vollenhoven R, et al. Systemic lupus erythematosus. Nature Reviews Disease Primers. 2016;2.

84. Barber MRW, Drenkard C, Falasinnu T, Hoi A, Mak A, Kow NY, et al. Global epidemiology of systemic lupus erythematosus. Nature Reviews Rheumatology. 2021;17(9):515-32.

85. Daniel J Wallace DDG. Clinical manifestations and diagnosis of systemic lupus erythematosus in adults UpToDate: UpToDate; [June 27, 2023]. Available from: https://www.uptodate.com/contents/clinical-manifestations-and-diagnosis-of-systemic-lupus-erythematosus-in-adults.

86. Abu-Shakra M, Urowitz MB, Gladman DD, Gough J. Mortality studies in systemic lupus erythematosus. Results from a single center. II. Predictor variables for mortality. J Rheumatol. 1995;22(7):1265-70.

87. Tsokos GC. Systemic lupus erythematosus. N Engl J Med. 2011;365(22):2110-21.

88. Yanaba K, Bouaziz JD, Matsushita T, Magro CM, St Clair EW, Tedder TF. B-lymphocyte contributions to human autoimmune disease. Immunol Rev. 2008;223:284-99.

89. Spronk PE, Limburg PC, Kallenberg CG. Serological markers of disease activity in systemic lupus erythematosus. Lupus. 1995;4(2):86-94.

90. Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Dennis GJ, James JA, et al. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. N Engl J Med. 2003;349(16):1526-33.

91. Shlomchik MJ, Madaio MP, Ni D, Trounstein M, Huszar D. The role of B cells in lpr/lpr-induced autoimmunity. J Exp Med. 1994;180(4):1295-306.

92. Domeier PP, Rahman ZSM. Regulation of B Cell Responses in SLE by Three Classes of Interferons. International Journal of Molecular Sciences. 2021;22(19).

93. Nicholas MW, Dooley MA, Hogan SL, Anolik J, Looney J, Sanz I, et al. A novel subset of memory B cells is enriched in autoreactivity and correlates with adverse outcomes in SLE. Clin Immunol. 2008;126(2):189-201.

94. Chang NH, McKenzie T, Bonventi G, Landolt-Marticorena C, Fortin PR, Gladman D, et al. Expanded population of activated antigen-engaged cells within the naive B cell compartment of patients with systemic lupus erythematosus. J Immunol. 2008;180(2):1276-84.

95. Grammer AC, Slota R, Fischer R, Gur H, Girschick H, Yarboro C, et al. Abnormal germinal center reactions in systemic lupus erythematosus demonstrated by blockade of CD154-CD40 interactions. The Journal of Clinical Investigation. 2003;112(10):1506-20.

96. Dörner T, Jacobi AM, Lee J, Lipsky PE. Abnormalities of B cell subsets in patients with systemic lupus erythematosus. Journal of Immunological Methods. 2011;363(2):187-97.

97. Gao N, Dresel J, Eckstein V, Gellert R, Störch H, Venigalla RK, et al. Impaired suppressive capacity of activation-induced regulatory B cells in systemic lupus erythematosus. Arthritis Rheumatol. 2014;66(10):2849-61.

98. Roozendaal R, Carroll MC. Complement receptors CD21 and CD35 in humoral immunity. Immunol Rev. 2007;219:157-66.

99. Menard L, Saadoun D, Isnardi I, Ng YS, Meyers G, Massad C, et al. The PTPN22 allele encoding an R620W variant interferes with the removal of developing autoreactive B cells in humans. J Clin Invest. 2011;121(9):3635-44.

100. Gies V, Schickel JN, Jung S, Joublin A, Glauzy S, Knapp AM, et al. Impaired TLR9 responses in B cells from patients with systemic lupus erythematosus. JCI Insight. 2018;3(5).

101. Manolakou T, Nikolopoulos D, Gkikas D, Filia A, Samiotaki M, Stamatakis G, et al. ATR-mediated DNA damage responses underlie aberrant B cell activity in systemic lupus erythematosus. Sci Adv. 2022;8(43):eabo5840.

102. Kansal R, Richardson N, Neeli I, Khawaja S, Chamberlain D, Ghani M, et al. Sustained B cell depletion by CD19-targeted CAR T cells is a highly effective treatment for murine lupus. Sci Transl Med. 2019;11(482).

103. Alexander T, Sarfert R, Klotsche J, Kühl AA, Rubbert-Roth A, Lorenz HM, et al. The proteasome inhibitior bortezomib depletes plasma cells and ameliorates clinical manifestations of refractory systemic lupus erythematosus. Ann Rheum Dis. 2015;74(7):1474-8.

104. De Luca F, Shoenfeld Y. The microbiome in autoimmune diseases. Clinical and Experimental Immunology. 2019;195(1):74-85.

105. Gill SR, Pop M, DeBoy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, et al. Metagenomic analysis of the human distal gut microbiome. Science. 2006;312(5778):1355-9.

106. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. Nature Reviews Immunology. 2009;9(5):313-23.

107. Handelsman J. Metagenomics: Application of genomics to uncultured microorganisms. Microbiology and Molecular Biology Reviews. 2004;68(4):669-+.

108. Qin JJ, Li RQ, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464(7285):59-U70.

109. Adak A, Khan MR. An insight into gut microbiota and its functionalities. Cellular and Molecular Life Sciences. 2019;76(3):473-93.

110. Weinstock GM. Genomic approaches to studying the human microbiota. Nature. 2012;489(7415):250-6.

111. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature. 2012;489(7415):220-30.

112. Rosenbaum JT, Silverman GJ. The Microbiome and Systemic Lupus Erythematosus. New England Journal of Medicine. 2018;378(23):2236-7.

113. Savage DC. MICROBIAL ECOLOGY OF THE GASTROINTESTINAL TRACT. Annual Review of Microbiology. 1977;31(1):107-33.

114. Huttenhower C, Gevers D, Knight R, Abubucker S, Badger JH, Chinwalla AT, et al. Structure, function and diversity of the healthy human microbiome. Nature. 2012;486(7402):207-14.

115. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. Nature Reviews Immunology. 2004;4(6):478-85.

116. Sender R, Fuchs S, Milo R. Are We Really Vastly Outnumbered? Revisiting the Ratio of Bacterial to Host Cells in Humans. Cell. 2016;164(3):337-40.

117. Medzhitov R. Recognition of microorganisms and activation of the immune response. Nature. 2007;449(7164):819-26.

118. Kamada N, Seo SU, Chen GY, Nunez G. Role of the gut microbiota in immunity and inflammatory disease. Nature Reviews Immunology. 2013;13(5):321-35.

119. Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science. 2005;307(5717):1915-20.

120. Zhang LS, Qing PY, Yang H, Wu YK, Liu Y, Luo YB. Gut Microbiome and Metabolites in Systemic Lupus Erythematosus: Link, Mechanisms and Intervention. Frontiers in Immunology. 2021;12.

121. Flint HJ, Scott KP, Louis P, Duncan SH. The role of the gut microbiota in nutrition and health. Nature Reviews Gastroenterology & Hepatology. 2012;9(10):577-89.

122. Hooper LV, Macpherson AJ. Immune adaptations that maintain homeostasis with the intestinal microbiota. Nature Reviews Immunology. 2010;10(3):159-69.

123. Schroeder BO, Bäckhed F. Signals from the gut microbiota to distant organs in physiology and disease. Nat Med. 2016;22(10):1079-89.

124. Lange K, Buerger M, Stallmach A, Bruns T. Effects of Antibiotics on Gut Microbiota. Digestive Diseases. 2016;34(3):260-8.

125. Saadat YR, Hejazian M, Bastami M, Khatibi SMH, Ardalan M, Vahed SZ. The role of microbiota in the pathogenesis of lupus: Dose it impact lupus nephritis? Pharmacological Research. 2019;139:191-8.

126. Yaigoub H, Fath N, Tirichen H, Wu CX, Li RS, Li YF. Bidirectional crosstalk between dysbiotic gut microbiota and systemic lupus erythematosus: What is new in therapeutic approaches? Clinical Immunology. 2022;244.

127. Zhang HS, Liao XF, Sparks JB, Luo XM. Dynamics of Gut Microbiota in Autoimmune Lupus. Applied and Environmental Microbiology. 2014;80(24):7551-60.

128. Macia L, Nanan R, Hosseini-Beheshti E, Grau GE. Host- and Microbiota-Derived Extracellular Vesicles, Immune Function, and Disease Development. International Journal of Molecular Sciences. 2020;21(1).

129. Neuman H, Koren O. The gut microbiota: a possible factor influencing systemic lupus erythematosus. Current Opinion in Rheumatology. 2017;29(4):374-7.

130. Shamriz O, Mizrahi H, Werbner M, Shoenfeld Y, Avni O, Koren O. Microbiota at the crossroads of autoimmunity. Autoimmunity Reviews. 2016;15(9):859-69.

131. Jandhyala SM, Talukdar R, Subramanyam C, Vuyyuru H, Sasikala M, Reddy DN. Role of the normal gut microbiota. World Journal of Gastroenterology. 2015;21(29):8787-803.

132. Belkaid Y, Hand TW. Role of the Microbiota in Immunity and Inflammation. Cell. 2014;157(1):121-41.

133. Chung HC, Pamp SJ, Hill JA, Surana NK, Edelman SM, Troy EB, et al. Gut Immune Maturation Depends on Colonization with a Host-Specific Microbiota. Cell. 2012;149(7):1578-93.

134. Chen YZ, Chaudhary N, Yang N, Granato A, Turner JA, Howard SL, et al. Microbial symbionts regulate the primary Ig repertoire. Journal of Experimental Medicine. 2018;215(5):1397-415.

135. Ma Y, Shi N, Li M, Chen F, Niu H. Applications of Next-generation Sequencing in Systemic Autoimmune Diseases. Genomics, Proteomics & Bioinformatics. 2015;13(4):242-9.

136. Sterlin D, Fadlallah J, Slack E, Gorochov G. The antibody/microbiota interface in health and disease. Mucosal Immunology. 2020;13(1):3-11.

137. Levy M, Kolodziejczyk AA, Thaiss CA, Elinav E. Dysbiosis and the immune system. Nature Reviews Immunology. 2017;17(4):219-32.

138. Olesen SW, Alm EJ. Dysbiosis is not an answer. Nature Microbiology. 2016;1(12):16228.

139. Hevia A, Milani C, Lopez P, Cuervo A, Arboleya S, Duranti S, et al. Intestinal Dysbiosis Associated with Systemic Lupus Erythematosus. Mbio. 2014;5(5).

140. Azzouz D, Omarbekova A, Heguy A, Schwudke D, Gisch N, Rovin BH, et al. Lupus nephritis is linked to disease-activity associated expansions and immunity to a gut commensal. Annals of the Rheumatic Diseases. 2019;78(7):947-56.

141. Rodriguez-Carrio J, Lopez P, Sanchez B, Gonzalez S, Gueimonde M, Margolles A, et al. Intestinal Dysbiosis Is Associated with Altered Short-Chain Fatty Acids and Serum-Free Fatty Acids in Systemic Lupus Erythematosus. Frontiers in Immunology. 2017;8.

142. He Z, Shao T, Li H, Xie Z, Wen C. Alterations of the gut microbiome in Chinese patients with systemic lupus erythematosus. Gut Pathog. 2016;8:64.

143. Greiling TM, Dehner C, Chen X, Hughes K, Iñiguez AJ, Boccitto M, et al. Commensal orthologs of the human autoantigen Ro60 as triggers of autoimmunity in lupus. Sci Transl Med. 2018;10(434).

144. Li Y, Wang HF, Li X, Li HX, Zhang Q, Zhou HW, et al. Disordered intestinal microbes are associated with the activity of Systemic Lupus Erythematosus. Clin Sci (Lond). 2019;133(7):821-38.

145. Zhang SX, Wang J, Chen JW, Zhang MX, Zhang YF, Hu FY, et al. The level of peripheral regulatory T cells is linked to changes in gut commensal microflora in patients with systemic lupus erythematosus. Ann Rheum Dis. 2021;80(11):e177.

146. van der Meulen TA, Harmsen HJM, Vila AV, Kurilshikov A, Liefers SC, Zhernakova A, et al. Shared gut, but distinct oral microbiota composition in primary Sjögren's syndrome and systemic lupus erythematosus. J Autoimmun. 2019;97:77-87.

147. Guo M, Wang H, Xu S, Zhuang Y, An J, Su C, et al. Alteration in gut microbiota is associated with dysregulation of cytokines and glucocorticoid therapy in systemic lupus erythematosus. Gut Microbes. 2020;11(6):1758-73.

148. López P, de Paz B, Rodríguez-Carrio J, Hevia A, Sánchez B, Margolles A, et al. Th17 responses and natural IgM antibodies are related to gut microbiota composition in systemic lupus erythematosus patients. Sci Rep. 2016;6:24072.

149. Gerges MA, Esmaeel NE, Makram WK, Sharaf DM, Gebriel MG. Altered Profile of Fecal Microbiota in Newly Diagnosed Systemic Lupus Erythematosus Egyptian Patients. Int J Microbiol. 2021;2021:9934533.

150. Toumi E, Goutorbe B, Plauzolles A, Bonnet M, Mezouar S, Militello M, et al. Gut microbiota in systemic lupus erythematosus patients and lupus mouse model: a cross species comparative analysis for biomarker discovery. Front Immunol. 2022;13:943241.

151. Luo XM, Edwards MR, Mu Q, Yu Y, Vieson MD, Reilly CM, et al. Gut Microbiota in Human Systemic Lupus Erythematosus and a Mouse Model of Lupus. Appl Environ Microbiol. 2018;84(4).

152. He J, Chan T, Hong X, Zheng F, Zhu C, Yin L, et al. Microbiome and Metabolome Analyses Reveal the Disruption of Lipid Metabolism in Systemic Lupus Erythematosus. Front Immunol. 2020;11:1703.

153. Man SM, Kaakoush NO, Mitchell HM. The role of bacteria and pattern-recognition receptors in Crohn's disease. Nature Reviews Gastroenterology & Hepatology. 2011;8(3):152-68.

154. Magne F, Gotteland M, Gauthier L, Zazueta A, Pesoa S, Navarrete P, et al. The Firmicutes/Bacteroidetes Ratio: A Relevant Marker of Gut Dysbiosis in Obese Patients? Nutrients. 2020;12(5).

155. Barlow GM, Yu A, Mathur R. Role of the Gut Microbiome in Obesity and Diabetes Mellitus. Nutrition in Clinical Practice. 2015;30(6):787-97.

156. Silverman GJ, Azzouz DF, Alekseyenko AV. Systemic Lupus Erythematosus and dysbiosis in the microbiome: cause or effect or both? Current Opinion in Immunology. 2019;61:80-5.

157. Lin L, Zhang JQ. Role of intestinal microbiota and metabolites on gut homeostasis and human diseases. Bmc Immunology. 2017;18.

158. Posadas-Romero C, Torres-Tamayo M, Zamora-Gonzalez J, Aguilar-Herrera BE, Posadas-Sanchez R, Cardoso-Saldana G, et al. High insulin levels and increased low-density lipoprotein oxidizability in pediatric patients with systemic lupus erythematosus. Arthritis and Rheumatism. 2004;50(1):160-5.

159. Yan B, Huang J, Zhang C, Hu X, Gao M, Shi A, et al. Serum metabolomic profiling in patients with systemic lupus erythematosus by GC/MS. Modern Rheumatology. 2016;26(6):914-22.

160. Wu TF, Xie C, Han J, Ye YJ, Weiel J, Li Q, et al. Metabolic Disturbances Associated with Systemic Lupus Erythematosus. Plos One. 2012;7(6).

161. Borba EF, Bonfa E. Dyslipoproteinemias in systemic lupus erythematosus: influence of disease, activity, and anticardiolipin antibodies. Lupus. 1997;6(6):533-9.

162. He JQ, Chan TL, Hong XP, Zheng FP, Zhu CX, Yin LH, et al. Microbiome and Metabolome Analyses Reveal the Disruption of Lipid Metabolism in Systemic Lupus Erythematosus. Frontiers in Immunology. 2020;11.

163. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature. 2013;504(7480):451-5.

164. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, et al. The Microbial Metabolites, Short-Chain Fatty Acids, Regulate Colonic T_{reg} Cell Homeostasis. Science. 2013;341(6145):569-73.

165. Xiang ST, Qu YQ, Qian SH, Wang RY, Wang Y, Jin YB, et al. Association between systemic lupus erythematosus and disruption of gut microbiota: a meta-analysis. Lupus Science & Medicine. 2022;9(1).

166. Choi SC, Brown J, Gong M, Ge Y, Zadeh M, Li W, et al. Gut microbiota dysbiosis and altered tryptophan catabolism contribute to autoimmunity in lupus-susceptible mice. Sci Transl Med. 2020;12(551).

167. Chen BD, Jia XM, Xu JY, Zhao LD, Ji JY, Wu BX, et al. An Autoimmunogenic and Proinflammatory Profile Defined by the Gut Microbiota of Patients With Untreated Systemic Lupus Erythematosus. Arthritis Rheumatol. 2021;73(2):232-43.

168. Ma Y, Xu X, Li M, Cai J, Wei Q, Niu H. Gut microbiota promote the inflammatory response in the pathogenesis of systemic lupus erythematosus. Molecular Medicine. 2019;25(1):35.

169. Weng NP, Snyder JG, Yu-Lee LY, Marcus DM. Polymorphism of human immunoglobulin VH4 germ-line genes. Eur J Immunol. 1992;22(4):1075-82.

170. van Es JH, Heutink M, Aanstoot H, Logtenberg T. Sequence analysis of members of the human Ig VH4 gene family derived from a single VH locus. Identification of novel germ-line members. The Journal of Immunology. 1992;149(2):492-7.

171. Cappione AJ, Pugh-Bernard AE, Anolik JH, Sanz I. Lupus IgG VH4.34 antibodies bind to a 220-kDa glycoform of CD45/B220 on the surface of human B lymphocytes. J Immunol. 2004;172(7):4298-307.

172. Bhat NM, Bieber MM, Teng NN. Cytotoxicity of murine B lymphocytes induced by human VH4-34 (VH4.21) gene-encoded monoclonal antibodies. Clin Immunol Immunopathol. 1997;84(3):283-9.

173. Pascual V, Victor K, Spellerberg M, Hamblin TJ, Stevenson FK, Capra JD. VH restriction among human cold agglutinins. The VH4-21 gene segment is required to encode anti-I and anti-i specificities. J Immunol. 1992;149(7):2337-44.

174. Hakomori S. Blood group ABH and Ii antigens of human erythrocytes: chemistry, polymorphism, and their developmental change. Semin Hematol. 1981;18(1):39-62.

175. Bhat NM, Bieber MM, Stevenson FK, Teng NN. Rapid cytotoxicity of human B lymphocytes induced by VH4-34 (VH4.21) gene-encoded monoclonal antibodies. Clin Exp Immunol. 1996;105(1):183-90.

176. Stevenson FK, Smith GJ, North J, Hamblin TJ, Glennie MJ. Identification of normal B-cell counterparts of neoplastic cells which secrete cold agglutinins of anti-I and anti-i specificity. Br J Haematol. 1989;72(1):9-15.

177. Potter KN, Li Y, Pascual V, Williams RC, Jr., Byres LC, Spellerberg M, et al. Molecular characterization of a cross-reactive idiotope on human immunoglobulins utilizing the VH4-21 gene segment. J Exp Med. 1993;178(4):1419-28.

178. Li Y, Spellerberg MB, Stevenson FK, Capra JD, Potter KN. The I binding specificity of human VH 4-34 (VH 4-21) encoded antibodies is determined by both VH framework region 1 and complementarity determining region 3. J Mol Biol. 1996;256(3):577-89.

179. Meffre E, Salmon JE. Autoantibody selection and production in early human life. J Clin Invest. 2007;117(3):598-601.

180. Pascual V, Capra JD. VH4-21, a human VH gene segment overrepresented in the autoimmune repertoire. Arthritis Rheum. 1992;35(1):11-8.

181. Bhat NM, Bieber MM, Chapman CJ, Stevenson FK, Teng NN. Human antilipid A monoclonal antibodies bind to human B cells and the i antigen on cord red blood cells. J Immunol. 1993;151(9):5011-21.

182. Silberstein LE, Jefferies LC, Goldman J, Friedman D, Moore JS, Nowell PC, et al. Variable region gene analysis of pathologic human autoantibodies to the related i and I red blood cell antigens. Blood. 1991;78(9):2372-86.

183. Bashford-Rogers RJM, Smith KGC, Thomas DC. Antibody repertoire analysis in polygenic autoimmune diseases. Immunology. 2018;155(1):3-17.

184. Børretzen M, Chapman C, Stevenson FK, Natvig JB, Thompson KM. Structural analysis of VH4-21 encoded human IgM allo- and autoantibodies against red blood cells. Scand J Immunol. 1995;42(1):90-7.

185. Potter KN, Li YC, Capra JD. The cross-reactive idiotopes recognized by the monoclonal antibodies 9G4 and LC1 are located in framework region 1 of two non-overlapping subsets of human VH4 family encoded antibodies. Scand J Immunol. 1994;40(1):43-9.

186. Thompson KM, Sutherland J, Barden G, Melamed MD, Randen I, Natvig JB, et al. Human monoclonal antibodies against blood group antigens preferentially express a VH4-21 variable region gene-associated epitope. Scand J Immunol. 1991;34(4):509-18.

187. Zheng NY, Wilson K, Wang X, Boston A, Kolar G, Jackson SM, et al. Human immunoglobulin selection associated with class switch and possible tolerogenic origins for C delta class-switched B cells. J Clin Invest. 2004;113(8):1188-201.

188. Kraj P, Friedman DF, Stevenson F, Silberstein LE. Evidence for the overexpression of the VH4-34 (VH4.21) Ig gene segment in the normal adult human peripheral blood B cell repertoire. J Immunol. 1995;154(12):6406-20.

189. Isenberg D, Spellerberg M, Williams W, Griffiths M, Stevenson F. Identification of the 9G4 idiotope in systemic lupus erythematosus. Br J Rheumatol. 1993;32(10):876-82.

190. Isenberg DA, McClure C, Farewell V, Spellerberg M, Williams W, Cambridge G, et al. Correlation of 9G4 idiotope with disease activity in patients with systemic lupus erythematosus. Ann Rheum Dis. 1998;57(9):566-70.

191. van Vollenhoven RF, Bieber MM, Powell MJ, Gupta PK, Bhat NM, Richards KL, et al. VH4-34 encoded antibodies in systemic lupus erythematosus: a specific diagnostic marker that correlates with clinical disease characteristics. J Rheumatol. 1999;26(8):1727-33.

192. Bhat NM, Lee LM, van Vollenhoven RF, Teng NN, Bieber MM. VH4-34 encoded antibody in systemic lupus erythematosus: effect of isotype. J Rheumatol. 2002;29(10):2114-21.

193. Mockridge CI, Rahman A, Buchan S, Hamblin T, Isenberg DA, Stevenson FK, et al. Common patterns of B cell perturbation and expanded V4-34 immunoglobulin gene usage in autoimmunity and infection. Autoimmunity. 2004;37(1):9-15.

194. Chapman CJ, Spellerberg MB, Smith GA, Carter SJ, Hamblin TJ, Stevenson FK. Autoanti-red cell antibodies synthesized by patients with infectious mononucleosis utilize the VH4-21 gene segment. J Immunol. 1993;151(2):1051-61.

195. Stevenson FK, Longhurst C, Chapman CJ, Ehrenstein M, Spellerberg MB, Hamblin TJ, et al. Utilization of the VH4-21 gene segment by anti-DNA antibodies from patients with systemic lupus erythematosus. J Autoimmun. 1993;6(6):809-25.

196. Liu S, Hou XL, Sui WG, Lu QJ, Hu YL, Dai Y. Direct measurement of B-cell receptor repertoire's composition and variation in systemic lupus erythematosus. Genes Immun. 2017;18(1):22-7.

197. Cambridge G, Isenberg DA, Edwards JC, Leandro MJ, Migone TS, Teodorescu M, et al. B cell depletion therapy in systemic lupus erythematosus: relationships among serum B lymphocyte stimulator levels, autoantibody profile and clinical response. Ann Rheum Dis. 2008;67(7):1011-6.

198. Paliwal S, Thakkar D, Chin WJ, Lee W, Neo JYJ, Hanson B, et al. LSO-006 Targeting pathogenic auto-reactive VH4-34 B cells with a rationally developed and highly specific anti-VH4-34 antibody offers a new therapeutic approach for VH4-34 autoimmune disorders. Lupus Science & Medicine. 2023;10(Suppl 1):A37-A.

199. Odendahl M, Jacobi A, Hansen A, Feist E, Hiepe F, Burmester GR, et al. Disturbed peripheral B lymphocyte homeostasis in systemic lupus erythematosus. J Immunol. 2000;165(10):5970-9.

200. Richardson C, Chida AS, Adlowitz D, Silver L, Fox E, Jenks SA, et al. Molecular basis of 9G4 B cell autoreactivity in human systemic lupus erythematosus. J Immunol. 2013;191(10):4926-39.

201. Ruff WE, Dehner C, Kim WJ, Pagovich O, Aguiar CL, Yu AT, et al. Pathogenic Autoreactive T and B Cells Cross-React with Mimotopes Expressed by a Common Human Gut Commensal to Trigger Autoimmunity. Cell Host Microbe. 2019;26(1):100-13 e8.

202. Palm NW, de Zoete MR, Cullen TW, Barry NA, Stefanowski J, Hao L, et al. Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. Cell. 2014;158(5):1000-10.

203. Sabouri Z, Schofield P, Horikawa K, Spierings E, Kipling D, Randall KL, et al. Redemption of autoantibodies on anergic B cells by variable-region glycosylation and mutation away from self-reactivity. Proc Natl Acad Sci U S A. 2014;111(25):E2567-75.

204. Slack E, Hapfelmeier S, Stecher B, Velykoredko Y, Stoel M, Lawson MA, et al. Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism. Science. 2009;325(5940):617-20.

205. Schoch CL, Ciufo S, Domrachev M, Hotton CL, Kannan S, Khovanskaya R, et al. NCBI Taxonomy: a comprehensive update on curation, resources and tools. Database (Oxford). 2020;2020.

206. Intestinibacter bartlettii [09/03/2023]. Available from: https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=261299.

207. Christensenellaceae bacterium [09/03/2023]. Available from: https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=2054177.

208. Anaerostipes hadrus [09/03/2023]. Available from: https://bacdive.dsmz.de/strain/5427.

209. del Rincon I, Zeidel M, Rey E, Harley JB, James JA, Fischbach M, et al. Delineation of the human systemic lupus erythematosus anti-Smith antibody response using phage-display combinatorial libraries. J Immunol. 2000;165(12):7011-6.

210. Grillot-Courvalin C, Brouet JC, Piller F, Rassenti LZ, Labaume S, Silverman GJ, et al. An anti-B cell autoantibody from Wiskott-Aldrich syndrome which recognizes i blood group specificity on normal human B cells. Eur J Immunol. 1992;22(7):1781-8.

211. Bleesing JJ, Brown MR, Novicio C, Guarraia D, Dale JK, Straus SE, et al. A composite picture of TcR alpha/beta(+) CD4(-)CD8(-) T Cells (alpha/beta-DNTCs) in humans with autoimmune lymphoproliferative syndrome. Clin Immunol. 2002;104(1):21-30.

212. Båve U, Alm GV, Rönnblom L. The combination of apoptotic U937 cells and lupus IgG is a potent IFN-alpha inducer. J Immunol. 2000;165(6):3519-26.

213. Blanco P, Palucka AK, Gill M, Pascual V, Banchereau J. Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. Science. 2001;294(5546):1540-3.

214. Majeti R, Xu Z, Parslow TG, Olson JL, Daikh DI, Killeen N, et al. An inactivating point mutation in the inhibitory wedge of CD45 causes lymphoproliferation and autoimmunity. Cell. 2000;103(7):1059-70.

215. Cyster JG, Healy JI, Kishihara K, Mak TW, Thomas ML, Goodnow CC. Regulation of B-lymphocyte negative and positive selection by tyrosine phosphatase CD45. Nature. 1996;381(6580):325-8.

216. Thomas MD, Clough K, Melamed MD, Stevenson FK, Chapman CJ, Spellerberg MB, et al. A human monoclonal antibody encoded by the V4-34 gene segment recognises melanoma-associated ganglioside via CDR3 and FWR1. Hum Antibodies. 1999;9(2):95-106.

217. Williams GW, Bluestein HG, Steinberg AD. Brain-reactive lymphocytotoxic antibody in the cerebrospinal fluid of patients with systemic lupus erythematosus: correlation with central nervous system involvement. Clin Immunol Immunopathol. 1981;18(1):126-32.

218. Anolik J, Sanz I. B cells in human and murine systemic lupus erythematosus. Curr Opin Rheumatol. 2004;16(5):505-12.

219. Sinha AA, Lopez MT, McDevitt HO. Autoimmune diseases: the failure of self tolerance. Science. 1990;248(4961):1380-8.

220. Shi L, Zhang Z, Yu AM, Wang W, Wei Z, Akhter E, et al. The SLE transcriptome exhibits evidence of chronic endotoxin exposure and has widespread dysregulation of non-coding and coding RNAs. PLoS One. 2014;9(5):e93846.

221. Maglione PJ, Simchoni N, Black S, Radigan L, Overbey JR, Bagiella E, et al. IRAK-4 and MyD88 deficiencies impair IgM responses against T-independent bacterial antigens. Blood. 2014;124(24):3561-71.

222. Manfredo Vieira S, Hiltensperger M, Kumar V, Zegarra-Ruiz D, Dehner C, Khan N, et al. Translocation of a gut pathobiont drives autoimmunity in mice and humans. Science. 2018;359(6380):1156-61.

223. Berer K, Mues M, Koutrolos M, Rasbi ZA, Boziki M, Johner C, et al. Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. Nature. 2011;479(7374):538-41.

224. Markle JG, Frank DN, Mortin-Toth S, Robertson CE, Feazel LM, Rolle-Kampczyk U, et al. Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. Science. 2013;339(6123):1084-8.

225. Proal AD, Albert PJ, Marshall TG. The human microbiome and autoimmunity. Curr Opin Rheumatol. 2013;25(2):234-40.

226. Forslund K, Hildebrand F, Nielsen T, Falony G, Le Chatelier E, Sunagawa S, et al. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. Nature. 2015;528(7581):262-6.

227. Wingfield B, Coleman S, McGinnity TM, Bjourson AJ. Robust Microbial Markers for Non-Invasive Inflammatory Bowel Disease Identification. IEEE/ACM Trans Comput Biol Bioinform. 2019;16(6):2078-88.

228. Pascual V, Victor K, Lelsz D, Spellerberg MB, Hamblin TJ, Thompson KM, et al. Nucleotide sequence analysis of the V regions of two IgM cold agglutinins. Evidence that the VH4-21 gene segment is responsible for the major cross-reactive idiotype. J Immunol. 1991;146(12):4385-91.

229. Chen JW, Rice TA, Bannock JM, Bielecka AA, Strauss JD, Catanzaro JR, et al. Autoreactivity in naïve human fetal B cells is associated with commensal bacteria recognition. Science. 2020;369(6501):320-5.

230. Feizi T. Structural and biological aspects of blood group I and i antigens on glycolipids and glycoproteins. Rev Fr Transfus Immunohematol. 1980;23(5):563-77.

231. Eda S, Yamanaka M, Beppu M. Carbohydrate-mediated phagocytic recognition of early apoptotic cells undergoing transient capping of CD43 glycoprotein. J Biol Chem. 2004;279(7):5967-74.

232. Feizi T, Monger E. Search for I antigen in human tissues. Nature. 1967;216(5119):1025-6.

233. Feizi T. Immunochemical studies of mammalian glycoproteins with blood group I activity. Clin Sci Mol Med. 1973;45(3):17P.

234. Feizi T, Turberville C, Westwood JH. Blood-group precursors and cancer-related antigens. Lancet. 1975;2(7931):391-3.

235. Feizi T. The I and i antigens on certain normal and pathologic tissues. Rev Fr Transfus Immunohematol. 1978;21(1):165-74.

236. Feizi T. The blood group Ii system: a carbohydrate antigen system defined by naturally monoclonal or oligoclonal autoantibodies of man. Immunol Commun. 1981;10(2):127-56.

237. Schutte ME, van Es JH, Silberstein LE, Logtenberg T. VH4.21-encoded natural autoantibodies with anti-i specificity mirror those associated with cold hemagglutinin disease. J Immunol. 1993;151(11):6569-76.

238. Smith G, Spellerberg M, Boulton F, Roelcke D, Stevenson F. The immunoglobulin VH gene, VH4-21, specifically encodes autoanti-red cell antibodies against the I or i antigens. Vox Sang. 1995;68(4):231-5.

239. Silberstein LE, George A, Durdik JM, Kipps TJ. The V4-34 encoded anti-i autoantibodies recognize a large subset of human and mouse B-cells. Blood Cells Mol Dis. 1996;22(2):126-38.

240. Bhat NM, Bieber MM, Spellerberg MB, Stevenson FK, Teng NN. Recognition of auto- and exoantigens by V4-34 gene encoded antibodies. Scand J Immunol. 2000;51(2):134-40.

241. Prendergast MM, Lastovica AJ, Moran AP. Lipopolysaccharides from Campylobacter jejuni O:41 strains associated with Guillain-Barré syndrome exhibit mimicry of GM1 ganglioside. Infect Immun. 1998;66(8):3649-55.

242. Nelson TA, Holmes S, Alekseyenko AV, Shenoy M, Desantis T, Wu CH, et al. PhyloChip microarray analysis reveals altered gastrointestinal microbial communities in a rat model of colonic hypersensitivity. Neurogastroenterol Motil. 2011;23(2):169-77, e41-2.

8. APPENDICES

Appendix-1: Research Ethical Approval

Yale University

300 George Street, Room 353F
New Haven, Connecticut 06511

Eric Meffre, Ph.D.
Adjunct Associate Professor of
Immunology and Medicine

Telephone 203 737-4535
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July 11, 2022

To whom it may concern,

This is to certify that Fatma Naz Cemre Kalayci (student ID N18247200), a MD/PhD student of Hacettepe University Tumor Biology and Immunology Program, whose Turkish adviser is Associate Professor Hande Canpinar, is working on her new PhD thesis “Systemic VH4-34-encoded antibody responses against commensal bacteria in patients with systemic lupus erythematosus."

Her project is approved by the Yale University Institutional Review Board (IRB).

Please feel free to reach out with any questions and concerns.

Yours sincerely,

Eric Meffre, PhD,
Email: eric.meffre@yale.edu

Appendix-2: Originality Report

turnitin

Dijital Makbuz

Bu makbuz ödevinizin Turnitin'e ulaştığını bildirmektedir. Gönderiminize dair bilgiler şöyledir:

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INSTITUTE OF HEALTH SCIENCES

SYSTEMIC VH4-34-ENCODED ANTIBODY RESPONSES
AGAINST COMMENSAL BACTERIA IN PATIENTS
WITH SYSTEMIC LUPUS ERYTHEMATOSUS

Fatma Naz Cemre KALAYCI, MD, PhD

Tumor Biology and Immunology Program
DOCTOR OF PHILOSOPHY THESIS

ANKARA
2023

F.Naz Cemre Kalaycı SYSTEMIC VH4-34-ENCODED ANTIBODY RESPONSES AGAINST COMMENSAL BACTERIA IN PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

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Appendix 3: Contributions of Collaborators

Jean-Nicolas Schickel[1],

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Also, we express our gratitude to Dr. Hedda Wardemann[8] for generously providing the sequences utilized in this study. Additionally, we extend our appreciation to Dr. L. Devine and C. Wang for their assistance with the cell sorting.

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Appendix-4: Funding sources

The research conducted in this study received financial support from the National Institutes of Health's National Institute of Allergy and Infectious Diseases, specifically through grant numbers AI061093 and AI071087, awarded to Dr. Eric Meffre.

Appendix-5: Publications

International Journal of *Molecular Sciences*

MDPI

Article

Androgen Deprivation Induces Transcriptional Reprogramming in Prostate Cancer Cells to Develop Stem Cell-Like Characteristics

Shiv Verma [1,2], Eswar Shankar [1,2], F. Naz Cemre Kalayci [3], Amrita Mukunda [4], Malek Alassfar [1], Vaibhav Singh [5], E. Ricky Chan [6], Gregory T. MacLennan [7] and Sanjay Gupta [1,2,8,9,10,*]

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Abstract: Enzalutamide, an antiandrogen, is approved for therapy of castration resistant prostate cancer. Clinical applications have shown that approximately 30% of patients acquire resistance after a short period of treatment. However, the molecular mechanisms underlying this resistance is not completely understood. To identify transcriptomic signatures associated with acquisition of drug resistance we profiled gene expression of paired enzalutamide sensitive and resistant human prostate cancer LNCaP (lymph node carcinoma of the prostate) and C4-2B cells. Overlapping genes differentially regulated in the enzalutamide resistant cells were ranked by Ingenuity Pathway Analysis and their functional validation was performed using ingenuity knowledge database followed by confirmation to correlate transcript with protein expression. Analysis revealed that genes associated with cancer stem cells, such as *POU5F1* (OCT4), *SOX2*, *NANOG*, *BMI1*, *BMP2*, *CD44*, *SOX9*, and *ALDH1* were markedly upregulated in enzalutamide resistant cells. Amongst the pathways enriched in the enzalutamide-resistant cells were those associated with RUNX2, hedgehog, integrin signaling, and molecules associated with elastic fibers. Further examination of a patient cohort undergoing ADT and its comparison with no-ADT group demonstrated high expression of POU5F1 (OCT4), ALDH1, and SOX2 in ADT specimens, suggesting that they may be clinically relevant therapeutic targets. Altogether, our approach exhibits the potential of integrative transcriptomic analyses to identify critical genes and pathways of antiandrogen resistance as a promising approach for designing novel therapeutic strategies to circumvent drug resistance.

Keywords: antiandrogens; castrate resistant prostate cancer; cancer stem cells; enzalutamide resistance; transcriptional reprogramming

9. CURRICULUM VITAE

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