B Cell Ontogeny and Stromal Regulation of Homeostatic Antibody Responses to Commensal Antigens in Humans

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A mis abuelos,

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ABSTRACT

The spleen contains a unique subset of innate-like marginal zone (MZ) B cells that rapidly mount protective antibody responses to invasive encapsulated bacteria. Here we show that human splenic MZ B cells also mount homeostatic antibody responses to commensal antigens undergoing systemic translocation from mucosal surfaces. This humoral response decreased upon splenectomy and correlated with splenic capture of commensal antigens and with the generation of MZ B cell-derived plasmablasts and plasma cells following GC-independent and -dependent pathways. Remarkably, commensal antigens targeted marginal reticular cells (MRCs) in addition to macrophages. Besides canonical stromal properties, MRCs expressed macrophage-like immunoactivating functions that promoted robust MZ B cell responses to microbial ligands. These responses required both contact-dependent and contact-independent signals from MRCs, including MAdCAM-1 and VCAM-1 adhesion molecules as well as cytokines such as BAFF and APRIL, respectively. Thus, MRCs may function as stromal activator cells that integrate the innate and adaptive arms of the splenic immune system to orchestrate a secondary line of systemic defense against commensal antigens of mucosal origin.

RESUM

La melsa conté un particular tipus de cèl·lules B de la zona marginal (MZ) que ràpidament produeixen anticossos contra bacteris invasius encapsulats. Nosaltres demostrem que el limfòcits B de la MZ porten a terme respostes humorals, en condicions homeostàtiques, enfront antígens comensals que arriben a la circulació sanguínia provinents de la superfície de les mucoses. Aquesta resposta humoral disminueix en pacients sense melsa i es correlaciona amb la captura d'antígens per part d'aquest òrgan, provocant la diferenciació de les cèl·lules B de la MZ a cèl·lules plasmàtiques productores d'anticossos específics de bacteris comensals. Això es duu a terme mitjançant dues vies: l'independent i la dependent de limfòcits T. Sorprenentment, la captura d'antígens comensals a la melsa involucra cèl·lules reticulars marginals (MRC) i els macròfags. A més de les propietats típiques de cèl·lules estromals, les MRC presenten funcions immuno-activadores similars a les dels macròfags, promovent les respostes de les cèl·lules B de la MZ contra lligants microbians. Aquestes respostes requereixen senyals dependents de contacte amb les MRC, a través de molècules d'adhesió com MAdCAM-1 i VCAM-1, i senyals que no depenen del contacte intercel.lular, com la producció de les citocines BAFF i APRIL. Per tant, les MRC de melsa operen com a cèl·lules estromals activadores que integren les respostes immunològiques innates i adaptatives per orquestrar una segona línia de defensa contra antígens comensals provinents de les mucoses.

PREFACE

Each year, blood-borne infections by encapsulated bacteria account for millions of deaths among neonates and infants. The generation of protection against these pathogenic bacteria involves splenic phagocytes and marginal zone (MZ) B cells, a population of innate-like lymphocytes that cooperate with follicular (FO) B cells to mount antibody responses against invasive bacteria. Currently, there are two types of vaccines that provide protection against encapsulated pathogens, but their efficacy varies with age and spleen function. Vaccines composed of unconjugated capsular polysaccharides (CPS) generate protective antibodies in adults but not infants, children or patients with congenital, functional or post-operatory asplenia. These limitations have been overcome with the development of vaccines containing protein-conjugated CPS, which are highly efficient in most individuals. However, these conjugated vaccines are more expensive, do not generate protection in T cell-deficient individuals, and contain CPS from fewer bacterial serotypes. Therefore, there is a continuous and active effort to develop new and more efficient vaccines against various encapsulated pathogens. To achieve this goal, it is imperative to better understand the mechanisms whereby splenic B cells generate antibodies. In this work, we elucidated the clonal architecture, reactivity and stromal regulation of homeostatic MZ B cell responses to commensal bacteria physiologically emerging from mucosal surfaces. These responses target highly conserved antigenic epitopes, including CPS, and therefore their study may provide valuable lessons as to how MZ B cells organize protective responses against pathogens.

ABBREVIATIONS

AID	Activation-induced cytidine deaminase
APRIL	A proliferation-inducing ligand
ASC	Antibody-secreting cells
BAFF	B-cell activating factor
Bcl-6	B cell lymphoma-6
BCMA	B cell maturation antigen
BCR	B cell receptor
BLIMP-1	B lymphocyte-induced maturation protein 1
BM	Bone marrow
BMSCs	Bone marrow stromal cells
BSAP	B-cell lineage specific activator protein
CCL	Chemokine ligand
CCR	Chemokine receptor
CD40L	CD40 ligand
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CLRs	C-type lectin receptors
CSR	Class switch recombination
CVID	Combined variable immunodeficiency
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
DAPI	4′-6-diamidine-2′-phenylindole
DC	Dendritic cell

DZ	Dark zone
EBV	Epstein–Barr virus
ELISA	Enzyme-Linked ImmunoSorbent Assay
FB	Fibroblasts
FC	Flow Cytometry
FDC	Follicular dendritic cell
FLT3	Fms-like tyrosine kinase 3
FO	Follicle
FRC	Fibroblastic reticular cells
GALT	Gut-associated lymphoid tissue
GC	Germinal center
GM-CSF	Granulocyte macrophage colony-stimulating factor
HLA	Human lecukocyte antigen
ICAM-1	Intercellular Adhesion Molecule 1
IEC	Intestinal epithelial cells
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IL	Interleukin
ILC	Innate lymphoid cell
ILF	Isolated lymphoid follicle
*i*NKT	Invariant natural killer T cells
IRF4	Interferon regulatory factor 4
LN	lymph node

LP	Lamina propria
LTi	Lymphoid tissue inducer
LTo	Lymphoid tissue organizer
LTα1β2	Lymphotoxin alpha1 beta2
LZ	Light zone
MAdCAM-1	Mucosal addressin cell adhesion molecule 1
MALT	Mucosa-associated lymphoid tissues
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MRC	Marginal reticular cell
MZ	Marginal zone
NGS	Next generation sequencing
NK	Natural killer
PAX5	Paired box 5
PB	plasmablast
PC	plasma cell
PD-1	Programmed death-1
PD-L1	Programmed death-ligand-1
PFZ	Perifollicular zone
PP	Peyer's patches
pre-BCR	pre-B cell receptor
PRR	Pattern recognition receptors
RAG	Recombination activating gene
RANKL	Receptor activator of nuclear factor κ-B ligand

RORγt	Retinoic acid-related orphan receptor γt
RP	Red Pulp
SED	Subepithelial dome
SHM	Somatic hypermutation
SLO	Secondary lymphoid organ
TACI	Transmembrane activator and CAML interactor
TCR	T cell receptor
TD	T-cell dependent
TdT	enzyme terminal deoxynucleotidyl transferase
T_{FH}	T follicular helper
T_{FR}	T follicular regulatory
TGF-β	Transforming growth factor β
TI	T-cell independent
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
T_{REG}	T regulatory
TSLP	Thymic stromal lymphopoietin
VCAM-1	Vascular cell adhesion molecule 1
WP	White pulp
XBP1	X-box-binding protein 1

CONTENTS

CHAPTER 1
INTRODUCTION

1. The Immune Sytem

The immune system is composed of physical barriers and biological processes that protect our body against illness. This protection involves the recognition and clearance of external agents such as toxic molecules or more complex microorganisms. Moreover, the immune system recognizes and eliminates apoptotic, necrotic or transformed host cells capable of inducing inflammation or tumour growth[1]. It is a sophisticated system equipped with receptors that have the capacity to sense and discriminate between healthy or harmful molecules[2,3]. However, inherited and/or environmental perturbations of this sytem can cause autoimmunity, inflammation or cancer[4].

The immune system includes two protective and highly integrated layers that generate increasingly specific protection[5]. Innate immunity provides a first line of nonspecific defence and it is constituted by granulocytes, monocytes, macrophages, dendritic cells (DCs) and natural killer (NK) cells that invade tissues upon infection or inflammation. These cells detect and kill pathogens through nonspecific germline-encoded pattern recognition receptors (PRRs), including Toll-like receptors (TLRs)[3].

Adaptive immunity is composed of highly specific T and B cells that recognize microbes through somatically recombined antigen receptors known as T cell receptor (TCR) and B cell receptor (BCR), respectively. These lymphocytes have the capacity to generate memory cells that patrol our organism and mount rapid and powerful protective responses upon secondary antigenic exposure[6].

2. B Cell Development

B cells orchestrate adaptive humoral immune responses by producing immunoglobulin (Ig) molecules commonly known as antibodies. These antibodies recognize specific conformational or linerar epitopes of microbial antigens. B cells were designated as such because they were originally identified in the bursa of Fabricius, where early B cells from birds undergo maturation[7].

In humans, B cell development initially occurs in the fetal liver and continuous in the bone marrow (BM) after birth[8]. Each newly generated B cell carries a transmembrane Ig receptor comprised of two identical heavy chains (H) and two identical light chains (L), which can be either κ or λ[9]. Both IgH and IgL chain molecules include an antigen-binding variable region encoded by recombined V_HDJ_H and V_LJ_L genes, respectively. The V, D and J segments of these genes are organized in multiple families within distinct IgH and IgL loci and their assembly into in-frame V_HDJ_H and V_LJ_L exons requires an antigen-independent diversification process known as V(D)J recombination[10,11]. B cell development proceeds through several intermediate stages that can be distinguished on the basis of the expression of various cell-surface markers, transcription factors and ordered patterns of IgH and IgL chain gene rearrangements **(Figure 1)**[12,13]. Progression through these stages involves a cross-talk between B cell precursors and bone marrow stromal cells (BMSCs), which guide B cell development through the expression of both membrane-bound and soluble growth and differentiation factors including interleukin (IL)-7, fms-like tyrosine kinase 3 (FLT3) and thymic stromal lymphopoietin (TSLP)[12,14]. Several transcription factors

regulate the early steps of B cell development, including the Paired-box 5 (Pax5) protein, also known as B-cell lineage specific activator protein (BSAP)[15].

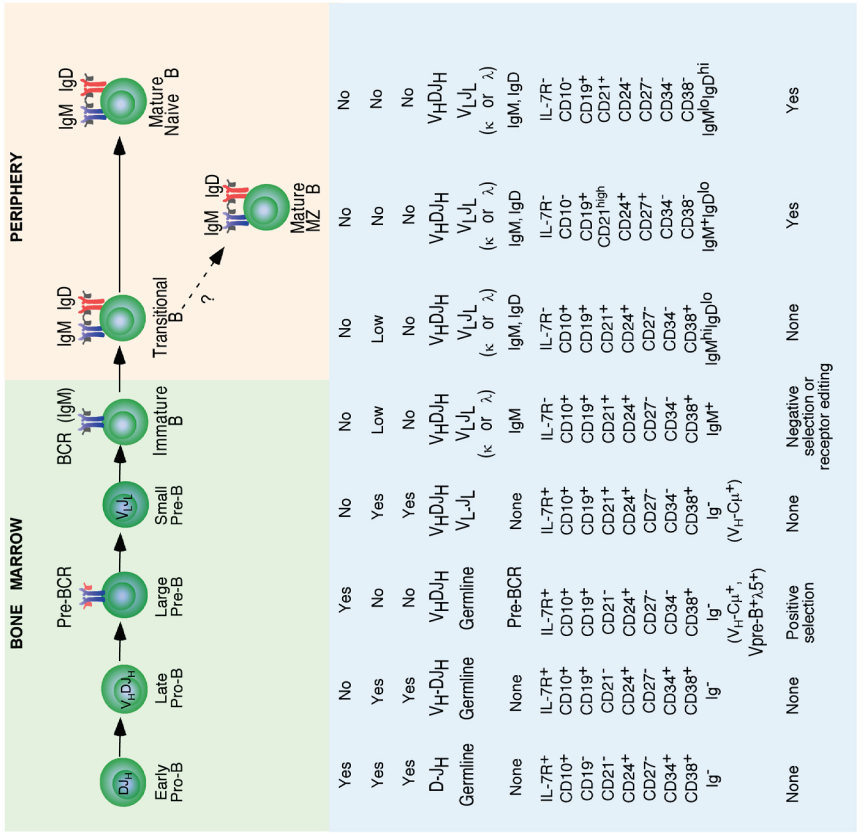


Figure 1. B cell development. Early B cell development occurs in an antigen-independent way through phenotypically distinct differentiation stages characterized by specific Ig gene rearrangement events. Pro-B cells emerge from common lymphoid precursor cells and include early pro-B (or pre-pro-B) and late pro-B (or

pro-B) cells that undergo DJ_H and V-DJ_H gene rearrangements, respectively. These DNA recombination events require RAG1 and RAG2 endonucleases and are associated with D gene diversification by nucleotide addition via the enzyme TdT. Late pro-B cells differentiate to large pre-B cells that express a surface pre-BCR molecule composed of a V_H-Cμ IgH chain and a pseudo (or surrogate) IgL chain formed by Vpre-B and λ5 proteins. Large pre-B cells with in-frame V_HDJ_H rearrangements undergo positive selection and further differentiate into small pre-B cells, which down-regulate surface pre-BCR expression, contain cytoplasmic V_H-Cμ protein and undergo V_L-J_L recombination via RAG proteins. Subsequent assembly of two IgH and two IgL chains leads to the formation of a complete surface BCR on immature B cells. In the presence of strong BCR signals from self-antigens, immature B cells undergo negative selection by clonal deletion. However, some autoreactive immature B cells can be rescued through receptor editing, which requires a new up-regulation of RAG protein expression. Then, immature B cells differentiate into transitional B cells that express both surface IgM and IgD through alternative splicing of a long V_HDJ_H-Cμ-Cδ mRNA. Transitional B cells exit the BM and further differentiate to either mature FO B cells or MZ B cells in SLOs. FO and MZ B cells initiate antibody production after differentiating to plasma cells in response to T cell-dependent or T cell-independent antigens, respectively.

a. V(D)J Recombination

Pro-B cells initially recombine D and J segments in the IgH locus to form a DJ segment that subsequently rearranges with a V segment to assemble a complete V_HDJ_H gene **(Figure 2)**. These recombination events randomly target individual members of multiple V, D and J gene families and require the induction of double-stranded DNA breaks in specific recombination signal sequences by a heterodimeric recombination activating gene (RAG) complex that includes RAG1 and RAG2 proteins[16,17]. The enzyme terminal deoxynucleotidyl transferase (TdT) increases the diversity of Ig genes genes by adding

N-nucleotides at the DJ junction of a recombined V_HDJ_H exon[18]. Productive V_HDJ_H recombination stops the expression of RAG proteins[11], leading to the transcription of the V_HDJ_H gene together with the constant (C) heavy chain μ (Cμ) gene to form a complete IgH chain[19]. Subsequent assembly of the IgH chain with surrogate invariant IgL chain, formed by V-preB and λ5 proteins, is followed by the surface expression of the pre-B cell receptor (pre-BCR) complex, which also includes invariant Igα and Igβ subunits with signalling function[20]. Signals emanating from the pre-BCR are a critical checkpoint for B cell development, because they regulate the expansion of pre-B cells and their further differentiation into immature B cells (positive selection)[21]. Immature B cells re-express RAG proteins to initiate the rearrangement of V and J segments from the IgL locus and form a complete Ig molecule[19]. Of note, RAG proteins target the Igλ locus when the Igκ locus fails to generate an in-frame (or productive) VJ rearrangement. Assembly of the IgL chain with the IgH chain is followed by the expression of a fully competent IgM receptor that functions as a surface B cell antigen receptor (BCR)[22].

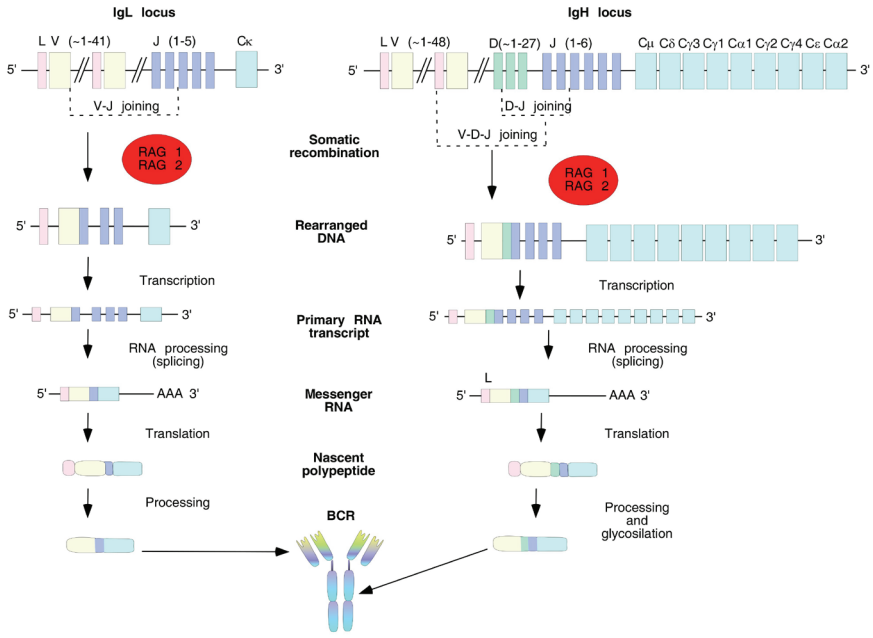


Figure 2. V(D)J recombination. The Ig loci include multiple cassettes of V, D and J (IgH locus and V and J (IgL loci termed Igκ and Igλ) gene segments that undergo random rearrangement during B cell development in the BM through a process involving RAG proteins. In the IgL loci, a V_L gene segment rearranges with a J_L gene segment to generate a V_LJ_L exon. Transcription of V_LJ_L and subsequent splicing of V_LJ_L mRNA to a Cκ or Cλ mRNA generates a V_LJ_L-C_L mRNA that is polyadenilated and then translated into a mature IgL chain protein. In the IgH locus, similar recombination and transcription events lead to the formation of a V_HDJ_H-C_H (Cμ in the earliest stages of B cell differentiation) mRNA that undergoes splicing and polyadenilation to generate a mature IgH protein. In each Ig locus, V gene transcription initiates at the level of a leader (L) sequence positioned upstream of each V segment. Ultimately, two identical IgH and two identical IgL chain proteins are assembled to generate a membrane-bound heterotetrameric protein termed BCR.

b. Checkpoints and Transitional B cells

Following Ig gene rearrangements, immature B cells expressing an autoreactive BCR undergo receptor editing, which involves the

replacement of the V segment in the recombined V_IJ_L gene with an upstream V segment through a RAG-mediated reaction[23]. An immature B cell unable to edit its autoreactive BCR is eliminated by apoptosis through a process referred to as clonal deletion[24]. After successfully passing through this tolerance checkpoint, immature B cells leave the BM as transitional B cells that co-express IgM and IgD through a process of alternative splicing of a long RNA that includes V_HDJ_H as well as Cμ and Cδ exons[25]. Transitional B cells are typically short-lived and functionally immature and express high levels of the developmentally regulated molecules CD10, CD24 and CD38, but not the memory B cell molecule CD27[26]. After colonizing peripheral lymphoid organs, transitional B cells become fully mature but antigenically naïve B cells expressing unmutated V(D)J genes together with surface IgM, IgD and CD19 but not CD24, CD27 and CD38 molecules **(Figure 1)**. At this time, stromal cells provide mandatory survival signals that maintain a highly diversified and fully functional repertoire of peripheral mature B cells capable to recognize virtually any antigens through a vast repertoire of BCR molecules[27,28].

3. Humoral Immunity

a. B Cell Activation and Differentiation

After colonizing secondary lymphoid organs (SLOs) such as the spleen, lymph nodes (LNs) and mucosal associated lymphoid tissues (MALTs), mature B cells undergo complex activaton and differentiation events in response to BCR stimulation by antigen[29]. The nature and presentation modalities of this antigen together with the quality of microenvironmental co-signals determine whether an

antigen-activated B cell will follow either a T-cell dependent (TD) or T-cell independent (TI) differentiation pathway **(Figure 3)**.

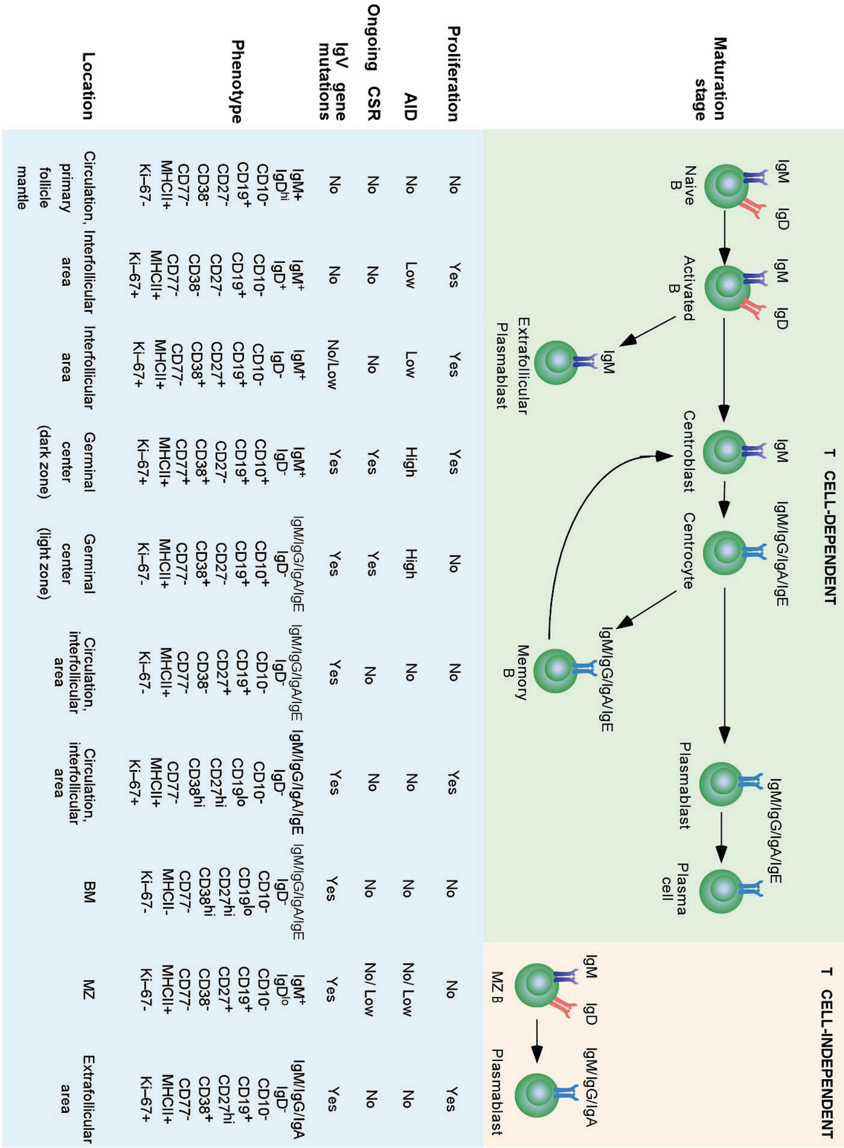


Figure 3. Development of mature B cells in SLOs. Mature B cells progress along phenotypically and genotypically distinct stages of differentiation after encountering antigen in SLOs. Naïve B cells populate the mantle zone of primary follicles and

predominantly differentiate through a TD pathway that involves protein antigen-driven cognate interaction with T_{FH} cells. Activated B cells emerging from this interaction either differentiate along an extrafollicular pathway that leads to the formation of short-lived IgM-PBs or enter the germinal center (GC) to become highly proliferating centroblasts. These cells undergo robust IgV gene somatic hypermutation (SHM) and, after completing class switch recombination (CSR) from IgM to IgG, IgA or very rarely IgE, become non-cycling centrocytes. Both SHM and CSR require the enzyme activation-induced cytidine deaminase (AID). Centrocytes further differentiate to either long-lived memory B cells, which recirculate and can reenter in a GC reaction, or short-lived IgM-PBs and long-lived plasma cells, which finally migrate to the BM. MZ B cells populate the MZ of the spleen and predominantly differentiate through a TI pathway that involves non-cognate interaction with innate immune cells. MZ B cells express moderately mutated IgV genes and differentiate into PBs, which secrete IgM but also class-switched IgG and IgA antibodies.

i. T Cell-Dependent B Cell Responses

In SLOs, naïve B cells typically occupy primary follicles surrounded by interfollicular areas enriched in T cells and DCs. When exposed to proteins antigens from invading pathogens, BCRs belonging to both IgM and IgD isotypes recognize and internalize this antigen[30-32] to initiate activating signals that lead to the down-regulation of IgD expression, followed by antigen processing into peptides that form immunogenic complexes by interacting with class-II human leukocyte antigen (HLA-II) molecules that translocate onto the cell surface. Of note, HLA-II is the human equivalent of major histocompatibility class (MHC)-II in mice.

By up-regulating the CCR7 chemokine receptor, antigen-activated B cells follow CCL19 and CCL21 chemokine gradients to colonize the T-B border of a follicle[33]. In this area, B cells establish a cognate

interaction with immature T-follicular helper (T_{FH}) cells specialized in providing powerful B cell helper signals, including CD40 ligand (CD40L), IL-21 and IL-4[34,35]. These T_{FH} cells originate from antigen-specific $CD4^+$ T cells establishing cognate interactions with DCs, a professional antigen-presenting cells (APC) type.

Dependening on the strength of BCR stimulation, helper signals from T_{FH} cells stimulate B cells to enter either a follicular or an extrafollicular differentiation pathway **(Figure 3)**[36]. In the extrafollicular pathway, activated B cells clonally expand and differentiate into short-lived IgM-expressing plasmablasts (PBs)[37]. In the follicular pathway, activated B cells migrate to the center of the follicle upon up-regulating the chemokine receptor CXCR5, which senses chemotactic CXCL13 signals released by follicular dendritic cells (FDCs). This process is accompanied by a concomitant CXCR5-dependent entry of mature T_{FH} cells into the follicle. In this pathway, activated B cells express the B cell lymphoma-6 (Bcl-6) transcription factor, which establishes a genetic program that ultimately triggers a germinal center (GC) reaction **(Figure 3)**[38]. In systemic SLOs, the GC reaction triggers B cell clonal expansion together with somatic hypermutation (SHM) and IgM-to-IgG class switch recombination (CSR), which lead to the generation of memory B cells and plasma cells (PCs) predominantly expressing IgG antibodies with high affinity for antigen.

Similar to spleen and LNs, Peyer's patches (PPs) and mesenteryc lymph nodes (MLNs) from the gut-associated lymphoid tissue (GALT) as well as follicular structures belonging to other compartments of the MALT contain B cells that initiate extrafollicular

or follicular TD responses[39-41]. In the GALT, GCs are constitutively active due to the continuous presence of an indigenous microbiota that predominantly induces B cell formation of class-switched IgA, a mucosal isotype specilaized in the containment of commensal bacteria and mucosal pathogens. While DCs from SLOs capture antigen from afferent lymphatics, DCs from PPs do so through specialized microfold (M) cells capable to internalize antigen direcly from the intestinal lumen. By generating T_{FH} cells, these antigen-pulsed DCs migrate to the interfollicular region of PPs to activate B cells by means of signals that include CD40L, IL-21 and TGF-β[39-41]. These B cells undergo SHM and IgM-to-IgA CSR in the GC to generate memory B cells and plasma cells expressing high-affinity IgA.

1. Germinal Center Reaction

The GC constitutes the hallmark of a TD antibody response against soluble protein antigens and particulate antigens, including microbes[42,43]. After up-regulating CXCR5 expression, early T_{FH} cells and activated B cells move to the follicle in response to CXCL13 from FDCs[44,45]. Similar to B cells, T_{FH} cells enter a Bcl6-dependent genetic program essential for the maintenance and development of the GC reaction[46-49]. In the early GC reaction, B cells differentiate into centroblasts and clonally proliferate to displace naive IgM^+IgD^+ B cells to the periphery of the follicle, thereby generating the mantle zone typical of an antigen-activated secondary follicle[49]. The rapid proliferation of centroblasts increases the size of the GC and, by day 7 after immunization, this structure becomes polarized into two distinct compartments known as the dark zone (DZ) and the light zone (LZ)[50] **(Figure 4)**.

In the DZ, centroblasts undergo not only clonal expansion, but also SHM and CSR, two Ig gene-diversifying processes highly dependent on the B cell-specific enzyme activation-induced cytidine deaminase enzyme (AID). While CSR diversifies the effector functions of an Ig molecule by replacing IgM with IgG, IgA or IgE, SHM provides a structural correlate for the selection of antibody mutants with higher affinity for antigen[51-54]. Centroblasts eventually migrate to the LZ of the GC, where they become smaller non-dividing centrocytes. In the LZ, centrocytes use their newly mutated BCR to recognize native antigen trapped on the surface of FDCs[55-57]. At the same time, centrocytes establish a cognate interaction with T_{FH} cells, which provide critical helper signals through CD40L and cytokines, including IL-21. Competition for appropriate T_{FH} cell help is the major factor for the selection of GC B cells expressing a BCR with higher affinity for antigen.

Centrocytes expressing a BCR with low affinity for antigen do not receive sufficient survival signals from T_{FH} cells and thereafter undergo apoptosis to be finally engulfed by resident phagocytes known as tingible body macrophages[49,58]. Instead, centrocytes with higher affinity re-enter the DZ of the GC to further ameliorate the "quality" of their BCR by undergoing additional rounds of proliferation and SHM[59]. Subsequently, these centrocytes re-enter the LZ to differentiate into memory B cells or immature PCs upon receving powerful but still poorly understood contact-dependent and contact-indepenent helper signals from T_{FH} cells in the context of a cognate interaction[60-64]. The iterative nature of DZ-LZ migration explains the phenomenon of affinity maturation, which consists in the

generation of B cells capable to increase their antigen affinity over time.

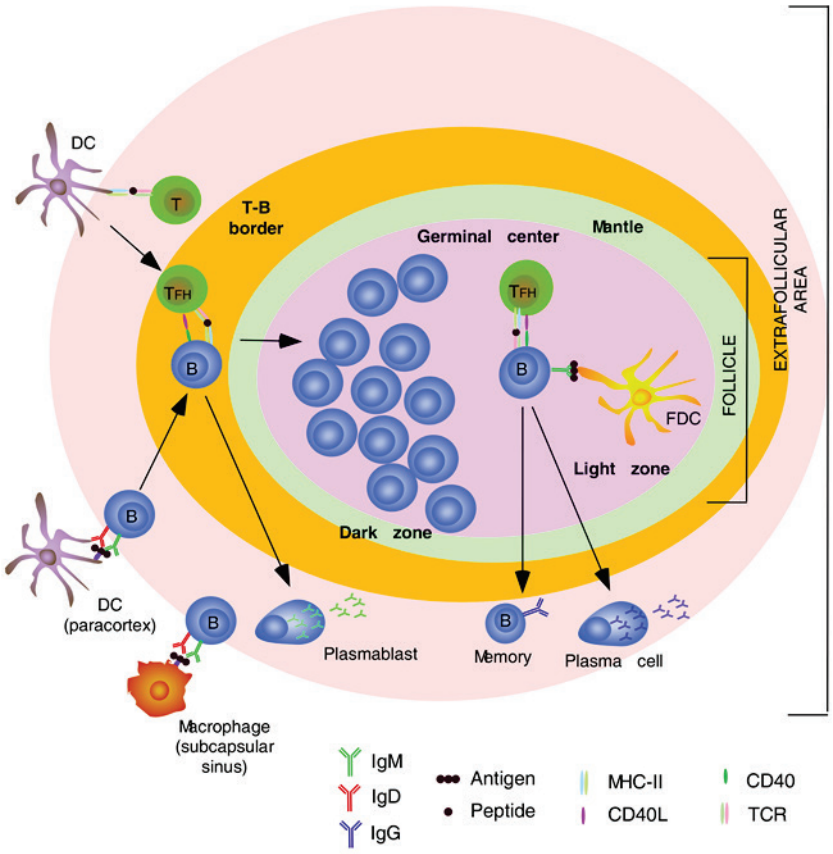


Figure 4. The germinal center reaction. Naïve B cells capture native antigen from subcapsular sinus macrophages and paracortical DCs through the BCR (both IgM and IgD molecules) and subsequently establish a cognate interaction with T_{FH} cells located at the boundary between the follicle and the extrafollicular area (T-B border). After activation by T_{FH} cells via CD40L and IL-21, B cells enter either an extrafollicular pathway to become short-lived PBs or into a follicular pathway to become germinal center centroblasts. In the DZ of the germinal center, centroblasts undergo extensive proliferation, express AID and induce SHM and CSR from IgM to IgG, IgA or IgE (the figure only shows IgG). After exiting the cell cycle, centroblasts differentiate into centrocytes that interact with FDCs located in the light

zone of the germinal center. FDCs expose immune complexes containing native antigen to the BCR. Centrocytes with low-affinity for antigen die by apoptosis, whereas centrocytes with high affinity for antigen differentiate to long-lived memory B cells or plasma cells expressing high-affinity and class-switched antibodies. Memory B cells recirculate, whereas plasma cells migrate to the BM.

a. Class Switch Recombination

Mature B cells expressing somatically recombined Ig V(D)J genes can change their antibody isotype through CSR **(Figure 5)**, an irreversible DNA recombination process that requires AID [65]. CSR modulates the effector functions of an antibody without changing its antigen specificity by replacing the Cμ gene encoding the C_H region of IgM with the Cγ, Cα or Cε gene encoding the C_H region of IgG, IgA or IgE, respectively.

AID specificlly targets intronic switch (S) regions located within the IgH locus located upstream of each C_H gene. These S regions guide CSR by recruiting AID after undergoing germline transcription. Both TD[66] and TI[67] signals induce AID expression and germline transcription by activating multiple transcription factors, namely nuclear factor-κB (NF-κB). These transcription factors activate promoters located upstream of a "cassette" including the intervening (I_H) exon, the S region and the C_H gene. Germline I_H-S-C_H transcription proceeds through the S region and terminates downstream of the C_H gene. The resulting primary transcript physically associates with the template DNA strand of the S region to form a stable DNA-RNA hybrid, which generates R loops in which the displaced non-template strand exists as a G-rich single-stranded DNA. AID deaminates cytosine residues on both strands of the S

region, thereby generating multiple DNA lesions that are ultimately processed into double-stranded DNA breaks.

Fusion of double-stranded DNA breaks at the two recombining S regions through the non-homologous end-joining pathway induces looping-out deletion of the intervening DNA (termed switch circle), thereby juxtaposing the V_HDJ_H exon to a new C_H gene. Of note, the detection of AID, spliced-out secondary germline transcripts and switch circles indicates the presence of actively ongoing CSR in B cells. Secondary germline transcripts derive from the splicing of primary transcripts to remove the intronic S region and join the I_H exon with C_H exons.

Although much debated, it is not clear whether the process of CSR occurs preferentially in the GC or in extrafollicular areas. The fact that CSR requires cell proliferation[68-71] and that the GC contains AID-expressing B cells initially suggested that the DZ was the site specialized in the induction of CSR[72]. However, AID was subsequently found in activated B cells and PBs located in extrafollicular areas of both tonsil and spleen[73]. In addition, mouse studies have shown that CSR can occur as early as 2 days after antigen stimulation, prior to GC formation and does not always require the presence of T cells, including T_{FH} cells[53,74]. Indeed, certain non-protein antigens such as polysaccharides with repetitive structure can induce CSR in the absence of T cells or GCs, probably through the activation of innate-like B cell subsets highly responsive to signals from myeloid and non-myelod cells, including DCs, monocytes, macrophages, neutrophils, stromal cells and epithelial cells[75-78].

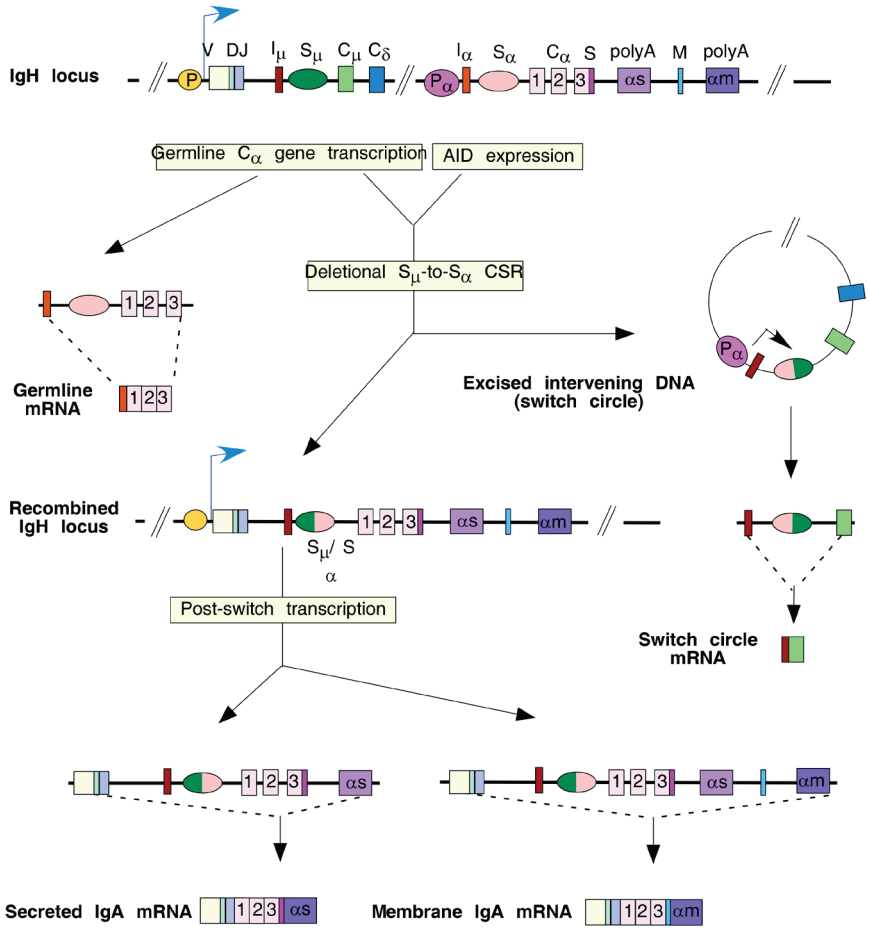


Figure 5. Class switch recombination. The IgH locus contains a rearranged V_HDJ_H exon encoding the antigen-binding domain of an immunoglobulin. B cells produce IgM and IgD through a transcriptional process driven by a promoter (P) upstream of V_HDJ_H. Production of IgG, IgA or IgE with identical antigen specificity but different effector function occurs through CSR. The diagram shows the mechanism of IgA CSR, but a similar mechanism underlies IgG and IgE CSR. Appropriate stimuli induce germline transcription of Cμ and Cα genes from a P located upstream of V_HDJ_H and through an intronic (I) exon and switch (S) region located upstream of Cμ and Cα exons. In addition to yielding sterile Iμ-Cμ and Iα-Cα mRNAs, germline transcription renders the Cμ and Cα genes substrate for AID, which deaminates cytosine residues on both DNA strands of the transcribed S

regions to generate multiple DNA lesions that are processed into double-stranded DNA breaks. Fusion of these breaks induces excision of the noncoding intervening DNA (switch circle) and rearrangement of the IgH locus by replacement of Cμ with the downstream Cα gene. Post-switch transcription of the IgH locus generates mRNAs for IgA protein.

b. Somatic Hypermutation

SHM is another AID-dependent Ig diversification process that occurs in the GC of SLOs[79]. SHM introduces point mutations in the complementarity determining regions (CDRs) of V(D)J genes, which form the antigen-binding pocket of an Ig molecule[80]. These mutations arise at a rate of 10^{-3}/basepair/generation, which is several orders of magnitude above the rate of spontaneous mutation, and provide the structural correlate for the selection of B cells with higher affinity for antigen.

SHM includes an initial phase that requires the mutagenic activity of AID, followed by a second phase involving recruitment of error-prone repair polymerases[81]. Error-prone DNA repair is performed by members of a family of low-fidelity translesional DNA polymerases that recognize DNA lesions and bypass them by inserting bases opposite to the lesion. Amino acid replacements introduced by SHM increase the affinity and fine specificity of an antibody, but do not usually modify the framework (FR) regions, which regulate the structural architecture of the antigen-binding site of an antibody molecule. Similarly, SHM does not induce amino acid replacements in the promoter and intronic enhancer, which regulate the transcriptional activity of the Ig locus.

SHM can have three different outomes: 1) the antibody does not change its affinity for antigen; 2) the antibody loses affinity for antigen; or 3) the antibody increases its affinity for antigen. This last outcome results in affinity maturation, which permits a more effective targeting and clearance of a pathogen[82]. Despite predominantly targeting rearranged V(D)J genes, SHM can also physiologically occur in non-Ig genes such as *BCL6*, which encodes a protein essential for the GC reaction[82, 83]. Furthermore, several mouse and some human studies indicate that certain antigens can trigger a lower degree of SHM compared to that induced within the specialized structure of the GC. This may explain why some carbohydrate-reactive B cells called marginal zone (MZ) B cells express Ig genes harbouring a low number of mutations, at least in humans. However, this issue remains poorly understood, possibly because MZ B cells are functionally heterogeneous and include clonotypes capable to enter the GC after recognizing protein antigens.

c. Memory B Cells

Pre-existing memory B cells are critical to mount fast secondary humoral responses to recall antigens. In addition to patrolling the circulation and SLOs, memory B cells form extrafollicular IgG-expressing aggregates as well as follicle-like IgM-expressing structures in draining LNs, at least in mice[6,62]. After secondary exposure to antigen, IgG^+ memory B cells rapidly generate antibody-secreting PBs, whereas IgM^+ memory B cells initiate a secondary GC reaction. These anamnestic responses are characterized by rapid B cell activation, proliferation and differentiation, with massive secretion of high-affinity antibodies[83]. In humans, typical GC-derived memory B cells

contain highly mutated Ig V(D)J genes encoding class-switched IgG, IgA or (less frequently) IgE receptors and express surface CD19, CD24 and CD27 but not IgD or CD38[84] **(Figure 3).** A similar phenotype can be detected in a small population of GC-derived memory B cells that contain hypermutated Ig V(D)J genes but express IgM instead of IgG, IgA or IgE. These IgM^+ memory B cells are distinct from IgM^+ MZ B cells, which co-express IgD, albeit in lower amounts compared to follicular B cells. The signals involved in the long-term survival of memory B cells remain unclear, but some studies point to an important role of nonspecific signals from microbial TLR ligands[85]. In contrast, other works indicate that antigen-specific signals involving help from T_{FH} cells, invariant natural killer T (*i*NKT) cells and even basophils are required[86,87].

d. Plasma Cells

Terminal B cell differentiation generates antibody-secreting cells (ASCs), which include actively dividing PBs and non-dividing PCs[72]. As mentioned earlier, TD responses can develop through either follicular or extrafollicular pathways **(Figure 3)**. In the extrafollicular TD pathway, antigen-activated B cells usually become short-lived PBs expressing moderate levels of the PC-inducing transcription factor B lymphocyte-induced maturation protein 1 (BLIMP-1). These PBs generate early IgM and some class-switched antibodies that afford some degree of protection despite showing low affinity for antigen[37]. In the follicular TD pathway, the GC reaction generates immature PCs with high affinity for antigen, which later migrate to the BM to become mature PCs tonically releasing antibodies into the circulation[6,74,86,88]. By doing so, GC-derived PCs provide a "secretory"

memory that integrates the "circulating" memory of GC-derived memory B cells.

In TD antibody responses, the transition from an activated B cell into a PC requires up-regulation of BLIMP-1, which induces a complex gene program involving silencing of transcription factors required for the maintenance of B cell identity, including paired box 5 (PAX5, also known as BSAP) and BCL-6. This program is coupled with up-regulation of interferon regulatory factor-4 (IRF-4), BLIMP-1 and X-box-binding protein-1 (XBP-1), which cooperate with BLIMP-1 to induce PCs[89-91]. Remarkably, recent studies show that PCs emerging from the GC reaction generate a negative feedback loop by inducing T follicular regulatory (T_{FR}) cells, which deliver inhibitory signals to GC-inducing T_{FH} cells. An additional negative feedback is provided by high-affinity antibodies released by PCs emerging from the GC, which compete with B cells expressing high-affinity BCRs for antigen, tereby turning off the GC reaction.

In general, long-lived PCs contain hypermutated Ig V(D)J genes, accumulate intracellular IgM, IgG, IgA or IgE, contain high levels of BLIMP-1[92-94], and express the syndecan-1 molecule CD138 along with very high levels of surface CD27 and CD38, but lack surface CD20, CD24, IgD and the nuclear proliferation-associated molecule Ki-67 **(Figure 3)**[86,95]. Long-lived PCs additionaly express surface CD19 along with variable levels of IgM, IgG, IgA or IgE, but tend to lose these molecules at a terminal maturation stage, particularly in the BM.

In TI antibody responses, the sequence of molecular events leading to the rapid generation and expansion of PBs is less understood.

Compared to follicular B cells, MZ B cells (and B-1 cells, at least in mice) not only express lower levels of PAX5, but also show some degree of constitutive BLIMP-1 expression, which may facilitate extremely rapid antigen-induced formation of PBs[96,97]. In this regard, a comparative analysis of the transcriptional profiles of splenic follicular B cells with that of MZ B cells shows that the latter feature a "pre-activated" state involving expression of several mechanistic target of rapamycin (mTOR)-linked gene products linked to the induction and survival of PBs (Sintes J., Gentile M. et al, Immunity, under revision).

In general, short-lived PBs contain unmutated, poorly mutated or highly mutated Ig V(D)J genes (the last case is typical of PBs emerging from memory B cells), accumulate intracellular IgM, IgG, IgA or IgE, express surface CD19 along with IgM, IgG, IgA or IgE[37], show very high levels of surface CD27 and CD38, contain Ki-67 and some BLIMP-1[92-94], but express little or no CD138, CD20, CD24 and IgD.

Both PCs and PBs require helper microenvironmental signals to maintain high viability and copius antibody secretion. While little is know regarding the survival requirements of PBs emerging from TI pathways, PCs generated by TD pathways need both contact-independent and contact-dependent survival signals generated by stromal cells, eosinophils and megakaryocytes from the BM. These signals include retaining chemokines such as CXCL12, pro-survival cytokines such as a proliferation-inducing ligand (APRIL) and IL-6, and surface structures such as vascular cell adhesion molecule 1 (VCAM-1)[85,98-100]. Interestingly, PC-inhibiting signals may be generated when antibodies required for a given immune response reach a high concentration in the serum. These antibodies can induce PC apoptosis

by cross-linking the inhibitory Fc receptor FcγRIIB[101].

ii. T Cell-Independent B Cell Responses

TD responses to a protein antigen require at least 5–7 days to develop, a delay that could prove fatal in the presence of blood-borne pathogens. To compensate for this limitation, TD responses are integrated by faster TI responses involving B-1 cells (in mice) and MZ B cells (in both mice and humans). These innate-like lymphocytes generate IgM and smaller amounts of class-switched IgG and IgA after recognizing highly conserved microbial products such as carbohydrates or lipids through both BCRs and TLRs[41,102-104]. Additional B-cell activating signals that support this TI pathway derive from macrophages, DCs, group 3 innate lymphoid cells (ILC3s) and neutrophils[78,105,106]. Although TI pathways usually generate unmutated antibodies that recognize multiple microbial determinants with low affinity, both human and mouse studies suggest that some TI responses can also give rise to weakly mutated antibodies with relatively high affinity for antigen[78,105,106].

Mouse B-1 cells constitute a distinct lineage of self-renewing B cells that occupy serosal cavities, spleen and intestine[103]. These cells generate "natural" (or pre-immune) immunity by spontaneously releasing polyspecific and largely unmutated IgM but also class-switched IgA and IgG3 that provide a first line of defense against microbes. Recent work has identified the existence of a possible human counterpart of mouse B-1 cells[107], but further studies are needed to confirm these findings. Similar to B-1 cells, MZ B cells express polyspecific and largely unmutated antibodies that recognize

TI antigens with low affinity, at least in mice[105]. In humans, MZ B cells can produce monospecific and weaakly or even highly mutated antibodies that recognize TI antigens with relatively high affinity[78,108]. In general, it seems clear that the human MZ B cell compartment is more heterogeneous that its mouse counterpart and likely includes MZ B cells that enter the GC to initiate typical TD responses. These B cells may later re-enter the MZ as classical memory B cells.

TI antigens can be divided in two distinct categories: classical TI-1 antigens include highly conserved microbial structures such as LPS and CpG DNA, which activate B cells by engaging TLRs with or without help from BCRs[109]. In contrast, classical TI-2 antigens include repetitive polysaccharides that activate B cells by extensively cross-linking BCRs. The additional involvement of C-type lectin receptors (CLRs) and possibly other carbohydrate-binding receptors remains unclear, but it is highly possible. In general, TI-2 antigens are large molecules with periodic epitopes that promote the cross-linking of multiple BCRs, but cannot be processed and uploaded onto HLA-II molecules[110]. Regardless of the mechanism, TI antigens rapidly induce the differentiation of B-1, MZ and some B-2 cells into short-lived PBs that generally secrete low-affinity and largely polyreactive antibodies, particularly in mice. This TI response bridges the temporal gap required for the induction of high-affinity antibodies by follicular B cells.

Despite being specialized in TI antibody responses, MZ B cells can also participate in TD antibody responses owing to their ability to capture antigen and shuttle from the MZ to the follicle. At this site, MZ B cells can either deposit antigen on FDCs or present

immunogenic peptides to T_{FH} cells[111,112]. This functional flexibility together with the open circulation of the human MZ may explain why some human MZ B cells express molecular fingerprints typical of a GC reaction, including hypermutated antibodies.

Besides the splenic MZ, the intestinal immune system supports TI antibody responses that predominantly include IgA and are highly integrated with TD responses. Indeed, mice lacking T cells retain a very significant IgA production[113-115]. These TI IgA responses usually take place within isolated lymphoid follicles (ILFs), but can also occur in PPs, MLNs and lamina propria (LP)[111].

In murine ILFs, DCs stimulated by commensal TLR ligands cooperate with ILC3-activated stromal cells to induce BAFF, APRIL, and TGF-β, which cooperate with commensal BCR and TLR ligands to elicit TI IgA CSR and production in B cells[111, 116-118]. In MLNs and PPs, this primitive TI pathway may further involve plasmacytoid DCs (pDCs), which are usually specialized in the production of type I interferon (IFN-I)[105,119]. Yet, pDCs from PPs and MLNs appear to produce little or no IFN-I. Instead, this cytokine mostly derives from stromal cells activated by commensal TLR ligands in a process that stimulates IFN-I-dependent BAFF and APRIL release by pDCs. Murine PPs and MLNs also contain FDCs that produce IgA-inducing factors such as BAFF, APRIL and TGF-β in response to commensal TLR ligands. This process is further increased by retinoic acid (RA), a derivative of dietary vitamin A.

In the LP and other sub-epithelial areas associated with PPs and ILFs, various subsets of macrophages and DCs sample luminal commensal

bacteria, which may be presented to local B cells[120,121]. These macrophages and DCs release BAFF, APRIL, TGF-β, IL-6, IL-10 and RA, which support local IgA production in a TI or even TD manner[113,116,122-125]. Recent findings suggest that TI reponses in the LP further involve iNOS-inducing signals from LTα1β2 expressed by ILC3s[126]. Of note, also intestinal epithelial cells (IECs) release BAFF, APRIL, RA and TGF-β in response to commensal TLR ligands and the cytokine epithelial growth factor (EGF). This process enhances plasma cell survival and differentiation in addition to local IgA CSR and production through a mechanism further involving TSLP, an epithelial cytokine that stimulates DC production of BAFF, APRIL and IL-10[76,127].

Clearly, the gut LP may induce IgA production through TI and TD pathways that parallel the TI and TD pathways that work in the extrafollicular area of the spleen. However, the relative importance of gut LP-induced IgA responses remains controversial, mostly because such responses are quantitatively less prominent than those occurring in PPs. Further controversy stems from the supposed low frequency of "class-switchable" B cells in the LP, which is thought to be mostly occupied by terminally differentiated PCs and memory B cells expressing IgA. However, besides confirming the IgA inductive properties of the LP, recent studies show that immature (BLIMP-1^{low}) PCs and even memory B cells retain the ability to undergo class switching, including sequential class switching. In general, IgA induction in the LP may help to rapidly control the concentration and functionality of highly conserved commensal antigens. Moreover, it may help to increase the functional flexibility of the human gut

immune system by triggering direct or sequential IgA2 class switching in pre-existing memory and immature (BLIMP-1^{low}) PCs expressing IgA1 or IgM. Furthermore, it may generate some degree of humoral protection against invading microbes until the emergence of more effective and specific IgA responses from classical follicular inductive sites[128].

4. Splenic B Cell Responses

a. Structure of the Spleen

The spleen is the largest blood filter organ of our body and is located at the left side of the abdomen, directly beneath the diaphragm and connected to the stomach[129,130]. Essentially, the spleen is organized as a tree of branching arterial vessels, in which the smaller arterioles end in a venous sinusoidal system. A fibrous capsule of connective tissue giving rise to internal trabeculae protects the surface of the spleen and supports its larger vasculature. The smaller branches of the arterial supply are sheathed by lymphoid tissue, which forms the white pulp (WP). In mice, the spleen is connected to the general circulation by the splenic artery[131,132]. This vessel branches into central arterioles surrounded by the periarteriolar lymphoid sheath (PALS). Central arterioles further branch into smaller follicular arterioles that cross the PALS and follicles of the WP to end as capillaries either in the marginal sinus (MS) or in the red pulp (RP)[131,132].

The MS is a low-resistance vessel with a fenestrated structure that separates the MZ from the PALS and follicles of the WP[131,132]. The blood contained in the MS slowly flows through the MZ and subsequently drains into the splenic cords and venous sinuses of the

RP to enter the general circulation[131,132]. As a result of this microcirculation, the mouse MZ can be considered to be an open compartment. In humans, the spleen receives blood from the splenic artery, which branches into central arterioles and penicillary arterioles[131,132]. These vessels are usually covered by the PALS, but not as often as in rodents.

Owing to the absence of a histologically defined MS, the blood flowing in penicillary arterioles directly drains into capillaries of the perifollicular zone (PFZ) and RP[131,132]. The PFZ is a well-defined area of decreased resistance that separates the MZ from the RP. Both the PFZ and the RP consist of an open circulatory system of blood-filled spaces known as splenic (or Billroth) cords, which have no defined endothelial delimitation and are continous with the venous sinusoidal vessels of the RP[131,132].

In rodents, some of the smallest arterial branches terminate in the MS, whereas others traverse the marginal zone (MZ) to form the venous system of the RP **(Figure 6)**. In humans, a large part of the blood ends in the PFZ and subsequently flows through the MZ[128] **(Figure 6)**. Given its strategic location at the interface with the circulatory system and the unusual structure of its lymphoid compartments, the spleen is an ideal site to mount rapid immune responses to blood-borne antigens, including pathogens such as encapsulated bacteria[126].

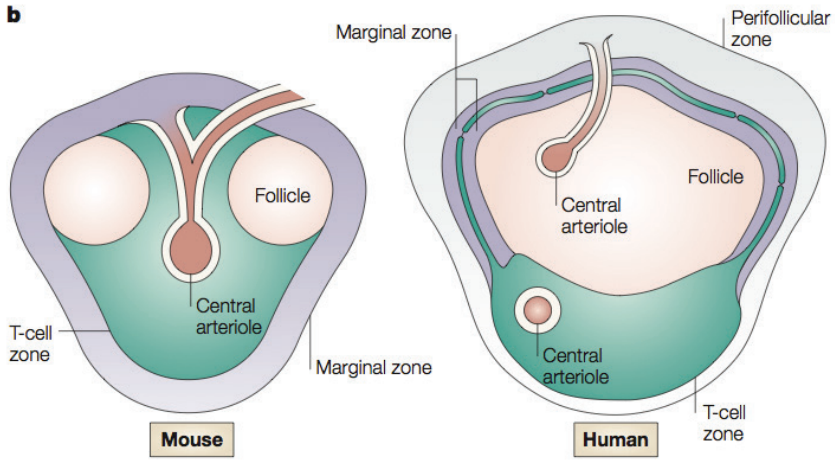


Figure 6. Structure of the white pulp in mice and humans. The main differences are found in the structure of the marginal zone, which surrounds the white pulp. Unlike mice, humans have an inner and and outer marginal zone, which is surrounded by a large perifollicular zone. In the perifollicular zone, some capillaries are sheathed by macrophages. These macrophages express sialic-acid-binding immunoglobulin-like lectin 1 (SIGLEC1, also known as CD169)[133,134]. Adapted from *Mebius R.E, et al., 2005*[131].

Red Pulp

The specialized structure of the RP, with its abundant phagocytic cells, permits the spleen to filter the blood and remove aged circulating hematocytes, including red blood cells. The red pulp is also important in iron recycling and clearance of blood-borne bacteria. These functions are mediated by specialized macrophages located in the splenic cords[135]. The flexible nature of this large venous system additionally permits the retention of viable circulating erythrocytes to decrease blood viscosity under resting conditions and form a ready-on-demand reservoir under stress conditions[136]. As they phagocytose red blood cells, splenic (and liver) macrophages release free

haemoglobin[137]. When undergoing over-accumulation in tissues, this iron-containing protein can cause inflammation and cell damage and thus it is promptly captured by RP macrophages through the scavenger receptor CD163[138]. By removing exceeding iron from the spleen, this process had the additional advantage of mitigating the survival of invading iron-dependent microbes[139-141]. These examples highlight only some of the multiple important biological functions exerted by RD macrophages.

The RP is also an anatomical site capable to provide suitable survival niches to PBs and PCs emergeing from the MZ and WP. After their emergence from splenic follicles, PBs initially migrate to RP areas distal from the MZ[142]. However, the precise nature of PB-PC-retaining niches and the cellular interactions involved in the creation of these niches remain unclear[37]. In general, the localization of PBs in the cords of the RP resembles that of PBs in the medullary cords of LNs. One possibility is that these specialized extrafollicular areas facilitate the rapid entry of PB-derived antibodies into the main bloodstream. Of note, PBs highy express CXCR4 and may migrate to the RP by navigating through chemotactic fields extablished by CXCL12, a chemokine highly expressed by stromal and endothelial cells located in the RP[143]. Up-regulationof CXCR4 by PBs coincides with down-regulation of CXCR5 and CCR7, two chemokines required for the retention of B cells in the WP. In mice, PBs require $CD11c^{hi}$ DCs to survive in the RP and differentiate into more mature PCs[144]. The presence of $CD11c^{hi}$ DCs in the T-cell zone (PALS) of the WP and bridging channels connecting the WP with the RP might be involved in this PB-to-PC transition. Remarkably, bridging channels temporarily

host ASCs emerging from the WP following antigenic challenge[145].

White Pulp

The WP contains well-compartimentalized T and B cell areas corresponding to peri-arteriolar lymphoid sheats (PALS) and lymphoid follicles. The correct organization and maintenance of the WP is controlled by specific chemokines that attract T and B cells to their specific compartments. PALS contain T cells that interact with DCs and transiting B cells, whereas follicles (contain B cells that undergo clonal expansion, CSR and SHM upon exposure to antigen. CXCL13 guides B cell migration to follicles[146], whereas CCL19 and CCL21 guide T cells and DCs to the PALS[147,148]. These chemokines are typically induced by local non-hematopoietic stromal cells in response to lymphotoxin-$\alpha 1\beta 2$ ($LT\alpha 1\beta 2$) and tumour-necrosis factor-α (TNFα) released by haematopoietic cell types, including B cells[149]. Needless to say, stromal cells express abundant LT-αR and TNFR1[150-152].

Engagement of LT-αR and TNFR1 by cognate ligands induces SC activation of nuclear factor-κB (NF-κB), which stimulates CCL19 and CCL21 expression. In contrast, CXCL13 derives from a $CD21^{+}CD35^{+}$ SC subset called FDCs and from additional stromal cells adjacent to FDCs[146]. Besides determing B cell migration into follicles, engagement of CXCR5 by CXCL13 elicits B cell expression of membrane-bound $LT\alpha 1\beta 2$. This TNF family member put in motion a classical positive feedbacl loop by triggering FDC maturation, including increased expression of CXCL13. A similar mechanism regulates the integrity of PALS, which contain fibroblastic reticular cells (FRCs) releasing T

cell-attracting CCL19 and CCL21[153]. These chemokine are also produced by DCs, albeit in lower ampounts compared to FRCs[153].

Marginal Zone

The elevated perfusion of the spleen permits this organ to provide efficient immune surveillance of the circulatory system through the MZ. Strategically interposed between the lymphoid tissue of the WP and the circulation, the splenic MZ contains B cells enmeshed within a stromal reticular network containing macrophages, DCs and some neutrophils[131]. All of these cells readily interact with circulating antigens as a result of the low flow rate of the blood passing through the MZ.

In mice, the fenestrated nature of the MS facilitates the entry of blood-borne antigens into the MZ[131]. At this site, MZ metallophilic macrophages (MMMs) expressing the adhesion molecule SIGLEC-1 (sialic-acid-binding immunoglobulin-like lectin 1, also known as MOMA-1) delimit the inner part of the MZ[154]. In addition, MZ macrophages (MZMs) expressing the C-type lectin receptor SIGNR-1 and the type I scavenger receptor MARCO (macrophage receptor with collagenous structure) inhabit the central and outer MZ[155-158] . Together with a subset of DCs, these macrophage subsets convey antigenic information to MZ B cells after capturing blood-borne antigens through ther uptake receptors (PRRs), including MOMA-1, SIGNR-1 and MARCO[159,160]. These structures may convey captured antigens to non-degradative intracellular vesicular compartments that may recycle to the cell surface for antigenic exposure to MZ B cells[30,161,162]. In an alternative pathway, blood-borne antigens are first

captured by circulating DCs and granulocytes and later transported into the MZ via the MS[163,164]. Unlike the mouse spleen, the human spleen has neither the MS nor specific macrophages[132,134]. One possibility is that antigens intreact with human MZ B cells through the PFZ, an area with open circulation patrolled by neutrophils with B cell helper function[78].

An important role in the organization and integrity of the MZ has been attributed to B cells. In mice, lack of B cells induced before or after birth causes disappearance of MMMs and MZMs[165], [166]. In LNs, a subset of ILC3s called lympoid tissue inducer (LTi) cells express LTα1β2 to stimulate SC production of chemokines and adhesion molecules needed for tissue development and organization[167,168]. Similar to LTi cells from developing LNs, B cells may play an important role in the organization of the splenic MZ. Indeed, B cells express LT-βR and produce chemokines such as CCL19 and CCL21, which may model MZ tissue organization in response to LTα1β2 from stromal cells and MS-associated endothelial cells. This process could occur when mature B cells colonize the MZ or transit through the MZ on their way to lymphoid follicles. This possibility correlates with the finding that MZMs require CCL19 and CCL21 for their localization in the MZ. This requirement is specific, because lack of CXCL13 seletively depletes a subset of mannose receptor-expressing DCs[159,169]. No chemokine has so far been identified for the recruitment and retention of MMMs, but their maintenance along the MZ requires MZ B cells[165]. Conversely, MZMs are needed for the retention of B cells in the MZ through a mechanism involving MARCO[170]. This implies that macrophages and B cells exert mutual functional

influences, at least in mice.

In mice, MZ B cells have been historically regarded as highly sessile, but recent evidence shows that these lymphocytes can cyclically shuttle between the MZ and follicles. This process involves a complex interplay between circulating lipids and chemokine receptors. Compared to naïve B cells, MZ B cells express more abundant $S1P_1$ and $S1P_3$ receptors, which facilitate B cell retention in the MZ through their interaction with a locally abundant lysophospholipid called sphingosine 1-phosphate (S1P)[171-173]. This interaction triggers down-regulation of $S1P_1$ and $S1P_3$ receptors, followed by CXCL13-guided migration of a large fraction of CXCR5-expressing MZ B cells to the follicle. Subsequent re-up-regulation of $S1P_1$ and $S1P_3$ receptors triggers the re-entry of B cells into the MZ under the influence of local S1P1.

This reversible follicular migration process allows MZ B cells to deposit blood-borne complement-opsonized antigens on the surface of FDCs. Of note, microbial products such lipopolysaccharide (LPS) further accelerate MZ B cell migration to the follicle[171]. Indeed, besides $S1P_1$ and $S1P_3$ receptors, MZ B cells express the $\alpha_L\beta_2$ integrin lymphocyte function-associated antigen-1 (LFA-1) and the $\alpha_4\beta_1$ integrin vascular cell adhesion molecule (VLA-4). These integrins retain B cells in the MZ by interacting with intercellular adhesion molecule-1 (ICAM-1) and vascular cell-adhesion molecule-1 (VCAM1), respectively, two molecules highly expressed by MZ-based macrophages and stromal cells[174]. By down-regulating LFA-1 and VLA-4 expression on MZ B cells, LPS facilitates their migration to the

follicle[171,174]. Thus, in mice, a complex and unique range of molecular interactions allows splenic MZ B cells to communicate with the follicle while retaining the possibility to migrate back to the MZ.

An additional layer of complexity derives from additional findings showing that $S1P_3$ expression by non-haematopoietic cells is absolutely required for the development of the MZ. Indeed, lack of $S1P_3$ causes loss of a subset of stromal cells called marginal reticular cells (MRCs), which typically express mucosal vascular addressin cell-adhesion molecule 1 (MAdCAM-1), a receptor for the integrin $\alpha_4\beta_7$ typically detected on high endothelium venules from the gut LP. Of note, MRCs form a single layer of stromal cells along the MS, are highly dependent on LTα1β2 from ILC3s and express MZ B cell-retaining receptors such as ICAM-1 and VCAM-1. In the absence of these MRCs, the mouse MZ becomes highly disorganized and completely dysfunctional, despite containing a higher number of B cells[175]. This implies that MAdCAM-1^+ MRCs are as important as MMMs and MZMs for the correct formation of the splenic MZ and for the function of its unique B cells. Of note, all of these findings refer to the biology of the mouse spleen, which may profoundy differ from that of the human spleen. In this regard, it must be anticipated that the human splenic MZ lacks professional macropages and its B cells may have full access not only to follicles but also to the general circulation.

b. Function of the Spleen

Besides removing damaged blood cells through RP-based

macrophages[131], the spleen clears blood-borne microbes through B cell responses orchestrated by distinct but highly integrated B cell subsets positioned in the MZ and WP. In the MZ, innate-like B cells mount early and explosive IgM but also IgG responses to highly conserved antigenic determinans in the absence of any help from T_{FH} cells[108]. These TI responses fill the temporal gap required by follicular B cells to mount classical TD reponses characterized by slowly developing induction of high-affinity IgG and immune memory. Given the topic of this thesis, we will mostly focus our discussion on MZ B cell responses. Before describing their functions in greater detial, we will highlight some of the differences that characterize mouse and human MZ B cells.

In mice, MZ B cells are mostly sessile and thus remain generaly confined to the anatomical area of the splenic MZ, which is internally delimited by the MS[160]. In humans, MZ B cells are free to enter the general circulation[84,176] and may thus colonize antigen entry sites different from the MZ, including the inner wall of the LN subcapsular sinus (SCS) as well as the sub-epithelial areas of tonsillar crypts and PPs[78,177-179]. These MZ-equivalent areas are poorly understood, but have a cellular composition similar to that of the splenic MZ and may therefore provide alternative functional niches for the development of MZ-like B cell responses. This might explain why splenectomized individuals partly recover key MZ B cell-associated functions, such as production of antibodies to encapsulated bacteria, several months following splenectomy[108]. An additional species-specific difference relates to the mutational status of Ig V(D)J genes. In mice, MZ B cells express mostly unmutated Ig V(D)J genes, but there is a relative lack

of systematic studies to ascertain whether mutations arise following immunization or infection. In humans, MZ B cells usually harbor weakly mutated Ig V(D)J genes, but some clones can also express a GC-like mutational profile, with a higher amount of mutations. These mutations appear early in life and further increase as the individual ages through mechanisms that remain poorly understood but may involve both GC-independent and GC-dependent pathways[176,180,181].

As for the activation mechanism of MZ B cells, this aspect has been mostly studied in mice but may be largely overlapping in humans. Particulate blood-borne antigens such as complement-coated bacteria activate MZ B cells after being captured by either circulating DCs and granulocytes or MZ-based macrophages, including MMMs and MZMs[163,182]. Soluble blood-borne antigens such as complement-coated microbial polysaccharides may directly interact with MZ B cells[183-185]. In both cases, complement proteins play a decisive role by interacting with signal-transducing complement receptors, such as CD21 and CD35, expressed by both macrophages and MZ B cells. In these latter, CD21 shows a very elevated expression and forms a complex with the BCR to amplify antigenic signals. Additional co-stimulatory signals from innate myeloid cells stimulate antigen-activated MZ B cells to rapidly differentiate into PBs by entering a rapidly developing TI pathway[111,161,186]. However, antigen-activated MZ B cells can also initiate slower TD responses after establishing cognate interactions with $CD4^+$ T cells, which may include T_{FH} cells. In the TI pathway, PBs rapidly migrate to the RP, where they undergo clonal expansion to release massive amounts of IgM. However, it must be remarked that MZ B cells and even PBs can further undergo CSR from IgM to

IgG or IgA[37,78,183]. While mouse MZ-derived PBs mostly produce IgG3, human MZ-derived PBs tendentially produce IgG2 in addition to IgG1. Notably, IgA responses have also been observed[37,78].Finally, some mouse infection models indicate that extrafollicular CSR is coupled to some degree of extrafollicular SHM, but it remains unclear whether the latter also involves MZ B cells[187].

i. Role of MZ B Cells in Infections

Individuals lacking the spleen as a result of surgery or congenital asplenia are at higher risk of pneumonitis, meningitis and fulminant septic syndrome, which are usually caused by encapsulated bacteria such as *Streptococcus pneumoniae, Haemophilus influenzae* and *Neisseria meningitides*[188-190]. Given that splenectomy causes depletion of circulating MZ B cells, these infections are generally attributed to impaired production of protective IgM and IgG to capsular polysaccharides by MZ B cells[180, 181, 18]. Of note, these defects are less severe in children than adults, suggesting that the pathogenesis of splenectomy-induced infections is complex and may vary in different ages[191].

Infections by encapsulated bacteria are also more frequent in individuals with inflammatory bowel disease (IBD) and correlate with progressive depletion of circulating MZ B cells, which could be secondary to inflammatory perturbation of the MZ caused by chronically increased systemic translocation of gut-derived commensal bacteria[192]. This process may also lead to MZ atrophy by determining functional exhaustion (anergy) and death of B cells. Alternatively, chronic production of pro-inflammatory cytokines such as TNF may

deplete MZ B cell precursors in the BM by increasing myelopoiesis at the expense of lymphopoiesis[193].

Similar pathogenetic mechanisms, including chronic microbial translocation, may explain the loss of MZ B cells and the increased rate of infection with encapsulated bacteria in individuals with secondary immunodeficiency caused by HIV-1 infection. Similar bacterial complications occur in patients with primary immunodeficiencies such as CVID. In these individuas, invasive pneumococcal disease and impaired IgM and IgG responses to capsular polysaccharides are often coupled with depletion of MZ B cells[192,194,195].

The key protective role of MZ B cells is further supported by a number of mouse models showing that lack of the spleen or genetic manipulations impairing the formation, survival, maintenance and/or activation of MZ B cells greatly reduce antibody responses to capsular polysaccharides from encapsulated bacteria[183,196]. Remarkably, mouse models, including T cell-deficient mice, further show that MZ B cells also enhance humoral responses against certain glycoproteins expressed by some enveloped blood-borne viruses[197,198].

ii. Role of MZ B Cells in Homeostasis

MZ B cells produce IgM but also IgG not only following infection, but also under homeostatic conditions[104,160]. In general, these antibodies recognize molecular signatures shared by foreign and autologous cells, including polysaccharides, phospholipids, glycolipids and nucleic acids. In addition to providing a first line of defense against invasive pathogens, this homeostatic humoral response may

promote tissue integrity by facilitating the removal of apoptotic and necrotic cells[104,160]. Besides recognizing pathogenic bacteria and self-antigens, antibodies homeostatically produced by MZ B cells bind antigenic determinants associated with commensal bacteria, including α-1,3-galactosyl residues but also polysaccharides, phopshopipids, etc[104,160,198,199]. Thus, it is plausible that MZ B cells generate a secondary line of systemic humoral defence against commensals breaching the mucosal barrier[104,160,199,200].

c. Spleen-Gut Communications

How MZ B cells interact with mucosal bacteria, including gut-derived commensals, remains poorly understood. Despite being segregated from the inner milieu of our body by a number of physical and biological barriers, commensals clearly influence both health and disease states, including the development and function of the immune system[201-206]. Although not essential for the formation of MZ B cells, commensals may enhance certain MZ B cell-derived antibody responses. Indeed, germ-free (GF) mice have impaired homeostatic and induced antibody responses to both artificial and microbial carbohydrates, including levan, dextran, peptidoglycan-associated polysaccharides and 3-fucosyllactosamine[204, 207].

In rodents, tiny amounts of commensal antigens such as LPS and peptidoglycan physiologically translocate from the intestinal lumen to the general circulation and eventually reach systemic lymphoid organs, including the spleen and BM, which are actively engaged in blood filtration[205,208]. In humans, physiological microbial translocation from mucosal surfaces could account for the detection of LPS and

peptidoglycan in blood, spleen, liver, BM and even brain[78, 208-210]. Of note, bacterial LPS has been even detected in gut-derived chylomicrons, which are circulating lipid-rich micellae generated by IECs for the absorption of cholesterol and tryglycerids[211,212]. It is tempting to speculate that chylomicrons and native or oxidezed forms of chylomicron-derived low-density lipoproteins (LDLs) may favor a "controlled" capture of LPS by splenic phagocytes, which indeed express a large array of lipid and apolipoprotein receptors.

In the spleen, blood-borne commensal antigens may lower the activation threshold of MZ B cells, thereby allowing them to build a ready-to-use antibody repertoire while acquiring a state of active readiness against pathogens[78, 199, 200 208]. This process may involve TLR engagement by commensal antigens and indeed humans with congenital defects of TLR signaling show a profound depletion of MZ B cells. This finding correlates with a nuber of recent studies from our group suggesting that the splenic environment is homeostatically exposed to microbial signals[78,106,213-215]. In particular, we have shown that splenic microbial products presumably originating from mucosal surfaces, including the TLR4 ligand LPS, induce the local reprogramming of circulating neutrophils (N_C cells) into MZ B cell-helper neutrophils (N_{BH} cells)[78,205]. Accordingly, GF mice and mice lacking TLR signalling showed reduced N_{BH} cells[104]. These cells may receive additional survival/activating signals from splenic ILC3s through a mechanism involving granulocyte and monocyte-colony stimulating factor (GM-CSF)[106]. Of note, splenic ILC3s express a gut-like phenotype and further enhance MZ B cell responses by establishing a reciprocal activation-inducing dialogue with MAdCAM-

1^+ MRCs. Ultimately, N_{BH} cells, ILC3s and MRCs cooperatively appear to enhance TI antibody responses through a TLR-enhanced mechanism involging activation of MZ B cells by BAFF, APRIL and NOTCH2 ligand signals[106].

The presence of homeostatic microbal products in the mouse spleen has been recently demonstrated by studies showing that some intestinal Gram-negative bacteria "leak" from the gut to the spleen, where they elicit homeostatic IgG3 responses that confer "natural" protection against systemic infection by *Escherichia coli* and *Salmonella* thorough a mechanism involving phagocytes[215]. This mechanism involved TLR4-dependent signals in addition to T cells. Thus, it seems clear that mucosal signals from commensal bacteria play an important role in the amplification of splenic MZ and possibly follicular B cells responses developing through TI or TD pathways.

d. Splenic MZ and FO B Cell Responses

i. Regulation of MZ B Cell Responses to TI Antigens

As mentioned earlier, complement proteins cooperate with the BCR complex to fully activate MZ B cells in response to complement-decorated TI antigens. Indeed, MZ B cells highly express the BCR-associated CD21 complement receptor through a NOTCH2-dependent signaling program essential for the maturation of MZ precursors into mature MZ B cells[216,217]. Besides BCR and complement receptors, TI-1 antigens such as LPS stimulate TLRs, which are highly expressed by MZ B cells compared to follicular B cells. Similaly, TI-2 antigens such as CPS may stimulate poorly characterized carbohydrate receptors in addition to the BCR and complement receptors. In

general, it appears that TI antigens use distinct molecular moieties to co-engage BCR, complement receptors and one or more PRRs, including TLRs, on MZ B cells[110,218,219].

In mice, particulate TI antigens such as encapsulated bacteria are exposed to MZ B cells after being captured by MMMs and MZMs. These MZ-specific macrophages capture TI antigens through various scavenger receptors and CLR family members[182,220]. Particulate TI antigens can be also captured by circulating neutrophils and $CD11c^{lo}$ DCs that may actually correspond to pDCs[163]. In mice, a distinct subset of $CD11c^{hi}$ DCs located in the RP further sustain TI antibody responses by providing BAFF and APRIL survival signals to PBs emerging from antigen-activated MZ B cells[116,144,163].

In humans, some studies indicate that the splenic MZ lacks MMMs and MZMs, raising questions as to how MZ B cells interact with blood-borne antigens. N_{BH} cells, DCs and macrophages organized around perifollicular capilarries could compensate for the lack of MZ-specific macrophages[106, 104, 221-223]. Similarly to macrophages and DCs, N_{BH} cells and ILC3s release BAFF and APRIL[78, 122, 114, 76,223], which promote MZ B cell activation and PC differentiation and survival **(Figure 7)**.

BAFF and APRIL are innate CD40L-like molecules produced by myeloid cells in response to TLR signals. In both mice and humans, BAFF and APRIL trigger IgM-to-IgG CSR by engaging transmembrane activator and CAML interactor (TACI), a TLR-inducible receptor highly expressed by MZ B cells[78,116,125,163,218]. By cooperating with signals from BCR ligands, TLR ligands and/or

cytokines, TACI support a powerful MZ B cell differentiation program that leads to the formation of PBs secreting IgM and some IgG. Remarkably, TACI signals through a TLR-like pathway involving the adaptor proteins MyD88, IRAK1, IRAK4, TNF receptor-associated factor 2 (TRAF2) and TRAF6[224]. By further cooperating with CD40 signals, TACI may integrate TI and TD signals to mount rapid but flexible MZ B cell responses **(Figure 7)**.

As discussed earlier, complement proteins are essential for the initiation of MZ B cell responses to TI antigens. Besides enhancing BCR signals, CD21 and CD35 interfere with MZ B cell expression of an inhibitory receptor called programmed cell death protein 1 (PD1)[225]. Of note, some complement fragments may further activate MZ B cells via CD40, although these finding needs further validation. Finally, MZ B cell may receive CD40-dependent signals from activated NK cells and *i*NKT cell[226], which further highlights a previously unsuspected complexity of MZ B cell-stimulating signals involved in the initiation of TI antibody responses.

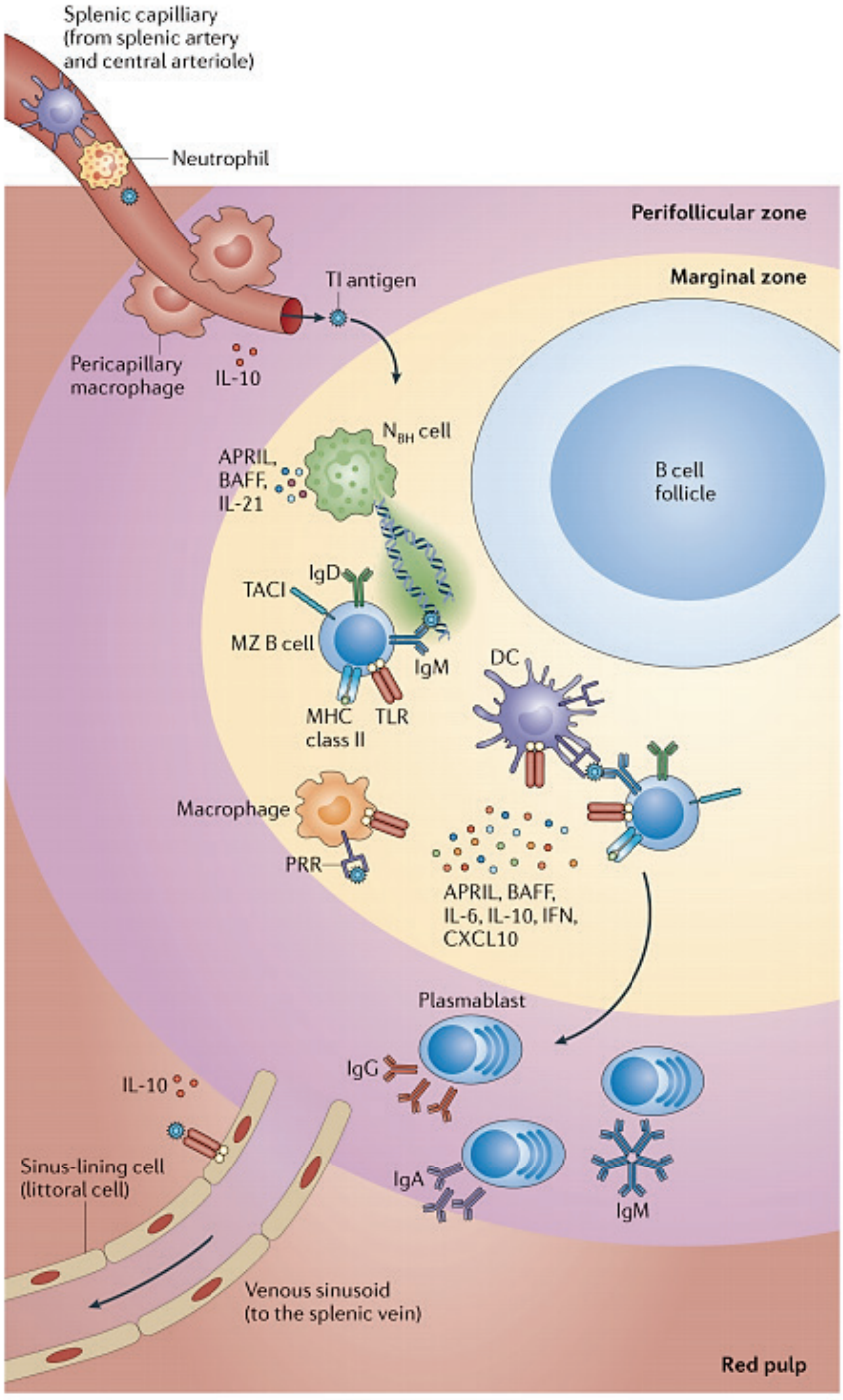

Splenic capilliary
(from splenic artery
and central arteriole)
Neutrophil
Perifollicular zone
Marginal zone
TI antigen
Pericapillary
macrophage
IL-10
N_{BH} cell
APRIL,
BAFF,
IL-21
B cell
follicle
IgD
TACI
MZ B cell
IgM
MHC
class II
TLR
DC
Macrophage
PRR
APRIL, BAFF,
IL-6, IL-10, IFN,
CXCL10
Plasmablast
IgG
IL-10
Sinus-lining cell
(littoral cell)
IgA
IgM
Venous sinusoid
(to the splenic vein)
Red pulp

Figure 7. TI MZ B cell responses in humans. TI antigens enter the MZ through the PFZ. In the outer MZ, antigens may be captured by neutrophil extracellular trap (NET)-like structures emanating from N_{BH} cells. These cells may differentiate from circulating neutrophils in response to IL-10 from perifollicular sinus-lining cells and macrophages and commensal TLR ligands. Antigen capture may also involve reticular cells, macrophages, sinus-lining cells and DCs. In addition to making Tl antigens available to B cell receptors (BCRs) and TLRs on MZ B cells, antigen-capturing cells release B cell-activating factor of the TNF family (BAFF) and a proliferation-inducing ligand (APRIL), which engage transmembrane activator and CAML interactor (TACI) on MZ B cells. N_{BH} cells might also release IL-21, thereby inducing class-switch recombination, somatic hypermutation and antibody production in MZ B cells. The generation of plasmablasts secreting IgM or class-switched IgG and IgA involves the production of IL-6, IL-10, IL-21 and CXC-chemokine ligand 10 (CXCL10) by antigen-capturing cells. Arrows indicate the putative path followed by antigens through the spleen. Adapted from *Cerutti et al, 2013*[105]

ii. Regulation of MZ B Cell Responses to TD Antigens

The composition of intruding antigens is likely crucial for determining whether MZ B cells enter a TI or a TD pathway. In general, blood-borne bacteria express not only TI antigens, such as TLR ligands and polysaccharides, but also TD antigens, such as outer-membrane proteins and zwitterionic polysaccharides[227]. Protein antigens and zwitterionic polysaccharides are processed to peptides and low-molecular-weight carbohydrates, respectively, and both are presented to $CD4^+$ T cells after being processed into MHC-II-containing intracellular compartments[228]. In this regard, MZ B cells express increased surface levels of MHC-II and T cell co-stimulatory molecules, including CD80 (B7.1) and CD86 (B7.2) compared to FO B cells. This expression is particularly evident after activation and

allows MZ B cells to present antigenic peptides more efficiently than FO B cells do, at least in mice[229]. The surprisingly efficient APC function of activated MZ B cells may partly relate to their continuous exposure to TLR ligands from commensal antigens, which are well-known inducers of MHC-II, CD80 and CD86.

After capturing and processing protein antigens, mouse MZ B cells establish cognate interactions with T_{FH} cells in the PALS. These MZ B cells subsequently enter the follicle to initiate a classical GC reaction that leads to the emergence of long-lived PCs and memory B cells expressing class-switched IgG with high affinity for antigen[111].

In mice, MZ B cells can also initiate extrafollicular TD responses that rapidly induce short-lived PBs secreting IgM and/or IgG. The isotypic composition of these T_{FH} cell-driven MZ B cell responses is regulated by unique subsets of DCs expressing distinct CLR family members. While MZ-based DCs express DC inhibitory receptor 2 (DCIR2, also known as CLEC4A4) and induce high-affinity IgG1 but not IgM via an extrafollicular pathway[186], PALS-based DCs express DEC205 (also known as LY75) and induce low-affinity or high-affinity IgM and IgG responses via extrafollicular and follicular pathways, respectively[230,231]. Finally, rapid extrafollicular TD antibody responses may further involve cognate interactions of lipid-specific MZ B cells with *i*NKT cells. Overall, these findings show that MZ B cells interact with multiple cells of the innate and adaptive immune system to mount TD antibody responses.

e. Splenic Innate Immune Cells with B Cell-Helper Function

Macrophages

Growing evidence indicates that macrophages contribute to the initition and maintenance of TD antibody responses in both mice and humans. Despite their poor APC activity, macrophages recognize, internalize, retain and expose particulate antigens through a variety of PRRs, including scavenger receptors[30,232]. In systemic LNs, subcapsular macrophages present particulate antigens or immunocomplexes to follicular B cells, thereby activating them through powerful BCR signals[233]. These activated B cells subsequently migrate to the T-B border to initiate the GC reaction[233]. Macrophages from systemic LNs initiate additionl TD responses by activating follicular B cells through multiple pathways involving DC-dependent and DC-independent presentation of CD1d-restricted glycolipid antigens to *i*NKT cells[234]. In addition to priming antigen-specific B cells, macrophages provide APRIL and IL-6 survival signals to PCs homing to the BM after emerging from the GC reaction. This process allows macrophages to sustain TD antibody production over prolonged periods of time[235].

Besides initiating TD antibody responses, macrophages enhance TI antibody production, including IgM-to-IgG CSR, by activating B cells through BAFF and APRIL[124,236]. Although sufficient to elicit CSR, macrophage-derived BAFF and APRIL require co-signals from TLR ligands and cytokines, including IL-6, IL-10 and TGF-β, to induce B-cell proliferation and PC differentiuation[124,236]. Additional evidence from human tonsils further emphasizes the complex signaling milieu

required by macrophages to initiate TI antibody responses. These studies show that BAFF and APRIL from $CD163^+$ macrophages induce IgM-to-IgG CSR as well as IgM and IgG production through a mechanism involving CXCL10, a chemokine also known as IP-10. While tonsillar macrophages produce CXCL10 in response to IL-6 released by antigen-activated B cells, engagement of CXCL3 on antigen-activated B cells by CXCL10 further augments the secretion of IL-6. This cooperation promotes the survival of B cell-derived PCs through STAT3-dependent signals from IL-6. A similar macrophage-orchestrated pathway may also enhance TD antibody production, as macrophages augment BAFF and APRIL secretion in response to IFN-γ and CD40L from T cells. It remains unknown whether similar macrophage-dependent mechanisms support antibody responsese in the human splenic MZ. Yet, these findings highlight the outstanding B cell-helper potential of macrophages from distinct anatomical districts.

In mice, splenic MZ B cells cooperate with MMMs expressing CD169 (SIGLEC-1, also known as MOMA-1), which binds sialic-acid-containing molecules expressed by microbes. The elevated expression of CD169 by these macrophages can be viewed as a strategy to concentrate blood-borne pathogens in this "front-line" area at the interface between the circulation and the immune system. Besides phagocytosing micribes, MMMs might activate CD1d-restricted *i*NKT cells to promote rapid antibody responses via extrafollicular B cells, which possibly include MZ B cells[237,238].

The mouse splenic MZ also contains MZMs, which express a DC-SIGN-related protein l called SIGNR1**.** This PRR binds complement-coated polysaccharides from both bacteria and viruses and thus may

be involved in the regulation of TI antibody responses against both these microbes. Another important PRR expressed by MZMs is MARCO, a scavenger receptor that recognizes several pathogens, including *Escherichia coli* and *Staphylococcus aureus*. Of note, SIGNR1 and MARCO have largely overlapping recognition patterns and both may enable presentation of particulate antigens to MZ B cells by macrophages[182,220], although this point has never been formally demonstrated. Indeed, both SIGNR1 and MARCO are required for the anatomical integrity of the splenic MZ, probably due to their ability to interact with both MZ B cells and stromal cells through poorly characterized counter-receptors.

In humans, the splenic MZ appears to contain neither MMMs nor MZMs, but scattered nonspecific macrophages expressing the scavenger receptor CD68 and the haemoglobin receptor CD163 can be detected, particularly in the outer portion of the MZ. Similar nonspecific macrophages are abundant in the PFZ and RP. Additional specific macrophages expressing CD169 have been shown to form pericapillary sheaths in the PFZ, an area of low resistance adjacent to the MZ. Together with neutrophils and DCs expressing CD11c and the endocytic receptor DEC205, also known as CD205, these perifollicular $CD169^+$ macrophages may contribute to antigen capture and activation of at least some MZ B cells[132,221-223]. However, this possibility has never ben formally demonstrated.

Dendritic Cells

DCs transport intact bacteria to the splenic MZ a few hours after capturing blood-borne antigens[163]. In addition to inducing TD

antibody responses against microbial proteins in splenic follicles, bacteria-transporting DCs can interact with MZ B cells in the bridging channels of the spleen to initiate TI antibody responses against microbial carbohydrates[164]. This process may involve activation of MZ B cells following BCR cross-linking by endocytosed TI antigen recycling to the surface of DCs along with DC production of the CD40L-related cytokines BAFF and APRIL. By acting in concert with antigen and cytokines such as IL-6, IL-10 and IFNs, BAFF and APRIL generate TACI-dependent signals that elicit IgM production, IgM-to-IgG CSR and PC differentiation[160].

Invariant Natural Killer T cells

As mentioned earlier, the regulation of follicullar B cell responses is not restricted to T_{FH} and T_{FR} cells, but also involves *i*NKT cells. *i*NKT cells are innate-like lymphocytes expressing an invariant V$\alpha14^+$ TCR that recognizes soluble glycolipids, such as α-galactosylceramide (α-GalCer), which is presented by the non-polymorphic MHC-I-like molecule CD1d[238-240]. After recognizing α-GalCer on DCs, *i*NKT cells can deliver noncognate help to B cells by inducing formation of efficient antigen presenting DCs and macrophages via CD40L and interferons[239,241]. The interaction of these DCs with T_{FH} cells is followed by a GC reaction that induces moderate IgG production by long-lived PCs, affinity maturation through SHM and immune memory[234].

Some studies show that *i*NKT cells can also establish cognate interactions with FO B cells[242,243]. Indeed, a subpopulation of *i*NKT cells up-regulates CXCR5 after interacting with α-GalCer presented by

CD1d-expressing B cells. Subsequent entry into the follicle stimulates *i*NKT cells to differentiate into NKT_{FH} cells through the activation of a Bcl6-dependent program that induces expression of CD40L, IL-21 and other canonical T_{FH} cell-associated molecules, including ICOS and PD-1[242,243]. The ensuing cognate interaction of NKT_{FH} cells with GC B cells elicits strong primary IgG production by short-lived follicular PBs but little affinity maturation and no immune memory.

*i*NKT cells may also enhance antibody production by inducing the formation of short-lived extrafollicular PBs. In this response, *i*NKT cells may establish a cognate interaction with CD1d-expressing B cells, including MZ B cells expressing lipid-specific BCRs[226,240]. The resulting CD1d-instructed *i*NKT cells up-regulate the expression of CD40L and release cytokines such as IFN-γ, which trigger IgM-to-IgG CSR and formation of extrafollicular lipid-specific PBs secreting IgM or IgG [226,240]. Thus, by establishing cognate or non-cognate interactions with follicular and extrafollicular B cells, *iNKT* cells activate TD and TI-like pathways that elicit rapid waves of high-affinity or low-affinity antibodies to invading microbes.

Neutrophils

Neutrophils are the first immune cells that migrate to sites of infection or inflammation[244,245]. Under homeostatic conditions, neutrophils also occupy PFZ areas of human and non-human primate spleens[78]. Also the mouse spleen contains perifollicular neutrophils, but they are about tenfold fewer than those found in humans and monkeys[78]. These neutrophils interact with perifollicular and MZ B cells through a non-inflammatory pathway that begins during fetal life and accelerates

after birth, a time that coincides with the colonization of mucosal surfaces by commensal bacteria[78].

In humans, splenic neutrophils constitutively release large amounts of APRIL and BAFF, which deliver powerful antibody-inducing signals to MZ B cells[78]. Accordingly, splenic neutrophils have been defined as as N_{BH} cells. Compared to N_C cells, N_{BH} cells have distinct phenotypic, transcriptional and functional properties[246], which reflect activation by local microenvironmental signals, including IL-10 and GM-CSF[104]. Consistent with this, the accumulation of N_{BH} cells in splenic PFZ areas coincides with postnatal deposition of discrete amounts of microbial TLR ligands of mucosal origin, such as LPS[78,205,247]. Conversely, GF mice lacking mucosal bacteria or mice lacking TLR signals show a decreased number of N_{BH} cells[104].

Together with cytokines, microbial products may contribute the differentiation of N_{BH} cells from circulating precursors. In the presence of LPS, human perifollicular sinus endothelial cells (SECs) stimulate the reprogramming of N_C cells into N_{BH} cells through IL-10[104,248]. These activated neutrophils stimulate antibody production while acquiring IL-10-dependent regulatory properties, which may enhance homeostatic humoral immune responses in a non-inflammatory environment[249]. As for GM–CSF, this cytokine is mostly released by ILC3s and stimulates the survival, activation, chemotaxis and B cell helper reprogramming of N_C cells in cooperation with IL-10[78]. The resulting N_{BH} cells upregulate AID expression in MZ B cells and induce IgM-to-IgG CSR through BAFF and APRIL[78]. Furthermore, N_{BH} cells enhance MZ B cell survival and trigger their differentiation into IgG-secreting PBs in a TI manner[104].

In mice, immature neutrophils can acquire APC properties by up-regulating MHC-II, CD80 and CD86 and releasing T-cell-activating cytokines in response to inflammatory stimuli. These neutrophils may enter the T-B border of the lymphoid follicle to initiate TD antibody production, possibly by activating T_{FH} cells[250-252]. In general, the crosstalk between B cells and neutrophils indicates that these granulocytes do not merely bind antigen-specific antibodies but also enhance antibody responses under homeostatic or inflammatory conditions. In humans, N_{BH} cells may cooperate with DCs, macrophages, monocytes, ILC3s and stromal cells to generate a variety of splenic niches capable to induce and support over time homeostatic and possibly post-immune PB and PC responses to a variety of microbial antigens[200].

Innate Lymphoid Cells

Besides mediating mucosal homeostasis, including IEC integrity, ILC3s regulate adaptive T and B cell responses, including antibody production by FO and MZ B cells[106,253-255]. Recent mouse studies indicate that ILC3 strategically located at follicular sites of lymphocyte entry promote the survival of memory $CD4^+$ T cells to enhance secondary IgG responses, including affinity maturation. This effect involves ILC3 expression of OX40 ligand (OX40L) and CD30 ligand (CD30L), two TNF family members that engage OX40 and CD30 receptors on memory $CD4^+$ T cells[256-258]. Thus, ILC3 may shape not only the magnitude, but also the affinity and duration of secondary TD antibody responses by controlling the survival of memory $CD4^+$ T cells.

Additional mouse studies show that a subset of splenic ILC3s internalize antigen and, in the presence of IL-1β, express MHC-II, CD80 and CD86 along with the T cell-activating cytokines IL-2, IFN-γ and TNF[255]. These changes allow ILC3s to present processed antigen to $CD4^+$ T cells, which thereafter undergo conal expansion to initiate TD antibody responses against protein antigens. When activated by cytokines or TLR ligands, also human ILC3 release IL-2 and up-regulate HLA-II expression[259], further corroborating the involvement of ILC3s in T cell responses.

In addition to enhancing TD antibody responses, ILC3s support TI responses against carbohydrate antigens[106] by helping the activation of splenic MZ B cells[105]. In humans, ILC3s inhabiting spelnic MZ and PFZ areas closely interact with $MAdCAM\text{-}1^+$ MRCs. Activation of these stromal cells by TNFα and LTα1β2 from ILC3s up-regulates MRC expression of ILC3-targeting survival factors, including IL-7. In the presence of IL-1β and IL-23 from local DCs and macrophages, ILC3 express BAFF, CD40L and Delta-like 1 (DLL1), a MZ B cell-inducing NOTCH2 ligand that also enhances the differentiation of MZ B cells into PBs[105,106]. In mice, splenic ILC3s express APRIL along with DLL1 and depletion of ILC3s hampers MZ B cell production of antibodies to TI antigens. In both humans and mice, ILC3s further help MZ B cells by co-opting N_{BH} cells via GM-CS[106]. In summary, it appears that ILC3s cooperate with multiple immune and non-immune splenic cell types to enhance both TD and TI antibody responses.

5. Splenic Stromal Cells

A remarkable feature of SLOs, including the spleen, is the localization of B and T cells in segregated tissue compartment with architectural and functional properties determined by complex networks of non-hematopoietic stromal cells of mesenchymal origin[260-262]. In addition to supporting the development and maintenance of SLOs through complex interactions with LTi cells, stromal cells provide structural scaffolds and chemokine-regulated antigen-conveying structures to SLOs[263]. Moreover, stromal cells form functional niches that deliver "nursing" signals to effector and regulatory immune cells engaged in the initiation, tuning and maintenance of defensive responses. Remarkably, growing evidence indicates that certain stromal cells also actively promote immune responses[262,264-266].

a. Mouse Stromal Cell Subsets

Stromal cells from SLOs, including spleen and LNs, are comprised of at least six subsets distinguishable for their distinct topographical, functional and phenotypic properties. Here, we will describe the SC subsets that make up the parenchyma of the spleen, including fibroblastic reticular cells (FRCs), follicular dendritic cells (FDCs), marginal reticular cells (MRCs) and fibroblasts (FBs). Stromal cells from LNs will be also discussed for comparison.

Lymphocytes and DCs colonize highly segregated compartments within SLOs thorugh chemokine-regulated pathways organized around SC-organized structures. B cells form orgrganized aggregates to form the cortical B cell zone of LNs and the follicular area of the WP of the spleen, whereas T cells form the T-cell zone in the PALS of

the spleen and paracortex of LNs. The T cell zone also contains DCs, which capture antigen and present immunogenic peptide-MHC-II complexes to T cells[267]. Two different subsets of stromal cells inhabit the B and T cell zones of SLOs: FDCs and FRCs, respectively **(Figure 8)**.

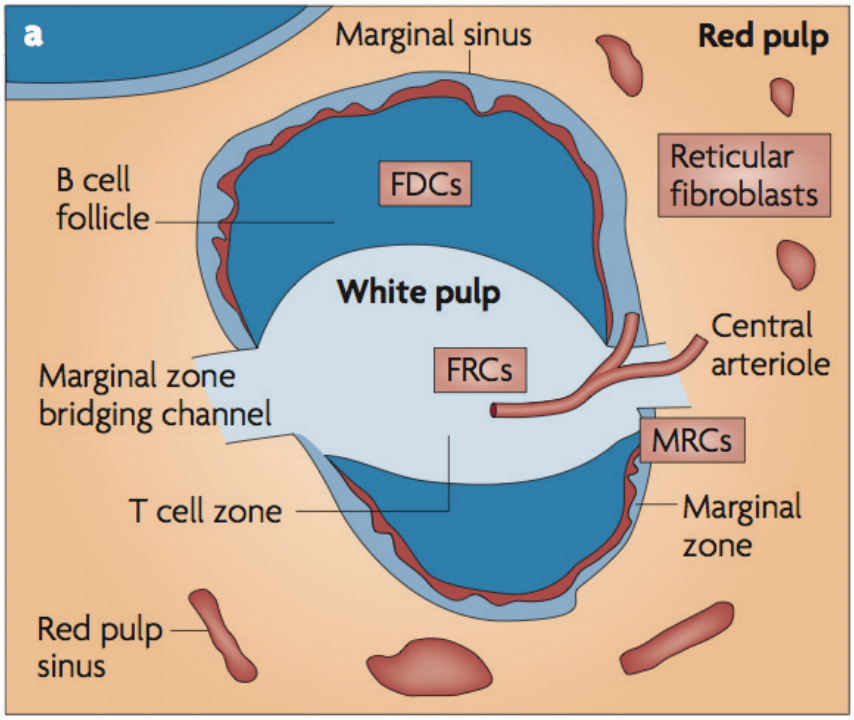


Figure 8. Architecture of the mouse spleen. a) The WP consists of T cell zones (also known as PALS) that contain networks of FRCs surrounding a central arteriole as well as B cell follicles includes a central network of FDCs. The MZ surrounding the WP is lined-up by MRCs, particularly at the edges of B cell follicles. Blood and leukocytes enter the spleen through branches of the central arteriole that drain into the MS and RP. In the cords of the RP, a dense network of reticular FBs and fibres make up an open circulation system, which is characterized by the lack of typical endothelial cell lining. Large numbers of macrophages phagocytose dying or damaged red blood cells in the RP (not shown). Immune cells enter the WP in T cell

zone areas adjacent to the MZ, known as bridging channels. Adapted from *Mueller S.N, et al. 2009*[260]

Follicular Dendritic Cells

FDCs form a dense network at the centre of the follicles that form the B cell zone of SLOs **(Figure 8)**. These stromal cells express the adhesion molecule VCAM-1, small amounts of MAdCAM-1 and a number of molecules that are common to all stromal cell subsets, including desmin and laminin. More importantly, FDCs strongly express antibody receptors (CD16, CD23, CD32) and complement receptors (CD21, CD35) that facilitate the capture of native antigen embedded within immune complexes that are subsequently exposed to FO B cells, including GC B cells[268].

Besides scanning the surface of FDCs to recognize cognate antigens, GC B cells generate powerful LT-mediated FDC differentiation signals following BCR engagement by antigen. But how do antigen-activated FO B cells migrate fro the T-B border to the center of the follicle to interact with FDCs? This migration involves FDC production of CXCL13, which generates a chemotactic gradient sensed by CXCR5 on activated FO B cells[269] **(Figure 9)**. A similar FDC-orchestrated mechanism mediated the recruitment of early T_{FH} cells, which is crucial for the initiation and maintenance of the GC reaction.

In the microenvironment of the GC, B and T_{FH} cells interact with FDCs in a highly organized manner[36]. While non-cycling B cells from the LZ interact with FDCs expressing CXCL13, cycling B cells from the DZ interact with stromal cells expressing CXCL12. Whether these

stromal cells represent a subset of FDCs or rather represent a distinct stomal population remains unclear. Certainly, CXCL13 and CXCL12 from from GC-based FDCs and stromal cells are important to regulate the interzonal migration of B cells that undelies the process of antibody affinity maturation[36].

Fibroblastic Reticular Cells

FRCs have been long viewed as structural elements involved in post-immune expansion and contraction of the LN as well as antigen transportation from the sub-capsular sinus into follicles. Subsequently, FRCs have been proposed to form specialized conduits capable of regulating the influx of cytokines and chemokines from the afferent lymph to the HEVs of LNs[270]. Later, this elegant function was formally demonstrated in both LNs[271] and spleen[272], heralding the dawn of a new era in our appreciation of the complex functions of stromal cells during the immune response.

FRCs from both spleen and LNs surround central arterioles and HEVs, respectively, forming a dense network filled with T cells[270] **(Figure 8)**. We now know from live imaging studies that T cells from these regions are in constant dynamic motion and move along FRC strands that function as chemokine-controlled guiding paths[273,274]. Indeed, FRCs produce collagen-rich reticular fibres that form a complex conduit-containing structure separate from the surrounding parenchyma.

In LNs, small molecules such as chemokines and soluble antigens from the afferent lymph enter this conduit network to gain rapid access to the T cell zone and HEVs[271]. Instead, large molecules unable

to enter the conduit system are either trapped by SCS macrophages or continue their travel around the SCS to finally drain into efferent lymphatic vessels. This process may constrain the nature of antigenic signals conveyed to T cells and DCs by FRC-generated conduits, possibly to prevent the "hijacking" of such conduits by pathogens.

In the spleen, FRCs from the T cell zone collect blood-borne molecules into conduit structures similar to those detected in LNs[272]. However, molecular exclusion differs in the spleen, since 70-kD dextran particles enter splenic but not LN conduits[271,272]. This raises the possibility that the conduit system is modeled to allow delivery of distinct antigenic signals to the T cell zones of different SLOs.

Similar to FBs from different tissues, FRCs typically express an antigen recognized by an antibody called ER-TR7[275]. In addition, FRCs express podoplanin (gp38), which allows the distinction of FRCs from other non-endothelial stromal cells[276]. FRCs are also readily identified by their abundant production CCL21 and CCL19 **(Figure 9)**, which are crucial for the delineation of T cell zones through the migration of T cells and DCs expressing CCR7[277].

In both humans and rodents, FRCs also express multiple extracellular matrix (ECM) components such as ER-TR7, fibrillin, laminin and fibronectin in addition to intracellular molecules usually found in certain FBs, including desmin and alpha-smooth muscle actin (α-SMA). These molecules may play distinct roles in FRC network formation, FRC contraction and FRC presentation of antigens or chemokines. Furthermore, FRCs express multiple integrin subunits and the adhesion molecules ICAM-1 and VCAM-1.

Similar to FDCs, FRCs may actively modulate the immune response. For instance, it has been recently demonstrated that mouse FRCs from both spleen and LNs promote cell-mediated immune responses to viruses. Furthermore, FRCs have been show to be essential for the concesrvation of a physiological follicular structure and for the maintenance of B cell homeostasis through the production of the B cell survival factor BAFF[278].

Marginal Reticular Cells

In mice, MRCs form a thin layer of stromal cells underneath the MS of the spleen and the SCS of LNs **(Figure 8)**. These MRCs have a phenotype distinct from that of FRCs and FDCs, as they express higher levels of MAdCAM-1, lack CCL19 and CCL21, and only weakly express CD21 and CD35[279]. However, similar to FRCs and FDCs, MRCs express CXCL13, ER-TR7, desmin, laminin and VCAM-1[279,280]. In the SCS, mouse MRCs further express TRANCE (RANKL)[279], a TNF family member essential for LN development[281,282] **(Figure 9)**. Interestingly, many MRCs from the SCS strongly express CXCL13 even when FDCs from small primary follicles show low or undetectable levels of this chemokine[283]. Probably, constitutive CXCL13 expression by SCS-associated MRCs is aimed at recruiting B cells to facailitate their exposure to antigens captured by local macrophages.

In general, little is known about the function of MRCs. Based on phenotypic similarities and synchronized localization during development, mouse MRCs may originate from anlagen-associated LTo cells and support the organization and function of SLOs as LTo

cells do[279]. In mouse LNs, MRCs may also deliver antigen from the SCS to lymphoid follicles by forming a conduit system as FRCs do[284,285]. Similarly, MRCs may cooperate with MMMs to facilitate antigen delivery to follicles in the mouse splenic MZ[286]. Based on these data, it is plausible that MRCs might be pecialized in the capture and delivery of antigens, at least in mice.

	LTo	MRC	FDC	FRC
MAdCAM-1	+	+	+†	-
CXCL13	+	+	+†	-
CR1/CD35	-	-	+	-
podoplanin/gp38	+	+	+†	+
ER-TR7 (reticular fiber)	+	+	-	+
CCL21	+	-	-	+
RANKL	+	+	-	-

Figure 9. Relationship between MRCs and other SC subsets in SLOs. Molecules expressed by mouse SC subsets. Expression levels of MAdCAM-1, CXCL13 and podoplanin (gp38) in FDCs are often weak or undetectable by immunohistochemistry (†). Adapted from *Katakai T., et al. 2012*[285]

Fibroblasts

Together with reticular fibres, FBs form a compact network in the antigen-filtering cords that characterize the structure of splenic PFZ and RD areas **(Figure 8)**. In these compartments, FBs may regulate immune responses by producing cytokines, including IL-6[287]. Besides enhancing the formation and survival of PCs, IL-6 and other cytokines

may regulate the phagocytic and anti-microbial properties of local macrophages. FB expression of multiple integrins may further assist the precise homing and retention of immune cells in the RP[288].

This splenic compartment lodges extrafollicular PCs expressing high levels of CXCR4 and LFA1, but low levels of follicle-retaining molecules such as CXCR5 and CCR7. By interacting with CXCR4 and LFA1, CXCL12 and ICAM-1 from FBs may enhance the retention and survival of PCs in the RP[143,287]. In the presence of malaria infection or endotoxemia, splenic FBs become extremely activated and physically merge to generate "barrier cells"[289,290]. This poorly understood process might restrict pathogen dissemination by constraining blood flow in the RP. Similar FB changes may control cell entry into or exit from sinusoids located around the WP in situation of acute stress.

FBs and reticular fibres form dense and loose networks also in the medullary cords and sinuses of LNs, respectively[291,292]. In principle, these FBs may regulate the function, survival and retention time of PCs as well as lymphocytes, macrophages, DCs and mast cells inhabiting medullary areas[291]. Consistent with this possibility, CXCL12 is largely restricted to the medulla, raising the possibility that FBs from LN medulla and splenic RP play similar roles in regulating PC retention and survival.

b. Immune Functions of Stromal Cells

Role of Stromal Cells in Lymphoid Organ Development

SLOs develop from the anlagen, an aggregate of mesenchymal and

hematopoietic cells associated with vessels or epithelium at a defined site and period in the fetus or infant[263]. A critical event in the development of SLO-associated anlagen is the accumulation of LTi cells, which interact with mesenchymal stromal cells known as LTo cells[293]. As mentioned earlier, LTα1β2 from LTi cells activates LTo cells via the LT-βR to induce ICAM-1, VCAM-1 and MAdCAM-1 adhesion molecules as well as CXCL13, CCL19, and CCL21 chemokines[167,294,295]. CXCL13 attract LTi cells via CXCR5, whereas CCL19, and CCL21 recruit T cells, B cells and DCs via CCR7[296-298]. As lymphocytes begin to accumulate after birth, the primitive SLO develops to forms segragated B and T cell compartments under the guidance of specific SC subsets[167,299].

As mentioned earlier, LTo cells express a phenotype similar to that of MRCs **(Figure 9)**. In developing LNs, LTo cells are more abundant in a peripheral region of the anlagen adjacent to a lympatic precursor of the SCS[168,279,296]. These LTo cells expand outwardly as lymphocytes progressively accumulate in the developing anlagen and ultimately form the MRC layer positioned underneath the SCS[279]. Similarly, LTo cells expand outwardly as lymphocytes accumulate around the central artery of the developing spleen and subsequently give rise to the MRC layer positioned around the MS. Overall, these observations suggest that LTo cells and MRCs are ontogenetically and functionally related and both play an important role in the shaping of SLOs.

Role of Stromal Cells in Antigen Capture

Mouse SLOs are characterized by unique structures that filter and transport antigens into lymphocyte-containing immune-inducing

compartments. LNs survey lymph-borne antigens by receiving a afferent lymphatic vessels that drains into the SCS. In contrast, the spleen surveys blood-borne antigens by receiving a central artery that drains in to the MS. In both organs, MRCs provide a structural support to the lymphatic endothelial cells that delimit the walls of the SCS and MS. In addition, MRCs further interact with $CD169^+$ macrophages that initiate immune reactions by delivering lymph/blood-borne antigens from the lumen of the sinus to adjacent lymphoid compartments[300,301]. Thus, in both LNs and spleen, MRCs may cooperate with macrophages to enhance front-line immune protection.

Although the precise immunological functions of MRCs remain elusive, these stromal cells may establish a conduit network that transports small soluble antigens to B cells and FDCs[284,299,302]. In addition, MRCs could model the localization, morphology and function of $CD169^+$macrophages, including their ability to capture particulate antigens and present them to B cells[300,301]. Accordingly, inhibition of MRC-targeting $LT\alpha1\beta2$ signals alters the correct positioning of antigen-capturing $CD169^+$ macrophages along the MS, which virtually disappears[303-305]. By expressing ICAM-1, VCAM-1 and CXCL13, MRCs may also orchestrate the migration of antigen-primed B cells and DCs to specific inductive sites and T cell areas, respectively.

Role of Stromal Cells in B and T Cell Functions

After entering the spleen and LNs, $CXCR5^+$ B cells crawl onto the local FRC network as they migrate toward follicles in response to

CXCL13 from FDCs[274]. Given that CXCL13 is also produced by peri-follicular MRCs, it is likely that B cells further interact with MRCs, but the functional impact of this interaction remains unknown. Together with FRCs, MRCs could modulate multiple functions of B cells, including their chemokine-guided movement across different regions of the follicle and quality of their response to antigen. More in general, MRCs, FRCs and FDCs likely deliver geographically and hierarchically organized signals to B cells as they move across distinct areas of SLOs.

Similar to B cells, T cells interact with MRCs and FRCs after entering LNs and with MRCs and FBs after entering the spleen. In the T cell zone, FRCs produce large amounts of CCL21[153], which generates robust retention signals in T cells by engaging CCR7. These CCR7 signals permit the permanence of T cells in SLOs for at least 12 hours, during which T cells screen the surface of DCs to eventually detect cognate antigens[306]. Similar to B cells[172], T cells exit SLOs through a mechanisn involving S1P, whose levels are much higher in blood and lymph compared to SLO tissues. It is likely that stromal cells facilitate the exit of T cells from SLOs by facilitating their progressive up-regulation of S1P1 receptors over time. The resulting increased S1P responsiveness over time allows T and B cells to overcome CCL21-mediated retention signals, thereby mediating lymphocyte entry into efferent lymph or venous blood, respectively[307].

But stromal cells may not limit their lymphocyte-modulating function by merelyreleasing chemokines. Indeed, stromal cell-expressed adhesion molecules such as integrins, VCAM-1 and podoplanin could generate traction forces that further influence lymphocyte migration

through different regions of SLOs. Sialic acid from splenic stromal cells plays a similar role, as it facilitates is lymphocyte trafficking to the WP[308]. Last but not least, expression of MHC-I and the inhibitory molecule PD-L1[309] may allow FRCs to finely tune T cell responses to antigen.

Role of Stromal Cells in DC and Macrophage Functions

The SLOs are professional immune surveillance structures that contain functionally distinct subsets of antigen-presenting DCs and antigen-capturing macrophages. The functional relationship of these innate immune cells with stromal cells is the focus a growing number of studies. While LNs contain both tissue-resident and migratory DCs, the spleen mostly includes tissue-resident DCs. These professional APCs accumulate next to HEVs in LNs and within bridging channels in the spleen, which facilitates the encounter of cognate antigens by T and B cells[310]. In LNs, resident DCs form a stable network by interacting with FRCs positioned within the T cell zone. In particular, the $CD8\alpha^+$ subset of DCs is tightly associated with CCL21-expressing FRCs[311,312]. In the spleen, resident $CD8\alpha^-$ DCs predominantly inhabit the MZ and bridging channels, where they probably interact with MRCs[317]. It remains unclear whether $CD8\alpha^-$ DCs occupy functionally equivalent areas of LNs.

Similar to lymphocytes, DCs use reticular networks of FRCs to orchestrate their migration to SLOs. In LNs FRCs stimulate DC maturation, including dendrite extension as well as antigen probing, capture and processing, by releasing CCL19 and CCL21, which bind

CCR7 on DCs[313]. CCL19 and CCL21 from FRCs further promote cognate DC-T cell interactions by stimulating the activation of T cells. This activation entails DC capture of antigen transported along conduits established by FRCs. In addition to DCs, macrophages from the SCS of LNs or MZ of the spleen capture antigen deposited on conduits generated by MRCs[314]. Whether MRCs directly transfer conduit-associated antigen to B cells remains to be established. In general, these findings clearly show that FRCs and MRCs facilitate the initiation of immune responses by establishing an intimate interplay withwith DCs and macrophages.

Role of Stromal Cells in Lymphocyte Responses

Infection, inflammation or immunization cause marked changes in SLOs, including spleen and LNs. In particular, there is an increased recruitment of CCR7-expressing T cells, B cells and DCs caused by local release of inflammatory cytokines and chemokines, including CCL19 and CCL21 from HEVs and FRCs[315]. Concomitaantly, there is a reduced egression of immune cells from inflamed SLOs for several days after infection, partly due to increased local release of IFN-I[316]. In LNs, these changes occur together with a rapid growth of HEVs triggered by vascular endothelial growth factor (VEGF), an angiogenic factor mostly produced by FRCs[317]. Of note, FRCs also undergo robust proliferation to accommodate the increased cellularity of antigen-stimulated SLOs.

At the peak of the immune response, FRCs down-regulate CCL19 and CCL21 expression, whereasand FDCs decrease CXCL13 expression[318]. These phenomena may be viewed as an FRC-controlled negative

feedback that constraints de novo recruitment of naïve lymphocytes and immature DCs when this recruitment is no longer needed. An additional advantage of decreased FRC expression of CCL19, CCL21 and CXCL13 is that memory and effector lymphocytes generated during the primary immune response may undergo more efficient expansion in a less competitive environment depleted of growth factor-consuming and stromal niche-occupying naïve lymphocytes. At a later stage, effector lymphocytes decrease CCR7 but increase CXCR3 expression, which facilitates their trafficking from SLOs to inflamed peripheral tissues expressing the CXCR3 ligand CXCL10. Overall, these findings indicate that FRCs and FDCs are central to the positive and negative regulation of SLO responses to infection, inflammation or immunization.

Remarkably, stromal cells can influence immune responses in additional and somewhat unpredictable ways. For example, FDCs and FRCs from intestinal but not peripheral SLOs express RA, a metabolite of dietary vitamin A[319]. By engaging intranuclear RA orphan receptors (RORs), RA increases the expression of α4β7 and CCR9 gut-homing receptor on effector T and B cells emerging from gut inductive sites, including mesenteric lymph odes (MLNs) and PPs. While α4β7 binds to MAdCAM-1 on HEVs from the gut effector site of the lamina propria (LP), CCR9 follows chemotactic gradients established by CCL25 produced by IECs. But this is not all. Indeed, FDCs from PPs and MLNs as well as poorly characterized stromal cells from gut isolated lymphoid follicles (ILFs) and LP but not peripheral LNs support TI and TD IgA responses in the gut, including IgM-to-IgA CSR and PC differentiation. This process involves

cooperative SC and DC production of B cell and IgA-inducing factors, including BAFF, APRIL, RA, TFG-β and IL-6, through a LTi cell-orchestrated mechanism enhanced by commensal TLR ligands[320]. Last but not least, some stromal cells could initiate adaptive cytotoxic responses by presenting antigen to T cells via HLA-II[321], whereas other stromal cells may attenuate T cell responses by expressing PDL-1[318]. Overall, these observations indicate that stromal cells regulate immune responses at multiple levels, far beyond the mere control of immune cell trafficking and survival.

Role of Stromal Cells in Lymphocyte Homeostasis

Mature lymphocytes are maintained at a constant level by steady-state release of lymphocytes newly generated by the thymus and BM, which counterbalances the death of aged or negatively selected lymphocytes. In the mouse, T cells survive for at least 8 weeks after emerging from the thymus, but this lifespan further decreases in the absence of antigenic signals[322]. Besides antigen, IL-7 plays a major role in T cell homeostasis and indeed this cytokine contributes to lymphocyte repopulation in lymphopenic SLOs[323]. This process occurs in the T cell zone of SLOs and may involve IL-7 and CCL19 production by FRCs[262].

Interestingly, IL-7 may also originate from MRCs positioned underneath the SCS[324]. Consistent with this possibility, also MRCs from human spleen express IL-7[106]. Remarkably, *in vitro* experiments indicate that T cells might be required to form mature FRC networks, raising the possibility that FRCs and T cells engage in a mutually beneficial crosstalk within SLOs[331]. Besides supporting T cell

homeostasis, stromal cells mediate B cell homeostasis through a mechanism that dioes not require IL-7[329]. Indeed, various radio-resistant non-hematopoietic cell types, including FDCs, express BAFF, which delivers mandatory survival signals by engaging BAFF-R on B cells[325,326]. Finally, poorly defined stromal cells from the spleen and BM support the survival of PBs and PCs through both contact-dependent and contact-independent signals, including the cytokines APRIL and IL-6.

Pathogen Interactions with Stromal Cells

The powerful function of stromal cells in immune responses may have a dark side, as it is becoming increasingly apparent that several intracellular pathogens target stromal cells, in particular FRCs. For instance, lymphocytic choriomeningitis virus (LCMV) infects FRCs in both spleen and LNs[309]. Besides the PALS, LCMV infects the RP, MZ and follicles from the spleen, suggesting viral targeting of FBs, MRCs and FDCs in addition to FRCs. Of note, LCMV impairs conduit formation by FRCs, which may contribute to chonic viral persistence. Multiple strains of LCMV cause FRC destruction and dysfunction[327] and similar detrimental effects on FRCs and FDCs have been observed upon infection with *Leishmania donovani*[328], simian immunodeficiency virus (SIV)[329], cytomegalovirus[330], as well as Ebola, Marburg or Lassa viruses[331].

The detrimental effects that these pathogens exert on these cells may have profound negative effects on antiviral immunity, hampering pathogen clearance while enhancing pathogen persistance. Interestingly, malaria parasites seem to preferentially infect FBs from

the splenic RP, which become dysfunctional[290]. In particular, infection prevents the formation of splenic barrier cells, which would otherwise restrict parasite access to the splenic cords. In summary, various pathogens seem to target distinct subsets of stromal cells. The full impact of SC infection by different pathogens is not fully understood, but it seems clear that pathogen-induced SC loss or dysfunction hampers the development of a protective immune response.

c. Unicity of Human Spleen and Role of Stromal Cells

Virtually all the functional information available on stromal cells has been gained from mouse studies. Except some phenotypic data, little is known about human stromal cells, including stromal cells from the human spleen. Publsihed immunohistologycal studies distinguished at least three phenotypically and morphologically subsets of stromal cells in the WP and MZ of human spleens[132,134,332,333].

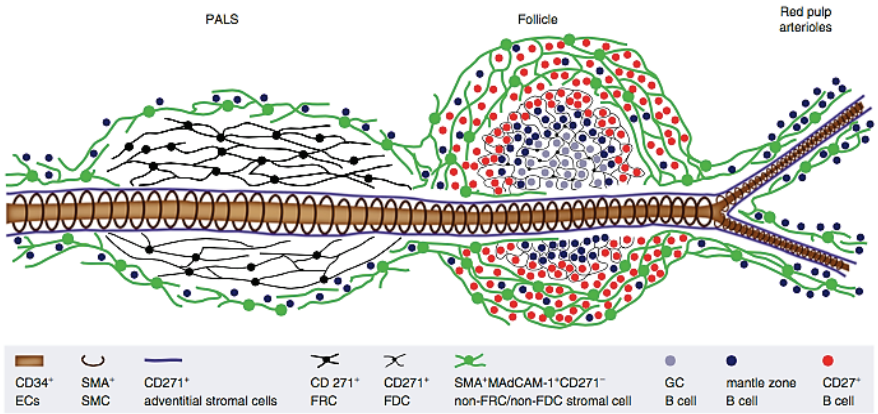


Figure 10. Schematics of PALS (left), secondary follicle (centre) and RP arterioles (right) from an adult human spleen stromal cell and B cell subsets but not T cells are depicted. PALS, follicles, vessels and cells are not shown on scale. The superficial stromal cells and FRCs have a sheet-like shape when tissue is sectioned tangentially.

The expression of α-SMA in non-FRCs-non-FDCs stromal cells possibly corresponding to MRCs is variable. Follicles may be crossed by lateral branches of central arterioles. Endothelial cells, ECs; smooth muscle cell, SMC; germinal centre; GC. Adapted from *Steiniger B. et al., 2015*[333]

Human FRCs inhabit the inner portion of PALS, which is primarily organized around arterioles branching from trabecular vessels **(Figure 10)**. This PALS contains $CD4^+$ and $CD8^+$ T cells along with conventional DCs and some pDCs[221,334]. The phenotype of human FRCs may differ from that of mouse FRCs, because immunohistological stainings failed to detect podoplanin[341]. However, human FRCs express the low-affinity nerve growth factor receptor CD271, which is also weakly positive in FDCs, as well as that CD141, CD90 and CD105[341]. In contrast, human FRCs express little or no MAdCAM-1 and α-SMA.

Human FDCs inhabit the mantle zone and GCs of follicles and express CD271 along with the complement receptors CD21 and CD35[341]. The Thy-1 antigen CD90 is only weakly expressed by FDCs from the mantle zone, whereas FDCs from the GC express some CD90. No more information is available regarding FDCs, although some studies indicate their important role in the survival, activation and differentiation of GC B cells.

Human stromal cells possibly corresponding to MRCs surround the surface of splenic follicles and PALS, inhabit the proper splenic MZ, express MAdCAM-1, CD90, CD105 (endoglin), CD141 and α-SMA, and produce CXCL12, CXCL13 and CCL21. In contrast to MRCs from the subcapsular area of human LNs, MRCs from human spleens

seem to lack podoplanin and RANKL (CD254), at least by tissue staining.

In the human perifollicular area, a SC network forms two distinct inner and outer areas: one proximal to and the second distal from the surface of adjacent lymphoid follicles[221]. The inner area includes some $CD205^+$ immature DCs and some pDCs, but does not contain known subsets of lymphocytes. In contrast, the outer area contains IgD^{lo} MZ B cells and a few scattered T cells. Notably, relatively rare IgD^{hi} B cells can be detected at the margins of the SC-containing MZ and at the periphery of SC-surrounded PALS. In these copartments, IgD^{hi} lymphocytes may represent FO B cells entering the RP to reach the general circulation or FO B cells migrating the T-B border, respectively.

In humans, a striking feature of MZ-associated stromal cells relates to the fact that they are associated neither with the MS, which is absent in human spleens, nor with MMMs and MZMs. The lack of professional MZ-based macrophages might be partly compensated by the presence of $CD169^+$ macrophages forming pericapillary sheats in the PFZ[134]. But overall, it is fair to say that the human MZ contains only rare macrophages, raising the possibility that other cell types take over the antigen-capturing function of these phagocytes.

Besides DCs, stromal cells might exert macrophage-like functions. This may be consistent with the observation that the human splenic MZ contains multiple SC layers expressing heterogeneous phenotypes. Larger SC layers typically contain MAdCAM-$1^{hi}CD90^+CD271^-$ cells that likely correspond to MRCs, whereas smaller SC layers include

MAdCAM-1$^{lo/-}$α-SMA^{+}CD141$^{+/-}$CD271^{-} cells that may be entirely different from MRCs or represent a specific differentiation/functional stage of MRCs. Of note, MAdCAM-1^{hi} MRCs are connected to large reticular fibers decorated by laminin and associated with some CD4^{+} T cells[130,335].

Despite the numerous species-specific differences[279,332], it is plausible that the stromal cells detected within the human splenic MZ represent the human equivalent of mouse MRCs **(Figure 10)**. One important question relates to the function of such stromal cells. Recent studies from our goup show that human splenic MRCs deliver contact-dependent and contact-independent survival signals to ILC3s, incuding IL-7[106]. Conversely, LTα1β2 and TNFα from ILC3s promote the activation of MRCs, including increased VCAM-1 and ICAM-1 expression[106]. Additional interactions of these cell types with MZ B cells promote homoestatic or post-immune TI IgM responses through a BAFF/APRIL-dependent mechanism further involving N_{BH} cells, a neutrophil subset coopted by ILC3s through GM-CSF[106].

In the following studies, we will ask whether human splenic MRCs enhance homeostatic IgM responses against commensal bacteria. We hypothesize that these MRCs exert macrophage-like antigen-capture functions and deploy macrophage-like signals to activate the differentiation of commensal-specific MZ B cells into clonally related IgM-secreting PBs and PCs. We further argue that this process may help the human spleen to build a secondary line of humoral defense aginst commensals breaching the bacterial barrier.

CHAPTER 2
AIMS

The overall goal of this project was to characterize the ontogeny, clonal architecture and regulation of homeostatic antibody responses to mucosal commensal antigens, including the interaction of splenic MZ B cells with marginal reticular cells (MRCs), an elusive stromal cell population inhabiting the marginal zone (MZ) of the human spleen.

AIM 1: To evaluate the role of splenic MZ B cells in systemic antibody responses to commensal antigens.

AIM 2: To elucidate the ontogeny of splenic PCs releasing commensal-specific antibodies, including their clonal relationship with MZ B cells.

AIM 3: To determine whether MRCs activate MZ B cells to induce their differentiation into antibody-secreting PCs.

CHAPTER 3
MATERIALS AND METHODS

Human Samples and Cell Lines

Mononuclear cells were isolated from histologically normal deceased organ donors or individuals undergoing post-traumatic splenectomy without clinical signs of infection or inflammation[78,106]. The use of tissue samples was approved by the Ethical Committee for Clinical Investigation of the Institut Hospital del Mar d'Investigacions Mèdiques (CEIC-IMIM 2011/4494/I). Sera samples were from fresh blood of healthy age-matched volunteers or splenectomized patients, provided by the Hospital Clínic de Barcelona. Fresh tissue samples and formalin-fixed and paraffin-embedded tissue sections were collected from the Mar Biobanc tissue repository with patient signed informed consent. All tissue samples were assigned coded identifiers and relevant clinical information remained concealed. Human Dermal Fibroblasts from adult (FB) skin was purchased from Sigma and cultured following manufacturer's instructions.

Mice

Wild-type (WT) C57BL/6 mice were obtained from Jackson Laboratory and housed under specific pathogen-free conditions. All animals were used at 8-14 wk of age. All experiments were approved by the Ethics Committee of the Barcelona Biomedical Research Park (PRBB) and performed according to Spanish and European legislation.

Sample Processing

Human splenocytes were obtained from fresh samples as reported in published studies[78,106]. To isolate human MRCs, splenic tissues were

enzymatically digested in Hank's balanced salt solution (Lonza) containing 1 mg/ml collagenase-IV (Invitrogen) and 50 ng/ml DNAse-I (New England BioLabs) at 37 °C for 45 min, followed by separation on Ficoll-Hypaque gradient. Splenocytes were resuspended in EGM medium (Lonza) and cultured in 0.1% gelatin (Sigma) pre-coated plates. 72 h later, adherent cells were obtained with Accutase cell detachment solution as instructed by the manufacturer (Millipore). Finally, $CD45^{-}$ $CD31^{-}$ adherent cells were sorted by flow cytometry and expanded up to three passages in EGM medium.

Flow Cytometry and Cell Sorting

Human splenic naïve B cells ($CD19^{+}CD38^{-}CD10^{-}IgD^{++}IgM^{+}CD27^{-}$), MZ B cells ($CD19^{+}CD38^{-}CD10^{-}IgD^{+}IgM^{++}CD27^{+}$), ME switched B cells ($CD19^{+}CD38^{-}CD10^{-}IgD^{-}IgM^{-}$), GC B cells ($CD19^{+}CD38^{+}CD10^{+}$), PBs ($CD19^{+}CD10^{-}CD38^{++}CD27^{++}MHCII^{+}$), PCs ($CD19^{+}CD10^{-}CD38^{++}CD27^{++}MHCII^{-}$), MRCs ($DAPI^{-}CD45^{-}CD31^{-}PDPN^{+}MadCAM\text{-}1^{+}$), macrophages ($DAPI^{-}CD14^{+}$), T cells ($DAPI^{-}CD3^{+}CD11c^{-}CD19^{-}$), DCs ($DAPI^{-}CD3^{-}CD14^{-}CD11c^{+}$) and BMSCs ($DAPI^{-}CD45^{-}CD31^{-}$) were incubated at 4°C with Fc-blocking reagent (Miltenyi Biotec) before the addition of the appropriate 'cocktails' of fluorochrome-labeled mAbs **(Table 1)** and were sorted with a FACSAria II (BD Biosciences) after exclusion of dead cells through 4′-6-diamidine-2′-phenylindole (DAPI) staining. The purity of cells sorted was consistently >95%. For the intracellular staining cells were stained with mAbs to specific surface molecules, fixed, permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen), and finally incubated with mAbs to human AID, Ki-67 and TLR9 **(Table**

1). Cells were acquired with LSRFortessa or LSRII (BD Biosciences) and data were further analyzed with FlowJo V10 software (TreeStar).

Cultures

Human splenic MRCs were plated in 24-well flat-bottomed plates and cultured for 24 or 48 h in EGM media, with or without 100 ng/ml LTα1β2 and 10 ng/ml TNFα (R&D Systems) or 1 μg/ml CpG ODN-2006 (Invivogen) or 100 ng/ml LPS (InvivoGen). In co-culture experiments MRCs were plated in 96-well U-bottomed plates for 24 h until confluence and MZ B cells were added at 1:20 ratio with or without 0.5 μg/ml CpG ODN-2006 (Invivogen) for 5 days. For blocking assays, 10 μg/ml α-MadCAM-1 (Biorad), 10 μg/ml α-ICAM-1 (R&D Systems) or/and 10 μg/ml α-VCAM-1 (R&D Systems) or 10 μg/ml isotype control IgG1 mAb (R&D Systems) were added.

Viability and Proliferation Assays

Cell survival was measured using the Annexin-V Apoptosis Detection Kit II (BD Pharmingen). Gates and quadrants were drawn to give ≤ 1% total positive cells in samples incubated with isotype control mAbs. Cell proliferation was assessed by CFSE (carboxyfluorescein diacetate succinimidyl ester) dilution staining using the CellTrace CFSE Cell Proliferation Kit (Invitrogen).

Generation of Epstein-Barr Virus Immortalized B Cell Lines

Purified B cell subsets were incubated with 2 mL of EBV culture supernatant (obtained from the B98-5 cell line) and 0.5 μg/mL of

CpG ODN-2006 (Invivogen) for 2 h in a 37°C, 5% CO2 incubator. Then, 3mL of R10 medium (RPMI1640, 10%FBS) supplemented with 0.5 μg/mL of CpG2006 and cyclosporine A (Sigma-Aldrich) (at 1 μg/mL final concentration) was added and the cell culture was extended for one week. After that, 5mL of R10 medium was added and the cell culture was extended until the cells form macroscopic clumps. EBV-transformed cells were cultured in R10 medium at 37ºC/5% CO_2 with regular feeding. Cell-free culture supernatants containing polyclonal immunoglobulins were stored at -80ºC.

Generation of Monoclonal Antibodies

1. Isolation of Single PBs-PCs from Human Spleen

PBs-PCs from human spleen were isolated by FACS. Briefly, $75x10^6$ splenocytes were stained using the antibodies indicated in **Table1**, and IgM^+ PBs-PCs ($CD19^+CD27^{hi}CD38^{hi}IgM^+$), IgG^+ PBs-PCs ($CD19^+CD27^{hi}CD38^{hi}IgG^+$) and IgA^+ PBs-PCs ($CD19^+CD27^{hi}CD38^{hi}IgA^+$) single cells were sorted on a FACSAria II (Becton Dickinson) into 96-well PCR plates containing 4 μl/well of ice-cold 0.5× phosphate-buffered saline (PBS) containing 10 mM DTT, 8 U RNAsin (Promega), 0.4 U Stop RNase Inhibitor RX (5 Prime). Plates were sealed, immediately frozen on dry ice and stored at -80ºC[336]. Four wells containing a single $CD3^+CD4^+$ T cell were also isolated as negative control.

2. Single cell Ig Gene Amplification and Cloning

The single cell RT-PCR was adapted from[336]. Total single cell RNA was reverse transcribed in 14 μl of total volume using 50 U of

Superscript III reverse transcriptase (Invitrogen), 150 ng of random hexamer primers, 0.5 µl of dNTP mix (10 mM of each nucleotide), 1 µl of 0.1 M DTT (Invitrogen), 0.5% v/v Igepal CA-630 (Sigma), 4 U RNAsin (Promega) and 6 U Stop RNase Inhibitor RX (5 Prime). The cDNA was synthetized at 42 °C for 10 min, 25 °C for 10 min, 50 °C for 60 min and 94ºC for 5 minutes.

IgH, Igλ and Igκ V gene transcripts were independently amplified by nested PCR in 96-well plates. The PCR reaction (total volume of 40 µl) contained 3.5 µl of cDNA, 20nM each primer or primer mix[336], 300 nM of each dNTP (Invitrogen), 2mM of $MgSO_4$ and 0.25 µl of Platinum Taq DNA Polymerase High Fidelity (Invitrogen). For nested PCR, 3 µl of the first PCR was used. Each round of PCR was performed for 50 cycles at 94 °C for 60 s, 60 °C (IgH/Igλ) or 62 °C (Igκ) for 60 s, 68 °C for 60 s.

PCR product was purified using the QIAquick PCR Purification Kit (Qiagen), digested using the following restriction enzymes: BshTI/Sal (IgH), BshTI /XhoI (IgL) and BshTI/Pf123II (IgK) (ThermoFisher Scientific) and ligated into the appropriated expression vector pN1-IgH, pN1-IgK or pN1-IgL by using the T4 DNA ligase (ThermoFisher Scientific). The vectors were sequenced and the cloned genes were analyzed by using the IMGT/V-Quest software[337]. Cloning of these PCR products into separate expression vectors was followed by co-transfection into human 293 cells cultured in Nutridoma medium (Roche). Three days post transfection; we recovered the supernatants from transfected 293 cells and performed ELISA to evaluate the reactivity of human recombinant mAbs secreted by 293 cells.

ELISA

Total IgM, IgG and IgA from culture supernatants were detected as reported[106]. To measure Ab-reactivity, ELISA plates were coated with specific antigens or bacteria **(Table 3)** and supernatants were used at 20 μg/ml antibodies concentrations and three 1:10 dilution in PBS. All ELISAs were developed with horseradish peroxidase-labeled goat anti-human IgM, IgG or IgA Fc Ab (Jackson ImmunoReseach) and TMB substrate reagent set (BD Bioscience). Optical dentisity (OD) absorvance at 450 nm was measured and Ab-reactivity was calculated after subtraction of background (OD_{450} of culture supernatants on PBS coated plates). Self-reactivity screenings were performed using QUANTA Lite™ ANA ELISA plates following the manufacturer's instructions (INOVA Diagnostics, Inc.).

Immunofluorescence Analysis

Frozen tissues from humans or mice were fixed as previously published[78,106] and stained with various combinations of antibodies **(Table 1 and 2)**. Biotinylated antibodies were detected with streptavidin–Alexa Fluor conjugates. Nuclear DNA was visualized with DAPI and coverslips applied with FluorSave reagent (Merck Millipore). Images were acquired either with a Leica TCS SP5 Upright confocal microscope (Leica) or a Nikon Eclipse Ni-E microscope (Nikon) and were further analyzed with ImageJ software. Hematoxilin-Eosin human splenic tissue sections were scanned and the follicles were counted using the Aperio ImageScope programme.

Immunohistochemistry Analysis

Formalin-fixed paraffin-embedded (FFPE) human tissue sections 3 μm in thickness were treated in xylene, decreasing alcohol gradient and distilled water was used for dewaxing and rehydration of the tissue. Heat induced epitope retrieval was performed for 15 minutes in Citrate buffer (pH 6) or Tris-EDTA buffer (pH 9). After epitope retrieval, tissue sections were permeabilized with 0.2% Triton X-100 in PBS, blocked with 5% bovine serum albumin and 5% Fc receptor blocking and stained with various combinations of antibodies to specific antigens **(Table 1)** using EnVision™ + Dual Link System-HRP (DAB+) for single stainings and EnVision G/2 Doublestain System Rabbit/Mouse (DAB+/Permanent Red) for double stainings (Dako). Sections were counterstained with hematoxylin.

RNA Extraction and Reverse Transcription

Total cellular RNA was isolated with the RNeasy Micro kit (Qiagen) following the manufacturer's protocol. Approximately 2 ng of RNA were reversed transcribed into cDNA using TaqMan® Reverse Transcription Reagents and Random hexamers (Thermo) as reported in previously published studies[78,106].

Quality Assessment of RNA Samples

Purity and integrity of the RNA were assessed by spectrophotometry and nanoelectrophoresis using the NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, USA) and the Nano lab-on-a-chip assay for total eukaryotic RNA using Bioanalyzer 2100 (Agilent Technologies, USA), respectively. Only samples with high purity and high integrity were subsequently used in microarray experiments.

Conventional and Quantitative Real-Time RT-PCR

For the quantification of human gene products, quantitative RT-PCR was done in 384-well plates with Power Sybr green PCR master mix (Thermo) and specific primer pairs **(Table 4)**. Gene expression was normalized to that of the gene encoding β-actin (*ACTB*) for each sample. For the analysis of Iμ-Cμ germline transcripts, conventional PCR was carried out using specific primers **(Table 5)** in a PCR volume of 50 μl with AmpliTaq Gold PCR mastermix (Invitrogen). Nested PCR analysis of Iα-Cμ and Iγ-Cμ circle transcripts was carried out using two pairs of specific primers (external and internal) and cycling conditions as follows. In the first round, the external primers were used at 95°C for 9 min in the denaturing step, 94°C for 30 sec, 60°C for 1 min for 30 cycles and 72ºC for 10min. In the second round, the internal primers were used at 95°C for 9 min in the denaturing step, 94°C for 30 sec, 60°C for 1 min for 25 cycles and 72ºC for 10min.

Gene Expression Profiling

For global transcriptome analysis RNA samples from BMSCs, MRCs and FBs were used. RNA samples were processed according to the manuals GeneChip WT PLUS Reagent kit (P/N 703174 Rev. 2) and Expression Wash, Stain and Scan User Manual (P/N 702731 Rev. 3) (Affymetrix Inc., USA). Whole transcriptome analysis was performed by using Affymetrix GeneChip Human Gene 2.0 ST Arrays (Affymetrix) in a GeneChip hybridization oven 640. Statistical analyses were performed using R (v 3.1.1, ref4) with the packages aroma.affymetrix, Biobase, limma, Vennerable and XLConnect.

Correction for multiple comparisons was performed by adjusting the p-value using false discovery rate (FDR) (Benjamini & Hochberg 1995). Genes with an adjusted p-value less than 0.05 and with an absolute fold change (FC) value above 2 were selected as significant. Functional gene categories were analyzed using Ingenuity Pathway Analysis (IPA, Qiagen). Transcriptional profiles were compared through Pre-ranked Gene Set Enrichment Analysis (GSEA) (http://www.broadinstitute.org/gsea/index.jsp); using the Molecular Signatures Database v5.1 gene set collections (H Hallmark gene sets, C2 Curated Gene sets, C5 GO gene sets from Biological process and C7 Immunologic signatures). This was performed with a ranked list of genes based on the -log10-transformed p-value with the sign of the logFC.

Next Generation Sequencing (NGS) of the Immunoglobulin Repertoire

For the amplification of Ig gene, aliquots of the resulting single-stranded cDNA products were mixed with HPLC-purified primers specific for VH1-VH6 framework region 1 (50 nM) and primers specific for constant regions Cα, Cμ and Cγ (250 nM) containing respectively the respective Illumina Nextera sequencing tags **(Table 6)** in a PCR volume of 25 μl (4 μl template cDNA) with High Fidelity Platinum PCR Supermix (Invitrogen). Amplification was performed with the following conditions: after an initial step of 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, ending with a final extension step of 72 °C for 5 min. Products were purified with miniElute PCR purification Kit (Qiagen) and Nextera indices were added via PCR with the following conditions: 72 °C for 3 min,

98 °C for 30 s, 5 cycles of 98 °C for 10 s, 63 °C for 30 s, and 72 °C for 3 min. Ampure XP beads (Beckman Coulter Genomics) were used for purification of the products, which were subsequently pooled and denatured. Single-strand products were paired-end sequenced on a MiSeq (Illumina) with the 500 cycle v2 kit (2 x 250 bp).

Analysis of Next Generation Sequencing Data

Paired-end raw sequencing reads were processed into donor-specific Ig-sequences and grouped in clonal families using a bioinformatic pipeline based on pRESTO[338], IgBLAST[339] and Change-O.[340] First, raw reads with a Phred score lower than 20 were filter out, and C-region and VH gene primers were masked. Next, corresponding paired-end reads were aligned and merged together (minimum overlap of 6 nucleotides) and annotated with donor, cell type and isotype origin. Finally, donor-specific Ig subsets were combined (prior to VDJ annotation) to estimate donor-specific clonal families. VDJ calling and assignment was performed using the default parameters for IgBLAST and querying the latest downloaded human IGH IMGT database (Feb 2016). Donor-specific clonal groups were inferred using a distance-based clustering method implemented in Change-O: sequences with the same VH and JH annotation, having the same CDR3 region length, and with Hamming distance higher than 85%, were considered to belong to the same clonal group or family[340]. Finally, germline sequence for each clone was reconstructed using the annotated VDJ information as implemented in Change-O. Donor-specific clonal groups were represented through Circos plots[341] to visualize the relationships between B-cell subsets at clonal level. VH and JH gene usage was estimated for each donor and B-cell subset, with bars

representing average values among donors. SHM levels were estimated by averaging the number of mutations (compared to the inferred germline sequence) of each clone belonging to a given B-cell subset and donor. Donor-specific clonally related B-cell lineage trees were estimated and plotted using IgTree software[342]. We have mapped the CDR3 of the monoclonal sequences (generated with Sanger) into our deep-sequencing reads.

Microbial Profile Analysis

To study the bacterial diversity and comparative community structure of human splenic DNA samples, in particular of $CD14^+$ macrophages we used the PhyloChip™ 16S microarray-based assay and bioinformatics methods. PhyloTech PhyCA-Stats™ analysis software package was used for multivariate statistical analysis. We focused our results into analysing the phylum and families contained in our samples. We compared our phylums with the ones present in stool, skin and oral cavities human samples; registered by the Human Microbiome Project (HMP)[343,344].

Detection of Bacterial 16S rRNA

To amplify genomic DNA from human samples, we first sorted out human splenic MRCs, T cells, DCs and macrophages as indicated above and purified the DNA following the QIAamp DNA Mini Kit (Qiagen). We amplified the bacterial DNA using the 16S rRNA primers from Illumina **(Table 7)** and following the Platinium High Fidelity PCR protocol (Invitrogen). Total *Ecoli* DNA was used as positive control.

Statistical Analysis

Differences between group-means were assessed with Prism 5.03 (GraphPad), using unpaired two-tailed Student *T* tests unless otherwise indicated and P values of less than 0.05 were considered significant.

Table 1. Antibodies to human antigens

Antigen	Label	Isotype	Clone	Manufacturer	Use
AID	-	m, IgG1, κ§	ZA001	Life Technologies	FC#
BAFF	PE∫	m, IgG1, κ	1D6	eBioscience	FC
BAFF-R	PE	m, IgG2a, κ	8A7	eBioscience	FC
BCMA	PE	g, IgG	pAb†	R&D	FC
CCR7	PE	m, IgG2a, κ	G043H7	Biolegend	FC
CCR9	PE	m, IgG2a, κ	L053E8	Biolegend	FC
CD10	PE	m, IgG1, κ	HI10a	Biolegend	FC
CD11a	PE	m, IgG1, κ	HI111	Biolegend	FC
CD11c	PE	m, IgG1, κ	X2	BD	FC
CD138	PE	m, IgG1, κ	DL-101	BD	FC
CD14	APC-Cy7^	m, IgG1, κ	HCD14	Biolegend	FC
CD141	-	m, IgG1, κ	1009	DAKO	IFA¶
CD141	PE	m, IgG1, κ	M80	Biolegend	FC
CD157	PE	m, IgG1, κ	SY11B5	eBioscience	FC
CD163	-	Rabbit	K20-T	Novus Biologicals	IFA
CD19	PE-Cy7	m, IgG1, κ	HIB19	Biolegend	FC
CD19	APC-Cy7	m, IgG1, κ	SJ25C1	BD	FC
CD20	FITC‡	m, IgG2b, κ	2H7	BD	FC
CD21	PE	m, IgG1	B-ly4	BD	FC
CD24	PE	m, IgG2a, κ	ML5	BD	FC
CD27	PE	m, IgG1, κ	O323	eBioscience	FC
CD27	PE-Cy7	m, IgG1, κ	M-T271	BD	FC
CD27	PerCP-Cy5.5º	m, IgG1, κ	M-T271	BD	FC
CD3	FITC	m, IgG1, κ	51.1	Biolegend	FC
CD3	APC-Cy7	m, IgG1, κ	SK7	BD	FC
CD31	AF647	m, IgG2a, κ	M89D3	BD	FC
CD36	PE	m, IgG2a, κ	5-271	Biolegend	FC
CD38	APC	m, IgG1, κ	HIT2	BD	FC
CD38	APC-Cy7	m, IgG1, κ	HIT2	Biolegend	FC
CD38	PerCP-Cy5.5	m, IgG1, κ	HIT2	BD	FC
CD4	APC	m, IgG1, κ	RPA-T4	BD	FC
CD4	FITC	m, IgG1, κ	OKT4	Biolegend	FC
CD45	PE-Cy7	m, IgG1, κ	HI30	Biolegend	FC
CD86	PE	m, IgG2a, κ	CD84.1.21	Biolegend	FC
CD90	-	Sheep	Af2067*	R&D	IFA
CD90	FITC	m, IgG1, κ	5E+10	Biolegend	FC
Collagen IV	-	Rabbit	pAb	Abcam	IFA
CXCL13	-	goat, IgG	pAb	R&D	IFA
CXCR4	PE	m, IgG2a, κ	12G5	Biolegend	FC
CXCR5	PE	m, IgG2a, κ	51505	R&D	FC
HLA-DR	AF700	m, IgG2a, κ	L243	Biolegend	FC

HLA-DR	PE	m, IgG2a, κ	L243	Biolegend	FC
ICAM-1	PE	m, IgG1, κ	HA58	Biolegend	FC
IgA	PE	m, IgG1	IS11-8E10	MACS	FC
IgA	DyLight649	goat, IgG	pAb	Jackson ImmunoR.	FC
IgD	-	Rabbit, IgG	IR517*	Dako	IFA
IgD	-	goat, IgG	2032-01*	Southern	IFA
IgD	FITC	goat, IgG	2032-02*	Southern	FC
IgG	PE	goat, IgG	pAb	Invitrogen	FC
IgG	PE	goat, IgG	pAb	Jackson ImmunoR.	FC
IgM	-	goat, IgG	H15000*	Invitrogen	IFA/IHC#
IgM	DyLight488	goat, IgG	pAb	Jackson ImmunoR.	FC
IgM	BV605	m, IgG1, κ	MHM-88	Biolegend	FC
IgM	PE	m, IgG1	SA-DA4	Southern	FC
IL-6R	PE	m, IgG1	UV4	Biolegend	FC
Integrin α4	PE	m, IgG1, κ	9F10	Biolegend	FC
Integrin β1	FITC	m, IgG1, κ	TS2/16	eBioscience	FC
Integrin β2	FITC	m, IgG1, κ	6.7	eBioscience	FC
Integrin β7	FITC	Rat, IgG2, κ	FIB504	Biolegend	FC
Ki-67	-	Rabbit, IgG	pAb	Abcam	IFA
Ki-67	PE	m, IgG1	20Raj1	eBioscience	FC
LTβR	PE	m, IgG1, κ	31G4D8	Biolegend	FC
MAdCAM-1	-	m, IgG1	314G8	Serotec	IFA
MAdCAM-1	FITC	m, IgG1	314G8	Serotec	FC
Podoplanin	PE	Rat, IgG2a, λ	NC-08	Biolegend	FC
RANK-L	PE	m, IgG2b, λ	MIH24	Biolegend	FC
TACI	PE	m, IgG2a, λ	11H3	eBioscience	FC
TNFR-I	PE	m, IgG1	16803	R&D	FC
TNFR-II	PE	m, IgG2a	22235	R&D	FC
VCAM-1	PE-Cy5	m, IgG1, κ	STA	Biolegend	FC
vWF	-	Rabbit	pAb	DAKO	IHC
α-SMA	-	Rabbit, IgG	EPR5368	Abcam	IHC
α-SMA	-	m, IgG2a, κ	1a4	DAKO	IHC

∫PE, phycoerythrin
^APC and Cy, allophycocyanin and cyanin
‡FITC, fluorescein
*Catalogue number
#FC, flow cytometry
ºPerCP, peridin chlorophill
¶IFA, immunofluorescence
#IHC, immunohistochemistry
§m, mouse
†pAb, polyclonal antibody

Table 2. Antibodies to mouse antigens

Antigen	Label	Isotype	Clone	Manufacturer	Use
B220	FITC‡	Rat, IgG2a, κ	RA3-6B2	eBioscience	IFA¶
MAdCAM-1	-	Rat, IgG2a, κ	MECA-367	Biolegend	IFA
MOMA-1	Biotin	Rat, IgG2a, κ	Polyclonal	Abcam	IFA

‡FITC, fluorescein
¶IFA, immunofluorescence

Table 3. Coating strategy for ELISA

Purified Antigens	Source	Company	Antigen concentration
Chitin	Shrimp cells	Sigma	50 μg/ml
Chitosan	Fungi	Sigma	50 μg/ml
CPS (serotypes 9N, 14N, 19F, 23F)	*Pneumococcus*	American Type Culture Collection	10 ng/ml
Escherichia coli	-	Invitrogen	1:1000
Lactobacillus	-	ATCC	1:1000
Laminarin (β-D-Glucan)	*Laminaria digitata*	Sigma	50 μg/ml
Lipoteichoic Acid (LTA)	*Bacillus subtilis*	Sigma	1 μg/ml
LPS	*Pseudomonas aureginosa*	Sigma	1 μg/ml
Mannan	*Saccharomyces cerevisae*	Sigma	50 μg/ml
Phosphorylcholine (PCh)	-	BioTools	2 μg/ml
Salmonella AT	-	ATCC	1:400
Staphylococcus aureus 29213	-	ATCC	1:1000
β-D-Glucan	*Barley*	Sigma	50 μg/ml

Table 4. Primers for human gene products

Target gene		Primer sequence
ACTB	S	GGATGCAGAAGGAGATCACT
	AS	CGATCCACACGGAGTACTTG
AICDA	S	AGAGGCGTGACAGTGCTACA
	AS	TGTAGCGGAGGAAGAGCAAT
IRF4	S	GCCAAGATTCCAGGTGACTC
	AS	ATCGTAGCCCCTCAGGAAAT
PRDM1	S	GTGGTATTGTCGGGACTTTGCAG
	AS	TCGGTTGCTTTAGACTGCTCTGTG
IL6	S	GGTACATCCTCGACGGCATCT
	AS	GTGCCTCTTTGCTGCTTTCAC
CXCL12	S	ACCGCGCTCTGCCTCA
	AS	CATGGCTTTCGAAGAATCGG
CXCL13	S	GCAGCCTCTCTCCAGTCCAA
	AS	TGGACACATCTACACCTCAAGCTT
TNFRSF17	S	GCTTGCATACCTTGTCAACTTCGATG
	AS	GAATCGCATTCGTTCCTTTCACTG
PAX5	S	TTGCTCATCAAGGTGTCAGG
	AS	CTGATCTCCCAGGCAAACAT
KI67	S	AAGCCCTCCAGCTCCTAGTC
	AS	TCCGAAGCACCACTTCTTCT
TNFRSF13	S	CGGAAAAGGAGAGCAGTGCTCA
	AS	GCCTAAGAGCTGGTTGCCACAT
TNFRSF13B	S	ACCGCGGGACTGAAAATCT
	AS	CACGCTTATTTCTGCTGTTCTGA
XBP1	S	AGGAGTTAAGACAGCGCTTGG
	AS	AGAGGTGCACGTAGTCTGAGTGCTG

Table 5. Primers for CSR

Target gene		Primer sequence
Iμ	S	GTGATTAAGGAGAAACACTTTGAT
Iα 1/2	S	CAGCAGCCCTCTTGGCAGGCAGCCAG
Iγ 1/2	S	CTCAGC CTCAGCCAGGACCAAGGAC
Cμ 243	AS	CACACCACGTGTTCGTCTG
Cμ 268	AS	GTTGCCGTTGGGGTGCTGGAC

Table 6. Primers for NGS

Target gene		Primer sequence
VH1a	S	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAGGTKCAGCTGGTGCAG
VH1b	S	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGSAGGTCCAGCTGGTACAG
VH1c	S	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCARATGCAGCTGGTGCAG
VH2	S	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAGGTCACCTTGARGGAG
VH3	S	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGTCCCTGAGACTCTCCTGT
VH4	S	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACCCTGTCCCTCACCTGC
VH5	S	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCAGCTGGTGCAGTCTGGAG
VH6	S	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAGGACTGGTGAAGCCCTCG
VH7	S	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAGGTGCAGCTGGTGCAA
Cμ	AS	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAGGAGACGAGGGGGAAAAGG
Cγ	AS	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCGATGGGCCCTTGGTGGA
Cα	AS	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAAGACCTTGGGGCTGGTCG

Table 7. Primers for 16S rRNA PCR

Target gene	Primer sequence	
16S Amplicon	S	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
	AS	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

CHAPTER 4
RESULTS

RESULTS

The Human Spleen Supports Homeostatic IgM Responses to Commensal Bacteria and Their Products

Splenic MZ B cells and B-1 cells are innate-like lymphocytes that produce microbe-reactive IgM antibodies following blood-borne infections or under homeostatic conditions[105,161]. By recognizing highly conserved molecular signatures shared by autologous apoptotic cells and invading microbes, IgM homeostatically released by innate-like B cells promote tissue integrity while promoting early clearance of intruding pathogens[345]. Of note, homeostatic IgM responses may also be important to control microbial products from commensals physiologically translocating from mucosal surfaces to the general circulation[205,206]. The mechanism underlying this process remains unknown, but commensals have been recently detected in the mouse spleen[215].

To determine whether the human spleen homeostatically generates IgM to commensals and their products, we performed ELISA using serum from healthy adult individuals and aged-matched splenectomized patients with no recent history of immunization or infection. Compared to controls, splenectomized patients had lower serum levels of IgM to *Escherichia coli*, *Staphylococcus aureus*, *Lactobacillus* and non-invasive *Salmonella typhymurium* **(Fig. 1)**, which inhabit the gut[346]. Splenectomized patients also showed decreased serum IgM to LPS from *Pseudomonas aureginosa*, lipoteichoic acid (LTA) from Gram-positive bacteria and chitin **(Fig. 1)**, a polysaccharide from fungal microorganisms inhabiting multiple mucosal surfaces[347].

Finally, splenectomized individuals had decreased serum levels of IgM to CPS from *Streptococcus pneumoniae* **(Fig. 1)**, a respiratory commensal that can cause fatal infections following spleen removal. These data indicate that human splenic B cells may be involved in homeostatic IgM responses to commensal microorganisms presumably originating from mucosal surfaces.

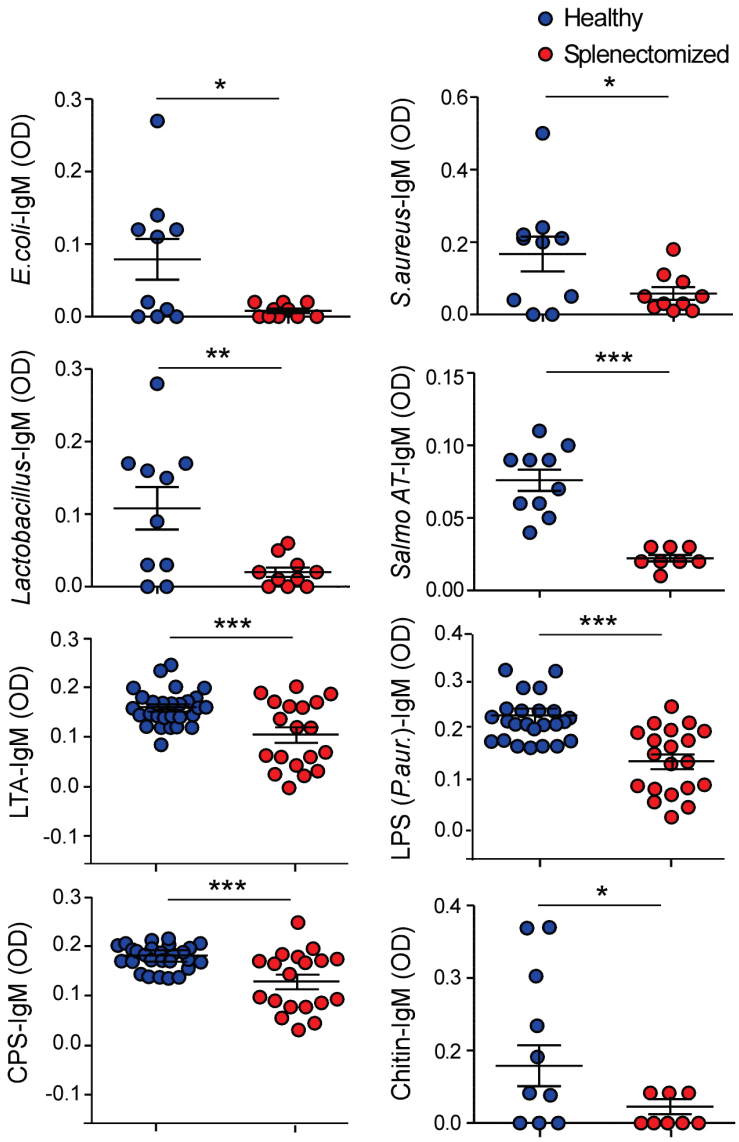


Figure 1. The human spleen enhances systemic IgM responses to commensal microbes. ELISA of serum IgM to *E. coli*, *S. aureus*, *Lactobacillus, Salmonella AT*, LTA (lipoteichoic acid), *LPS* (from *P. aureginosa*), CPS and chitin from healthy donors (HD) and age-matched splenectomized patients. Data summarize at least eight age-matched donors per group. Error bars represent s.e.m. *P<0.05, **P<0.01, ***P<0.001 (two-tailed unpaired Student's t-test).

Human Splenic IgM-Expressing PCs May Emerge from Either GC-Independent or GC-Dependent Pathways

Having shown that the human spleen supports homeostatic IgM responses to microbes, we verified whether these responses correlate with homeostatic splenic induction of PCs. PCs usually differentiate from naïve B cells undergoing maturation in GC-containing secondary follicles. These long-lived PCs usually migrate to the BM, where they tonically release antibodies into the general circulation. Unlike naïve B cells, splenic MZ B cells are more versatile and can undergo PC differentiation through either extrafollicular GC-independent pathways or follicular GC-dependent pathways. The choice of either of these pathways likely relates to the nature of the antigen. While blood-borne TI antigens such as carbohydrates and lipids preferentially stimulate GC-independent responses, blood-borne protein antigens mostly elicit GC-dependent responses.

First, we ascertained whether splenic tissues from adult individuals contained PCs along with lymphoid follicles containing GCs. Conventional microscopy of adult spleens showed that splenic tissue included more GC-less primary follicles than GC-containing secondary follicles **(Fig. 2A)**. A retrospective analysis of archived

splenic samples from children and adults demonstrated that the frequency of primary follicles and secondary follicles progressively increased and decreased with age, respectively **(Fig. 2A)**. This observation may reflect the fact that GC-inducing immunizations are frequent in the first years of life, but become rarer in adults.

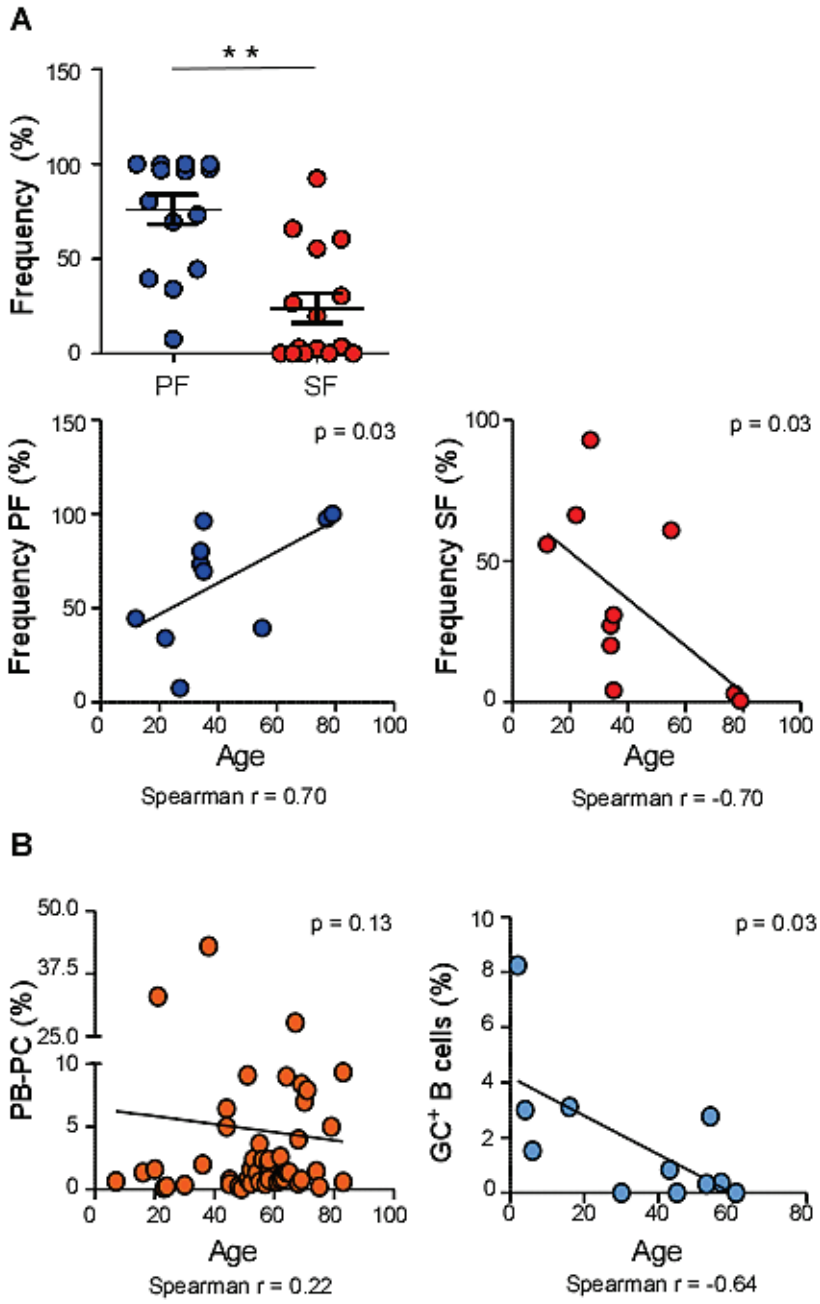
Next, we ascertained the relationship between splenic PCs and GCs. Flow cytometry demonstrated that the frequency of splenic $CD19^{+}CD27^{hi}CD38^{hi}$ PCs did not correlate with that of GC-containing secondary follicles **(Fig. 2B)**. Actually, PCs remained stable or even increased in the spleen of aged subjects harboring no or few GC-containing secondary follicles **(Fig. 2B)**. Besides showing increased PC frequency, spleens from adult subjects showed fewer $CD19^{+}CD10^{+}CD38^{+}$ GC B cells compared to spleens from younger individuals. Overall, these findings suggest that at least some splenic PCs emerge in a GC-independent manner. An alternative explanation is that splenic PCs survive for long periods of time following the demise of their inductive GCs.

To better understand this issue, further phenotypic studies aimed at gaining new insights into the origin and function of splenic PCs. Compared to $CD19^{+}CD27^{+}CD38^{-}$ B cells, which include a mix of MZ and ME B cells, splenic PCs strongly expressed canonical PC-associated molecules such as CD138 and BCMA, up-regulated the expression of the PC-inducing receptor IL-6R, but down-regulated the expression of the paradigmatic B cell-identity molecules CD20, CD21, CD24, BAFF-R and TACI **(Fig. 2C)**. Together with conserved CD19 and robust IgM expression, this phenotype indicates that splenic PCs

express an immature maturation stage, which may reflect recent differentiation from antigen-activated B cells.

Consistent with this possibility, splenic PCs expressed the integrin CD11c, the co-stimulatory receptor CD86 and the antigen-presenting molecule MHC-II **(Fig. 2C)**, three surface structures usually found on antigen-activated B cells, including recently induced and short-lived PBs. Finally, while the vast majority of splenic PCs expressed IgM, a fraction of them expressed IgG, possibly as a result of IgM-to-IgG class switching events occurring inside or outside lymphoid follicles. Very few IgA class-switched PCs were also present, albeit less abundant than IgG class-switched PCs.

In additional phenotypic studies, we attempted to gain more information regarding the migration history and geography of splenic PCs. These cells showed down-regulation of the follicle-retaining receptor CXCR5, which binds the chemokine CXCL13, and sustained up-regulation of the stroma-retaining CXCR4 receptor **(Fig. 2C)**, which binds the chemokine CXCL12. This chemokine receptor profile suggests that splenic PCs exit CXCL13-expressing follicular and MZ areas to colonize CXCL12-rich stromal niches within extrafollicular compartments that may include peri-MZ and RP areas.


A
* *
Frequency (%)
150
100
50
0
PF
SF
Frequency PF (%)
p = 0.03
Age
Spearman r = 0.70
Frequency SF (%)
p = 0.03
Age
Spearman r = -0.70
B
PB-PC (%)
50.0
37.5
25.0
10
5
0
p = 0.13
Age
Spearman r = 0.22
GC+ B cells (%)
p = 0.03
Age
Spearman r = -0.64

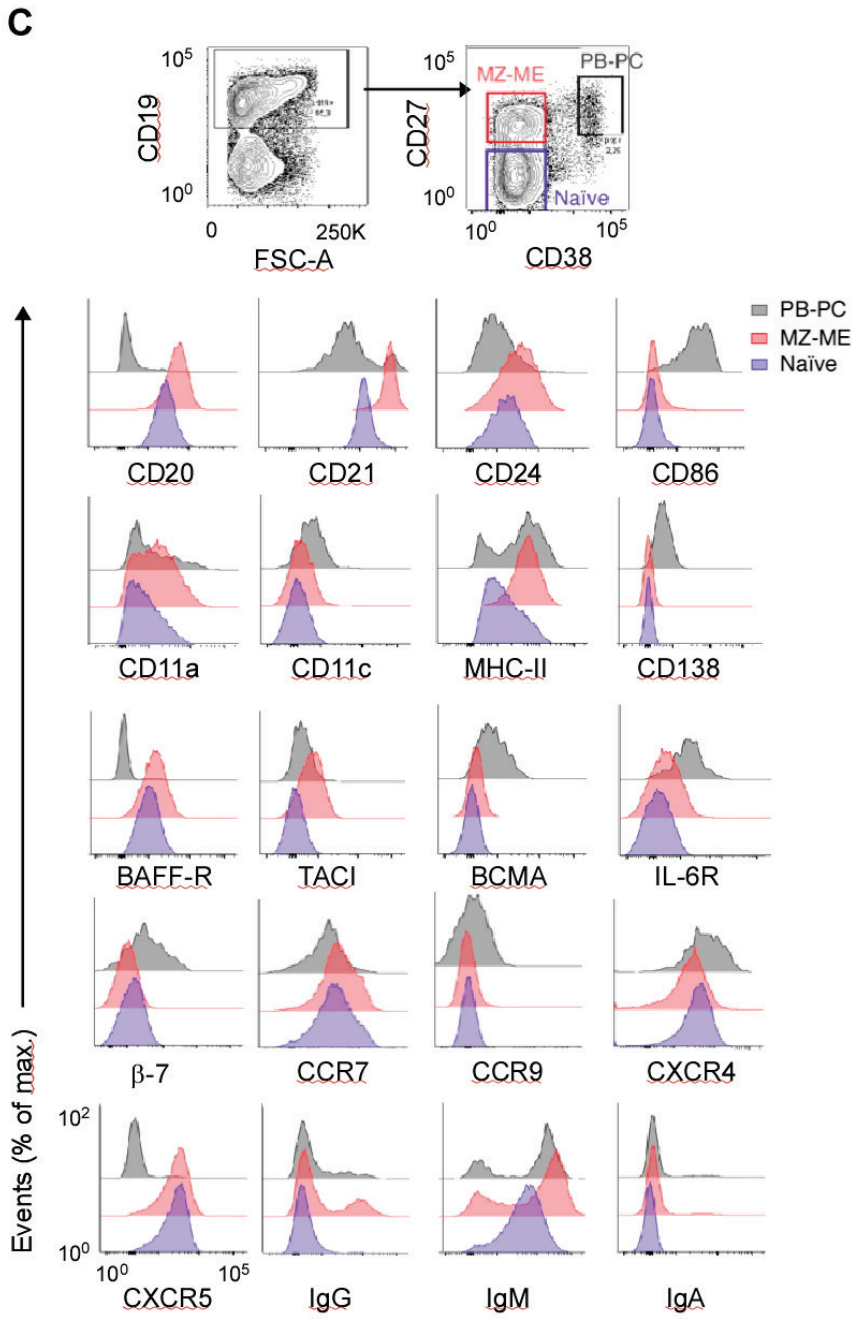


Figure 2. The human spleen contains PCs possibly emerging from both GC-independent and GC-dependent pathways. (A) Microscopic measurement of GC-less primary follicles (PF) and GC-containing secondary follicles (SF) in human

H-E-stained spleens from infants, children and adults. (B) Flow cytometric analysis of viable ($DAPI^-$) $CD19^+CD10^+CD38^+$ GC B cells and $CD19^+CD27^{hi}CD38^{hi}$ PBs-PCs in splenic tissues. (C) Flow cytometric analysis of CD20, CD21, CD24, CD86, CD11a, CD11c, MHC-II, CD138, BAFF-R, TACI, BCMA, IL-6R, β-7, CCR7, CCR9, CXCR4, CXCR5, IgG, IgM and IgA on viable ($DAPI^-$) $CD19^+CD27^{hi}CD38^{hi}$ PCs-PBs and, for comparison, naïve $CD19^+CD27^-CD38^-$ B cells and MZ-ME $CD19^+CD27^-CD38^-$ B cells. H-E, hematoxilin-eosin; FSC-A, forward scatter area; MZ, marginal zone; ME, memory; PC, plasma cell; PB, plasmablast. Data include at least ten different experiments (A, B) or are from one of four experiments with similar results (C).

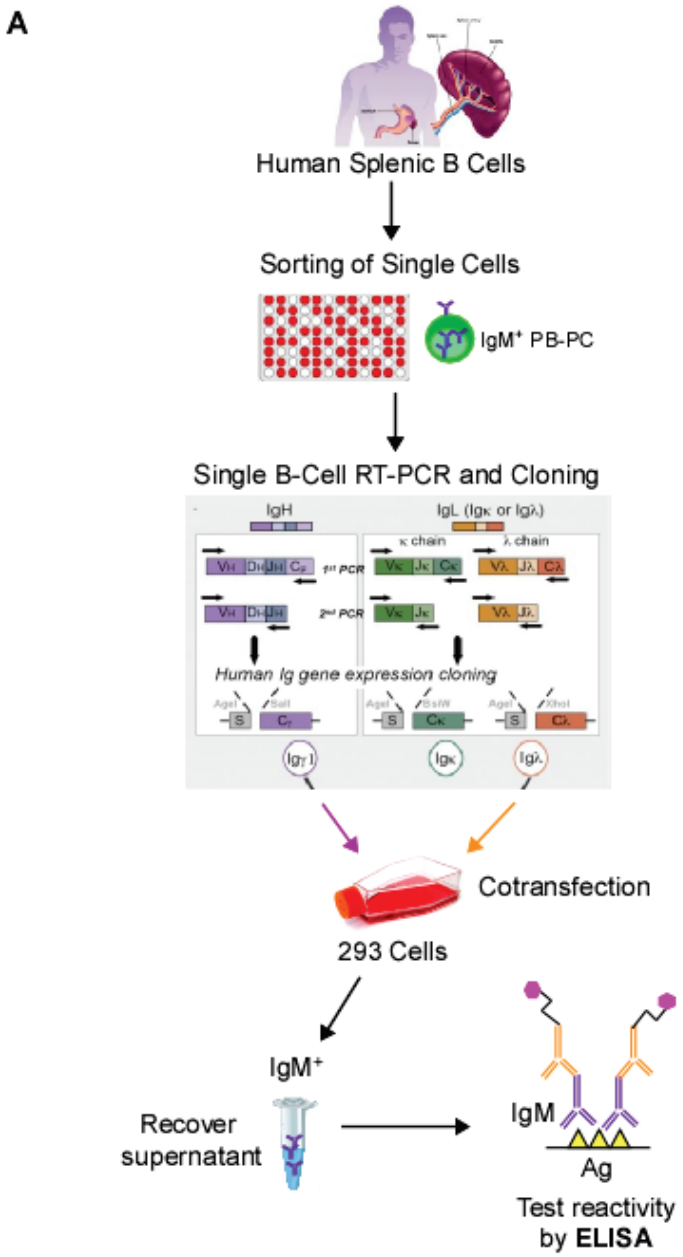
Surprisingly, splenic PCs also showed a relatively conserved expression of CCR7 **(Fig. 2C)**, a CCL19/CCL21 receptor required for B cell homing to SLOs, including the spleen. This finding indicates that at least part of splenic PCs may not home to the BM but rather remain localized in the spleen.

Finally, splenic PCs expressed more β7 and CCR9 **(Fig. 2C)**, which are MAdCAM-1 and CCL25 receptors usually induced by PCs to colonize the small intestine[348]. However, an alternative explanation is that splenic PCs up-regulate β7 and CCR9 to colonize and interact with splenic niches expressing MAdCAM-1 and perhaps CCL25. Collectively, these findings suggest that the adult spleen generates PCs through either GC-independent or GC-dependent pathways. A large fraction of these PCs could emerge from local stimulation of pre-existing effector B cells, including MZ and possibly memory B cells.

Human Splenic PCs Produce IgM Antibodies Recognizing Commensal Bacteria

Given that the human spleen homeostatically produces commensal-specific IgM and contains recently induced PCs, including PBs, we took advantage of a well-established antibody cloning-expression strategy to ascertain whether IgM from single-sorted splenic $IgM^{+}CD19^{+}CD27^{hi}CD38^{hi}$ PBs and/or PCs (hereafter referred to as PBs-PCs) recognized commensal bacteria. Briefly, we single-sorted PBs-PCs and performed RT-PCR to amplify VμDJμ-Cμ antibody H chain and matched VκJκ and VλJλ antibody L chains **(Fig. 3A)**. Cloning of these PCR products into separate expression vectors was followed by co-transfection into human 293 cells cultured in Nutridoma medium. Three days post transfection; we recovered the supernatants from transfected 293 cells and performed ELISA to evaluate the reactivity of human recombinant monoclonal antibodies (mAbs) secreted by 293 cells.

We screened a total of 15 mAbs from splenic IgM^{+} PBs-PCs and 10 of these mAbs recognized *Escherichia coli, Staphylococcus aureus* and *Lactobacillus* **(Fig. 3B)**. Of these 11 antibodies, mAb 9 recognized each of the three tested bacteria, whereas mAbs 20 and 21 recognized *Lactobacillus* and *Staphylococcus aureus*, respectively, but not the remaining two bacteria **(Fig. 3B)**. These data indicate that at least some commensal-specific IgM antibodies produced by human spleens emerge from locally generated PBs-PCs.


A
Human Splenic B Cells
Sorting of Single Cells
IgM+ PB-PC
Single B-Cell RT-PCR and Cloning
IgH
IgL (Igκ or Igλ)
κ chain
λ chain
1st PCR
2nd PCR
Human Ig gene expression cloning
Cotransfection
293 Cells
IgM+
Recover supernatant
IgM
Ag
Test reactivity by ELISA

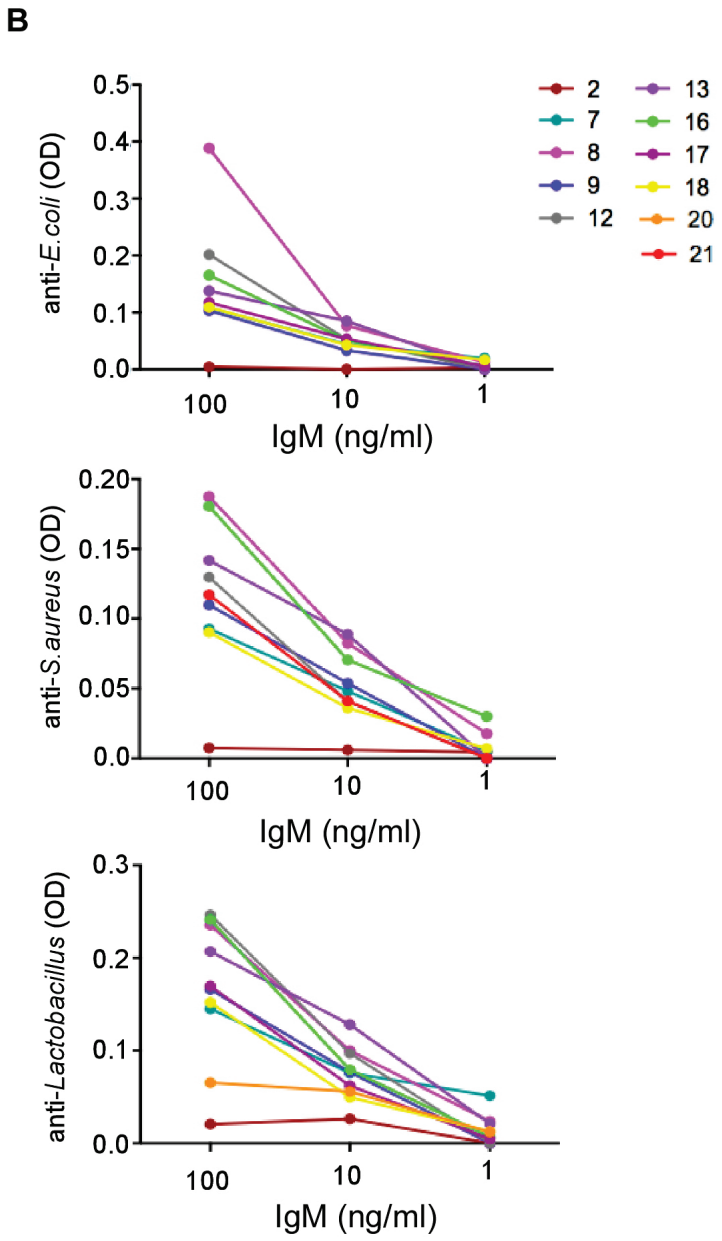
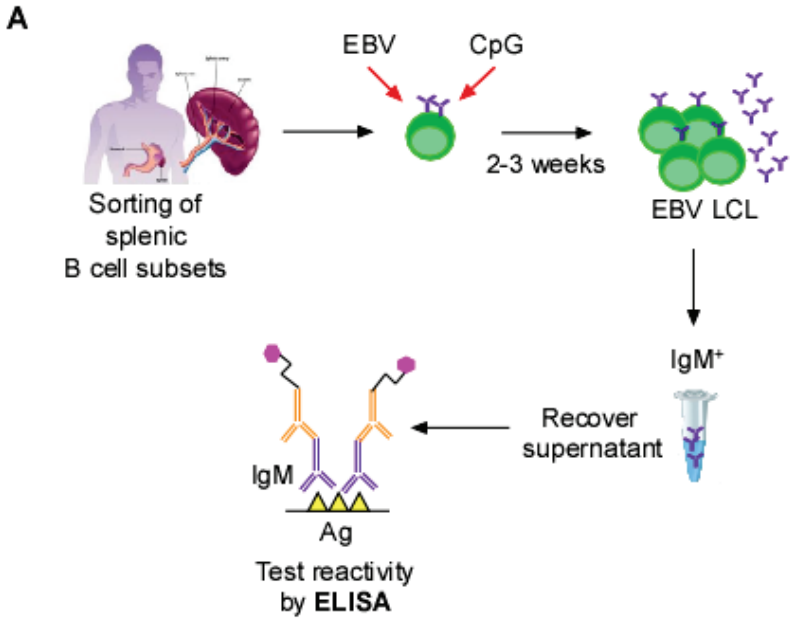


Figure 3. Human splenic PCs secrete IgM antibodies that recognize commensal antigens. (A) Experimental design used to generate human recombinant mAbs from single-cell sorted IgM$^+$ PBs-PCs. (B) ELISA of reactivity of 11 mAbs against *Escherichia coli*, *Staphylococcus aureus* and *Lactobacillus*.

Human Splenic MZ B Cells Produce IgM Antibodies Recognizing Commensal Products

To ascertain whether human splenic MZ B cells recognize bacteria as splenic IgM^+ PCs do, we set up an ELISA-based strategy and determined whether IgM from the supernatants of EBV-transformed MZ B cells could recognize bacterial antigens **(Fig. 4A)**. In similar assays, naïve B cells, which are not primed by antigen but express variable degrees of autoreactivity, were used as control.



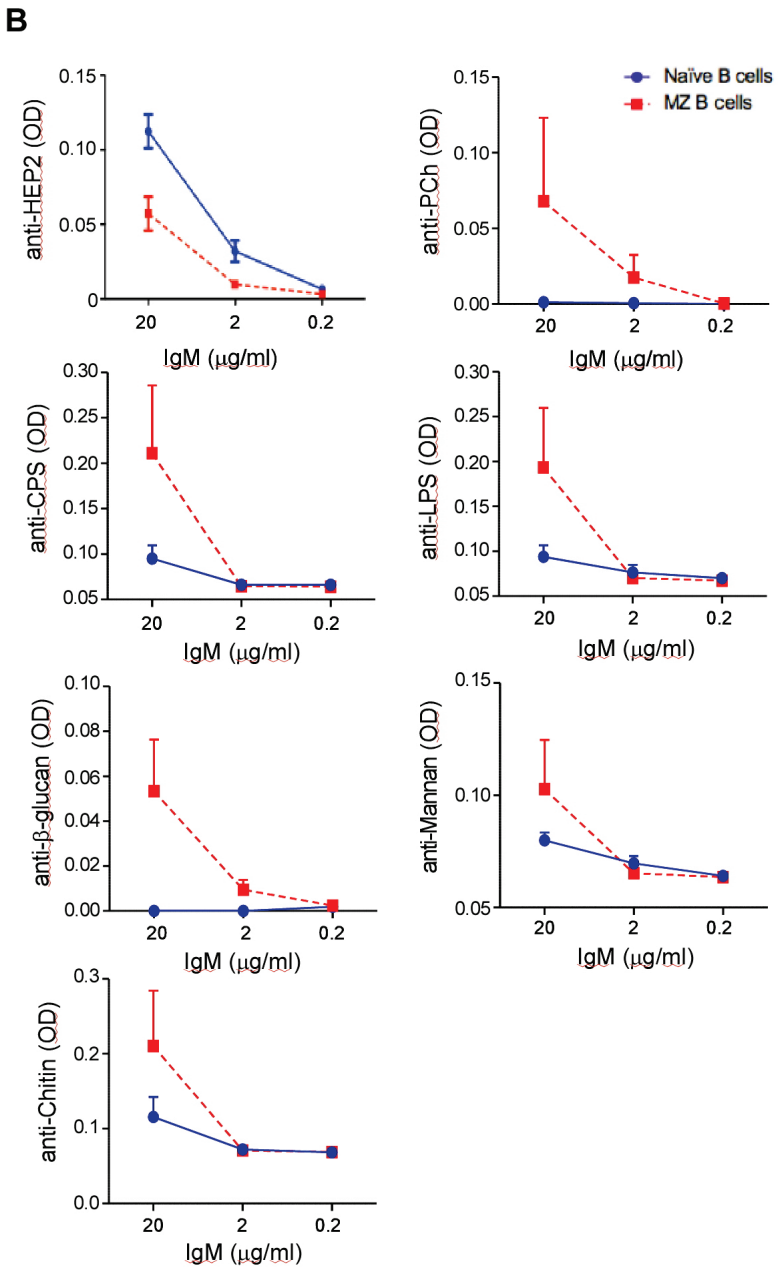


Figure 4. Human splenic MZ B cells produce IgM antibodies to commensal antigens. (A) Experimental design used to test the reactivity of IgM from EBV-transformed splenic MZ and naïve B cells. (B) ELISA analysing the reactivity of IgM antibodies from MZ and naïve B cells against Hep-2 cell lysates, PCh, CPS, LPS, β-

glucan, mannan, and chitin. EBV, Epstein-Barr virus; LCL, lymphoblastoid cell line; Ag, antigen; OD, optical density; PCh, phosphorylcholine; CPS, capsular polysaccharides; LPS, lipopolysaccharides. Error bars represent s.e.m. from three independent donors.

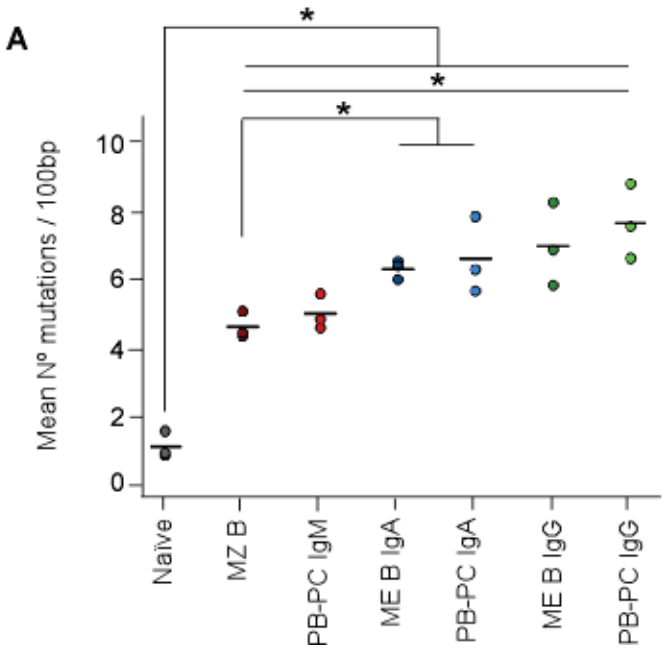
IgM antibodies from MZ but not naïve B cells recognized the bacterial lipid phosphorylcholine (PCh) as well as CPS from *Streptococcus pneumoniae* and LPS from Gram-negative bacteria, including *Pseudomonas aeruginosa* **(Fig. 4B)**. In addition, IgM antibodies from MZ but not naïve B cells reacted against β-glucan, mannan and chitin **(Fig. 4B)**, which are carbohydrates presumably originating from commensal fungi[349,350]. Furthermore, IgM from MZ B cells showed some degree of reactivity against autologous cell antigens, but this autoreactivity was not as high as that of IgM from naïve B cells **(Fig. 4B)**, which are physiologically enriched in self-reactivity. Though more reactivity studies comparing MZ B cells with PBs and PCs will be needed, these data suggest that splenic MZ B cells may represent the precursors of commensal-reactive PBs-PCs homeostatically secreting IgM.

MZ B cells Are Closely Affiliated with IgM$^+$ PCs Arising from Either GC-Independent or GC-Dependent Pathways

Given that IgM from splenic MZ B cells recognize bacterial antigens as IgM antibodies from PBs-PCs do, we hypothesized that MZ B cells and IgM$^+$ PBs-PCs have a common ontogeny. To clarify this point, we performed high-throughput next generation sequencing (NGS) of the Ig gene repertoire from splenic MZ B cells and IgM$^+$ PBs-PCs from

identical donors. In these experiments, naïve B cells were analyzed for comparison together with class-switched ME B cells and PBs-PCs expressing IgG or IgA. This additional analysis was important to establish the possible origin of IgM^+ PBs-PCs from naïve B cells and determine whether MZ B cells can enter GC reactions yielding highly mutated and class-switched clones.

First, we compared the mutational profile of VDJ regions from each splenic lymphocyte population. MZ B cells and IgM^+ PBs-PCs showed a comparable mutational load, which was higher than that of naïve B cells but lower than that of class-switched ME B cells and class-switched PBs-PCs, which were highly mutated **(Fig. 5A)**. Consistent with these findings, both MZ B cells and IgM^+ PBs-PCs included a higher proportion of unmutated clones compared to class-switched ME B cells and class-switched PBs-PCs **(Fig. 5B)**.



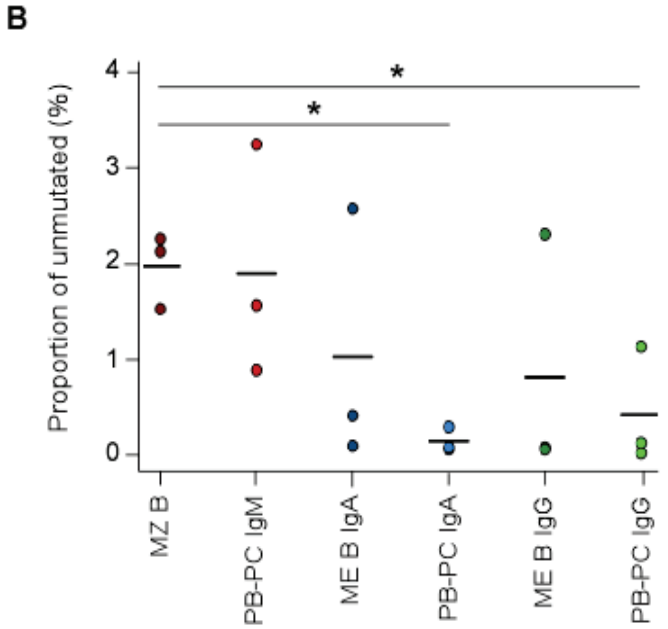


Figure 5. Human splenic MZ B cells have a mutational profile comparable to that of IgM$^+$ PBs and PCs. (A) Mean number of mutations per sequence and donor (expressed as mean nº mutations/100 bp) in splenic naïve B cells (grey), MZ B cells (dark red), IgM$^+$ PB-PCs (light red), IgA$^+$ ME B cells (dark blue), IgA$^+$ PBs-PCs (light blue), IgG$^+$ ME B cells (dark green), and IgG$^+$ PBs-PCs (light green). (B) Proportion of unmutated sequences in cell populations shown in (A). Unmutated sequences are defined as having zero mutations when compared to putative germline counterparts. MZ, marginal zone; ME, memory; PBs-PCs, plasmablasts-plasma cells. Thick line indicates the mean. *P <0.05 (two-tailed unpaired Student's *t*-test). Data include three biological replicates within each subset.

Given that SHM usually occurs in the GC, our findings suggest that MZ B cells and IgM$^+$ PBs-PCs may represent the end product of an early GC reaction that develops without completing SHM and class switching. An alternative but not mutually exclusive possibility is that MZ B cells and IgM$^+$ PBs-PCs emerge from a GC-independent

pathway capable to induce some degree of SHM, possibly in extrafollicular areas [84,105,351,352].

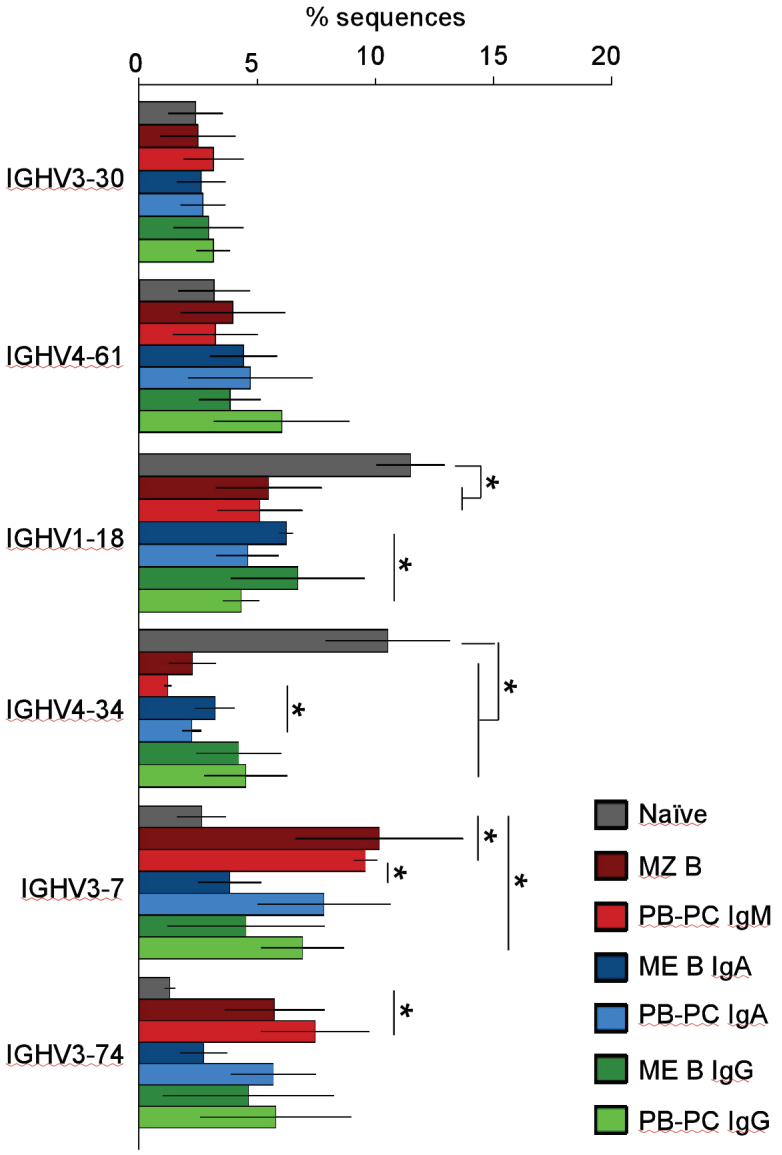


Figure 6. Human splenic MZ B cells have a VDJ gene repertoire comparable to that of IgM⁺ PBs and PCs. Donor IGHV gene usage (expressed as relative frequency) in sorted splenic naïve B cells (grey), MZ B cells (dark red), IgM⁺ PB-PCs (light red), IgA⁺ ME B cells (dark blue), IgA⁺ PBs-PCs (light blue), IgG⁺ ME B cells

(dark green), and IgG$^+$ PBs-PCs (light green). The graph includes examples of IGHV genes differentially used by the populations under study. IGHV, Ig heavy chain variable gene; MZ, marginal zone; ME, memory; PBs-PCs, plasmablasts-plasma cells. Error bars represent S.D.; *P <0.05 (two-tailed unpaired Student's *t*-test). Data includes three biological replicates for each cell subset.

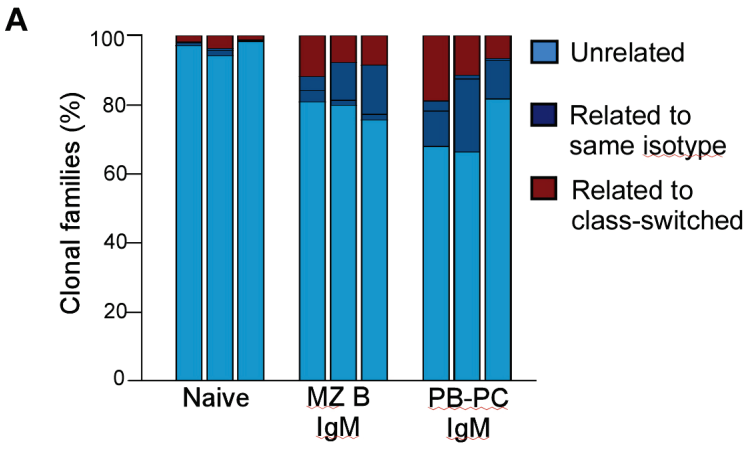
The possible GC-independent origin of at least some MZ B cells and IgM$^+$ PBs-PCs was consistent with their expression of an IgH variable region (IGHV) gene repertoire largely comparable with that of naïve B cells **(Fig. 6)**. Yet, striking differences were also present, including depletion of IGHV1-18 and IGHV4-34 genes and enrichment in IGHV3-7 and IGHV3-74 genes in MZ B cells and IgM$^+$ PBs-PCs compared to naïve B cells **(Fig. 6)**. Of note, IGHV3-7 and IGHV3-74 genes were also enriched in highly mutated ME B cells and PBs-PCs expressing class-switched IgG or IgA **(Fig. 6)**. In agreement with earlier data, these findings consolidate the possibility that the human spleen generates commensal-reactive IgM antibodies through homeostatic MZ B cell responses involving GC-independent or GC-dependent pathways.

Human Splenic IgM$^+$ PCs Emerge from Clonally Organized Responses Locally Induced by MZ B Cells

Having shown comparable reactivity, VH gene usage and mutational loads in human splenic MZ B cells and IgM$^+$ PBs-PCs and knowing that MZ B cells are a primary source of IgM antibodies to blood-borne antigens, we performed NGS of Ig VH genes to determine whether human splenic IgM$^+$ PBs-PCs arise from local MZ B cell precursors in a clonally organized response. A similar strategy was

used to ascertain the clonal relationship between splenic MZ B cells and class-switched ME B cells and PBs-PCs expressing IgG or IgA.

NGS demonstrated that virtually all of the clonal families arising from splenic naïve B cells were unrelated to other B cell subsets **(Fig. 7A)**. In contrast, 20% of clonal families formed by MZ B cells and IgM^+ PBs-PCs were affiliated to non-switched clonotypes expressing IgM. An additional 10% of clonal families formed by MZ B cells and IgM^+ PBs-PCs were affiliated to class-switched clonotypes expressing IgG or IgA **(Fig. 7A)**. Circos plot analysis showed that two of the five largest clonal families formed by MZ B cells harbored a large number of Ig VH gene mutations **(Fig. 7B)**. These families were clonally affiliated to highly mutated IgM^+ or IgG^+ PBs-PCs as well as IgA^+ ME B cells **(Fig. 7B)**.



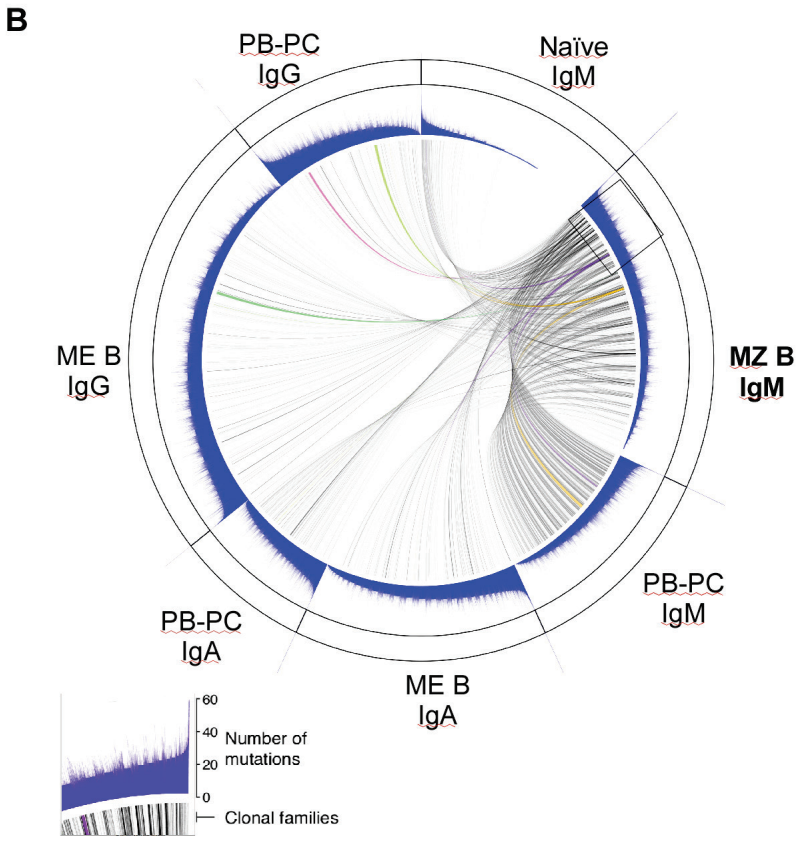


Figure 7. Human splenic IgM⁺ PBs-PCs locally emerge from clonally organized humoral responses initiated by MZ B cells. (A) Proportion of clonal families within splenic naïve B cells, MZ B cells and IgM⁺ PBs-PCs either unrelated to other clonal families or related to different cell types of identical or different isotype. (B) Circos plot representing SHM and clonal relationships within naïve B cells, MZ B cells, IgM⁺ PBs-PCs, IgA⁺ ME B cells or PBs-PCs, and IgG⁺ ME B cells or PBs-PCs from one of three representative donors. The outer track illustrates the number of mutations per sequence. Its inner edges illustrate clonal relationships between MZ B cells and other B cell and PB-PC subsets. The five largest clonal families are indicated with different color codes.

Three of the remaining largest clonal families of MZ B cells were characterized by a low number of mutations and were affiliated to IgM^+ or IgG^+ PBs-PCs as well as IgG^+ ME B cells **(Fig. 7B)**. In general, this NGS approach allowed us to conclude that clonally expanded MZ B cells were more frequently related to IgM^+ PB-PC clonotypes. However, a few of the largest clonal cluster of MZ B cells were also affiliated with class-switched PBs-PCs or ME B cells expressing IgG or IgA. Thus, we propose that a fraction of MZ B cells locally expands to differentiate into clonally related IgM^+ PBs-PCs and class-switched PBs-PCs or ME B cells via a classical GC-dependent pathway inducing robust SHM with or without CSR. An alternative GC-independent pathway inducing weak SHM may further diversify the splenic PB-PC repertoire.

Human Splenic MZ B Cells Locally Generate Clonal Families of IgM^+ As Well As Class-Switched PBs and PCs.

To further illustrate the GC-dependent or GC-independent nature of the differentiation processes locally targeting splenic MZ B cells, we generated Ig VH lineage trees from clonal families encompassing MZ B cells affiliated with ME B cells and/or PBs-PCs. Small Ig VH gene trees were selected to facilitate the visualization of gene alignments against inferred germline (GL) sequences.

By using this approach, we identified two paradigmatic clonal families that included VH sequences from splenic MZ B cells and IgM^+ PBs-PCs. The first example shows a MZ B cell clonally related to an IgM^+ PB-PC harboring only two Ig VH gene mutations compared to the

putative GL progenitor **(Fig. 8A)**. This example may reflect a MZ B cell that clonally differentiates into an IgM^+ PB-PC through a GC-independent pathway. The second example shows a MZ B cell harboring 16 Ig VH gene mutations that clonally differentiates into an IgM^+ PB-PC after accumulating 3 additional Ig VH gene mutations **(Fig. 8B)**. This case may reflect a MZ B cell that enters a GC-dependent pathway to become a memory B cell that further differentiates into an IgM^+ PB-PC.

A

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CLUSTAL O(1.2.1) multiple sequence alignment

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Marginal M - Spleen       GTGGACTCTGTGAAGGGCCGATTCACCATCTCCAGAGACAACGCCAAGAACTCACTGTAT
Plasma Cell M - Spleen    GTGGACTCTGTGAAGGGCCGATTCACCATCTCCAGAGACAACGCCAAGAACTCACTGTAT
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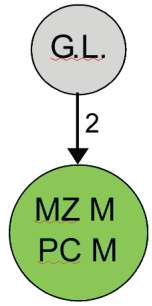
CDR regions
IgM primer

(reverse complement)



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Marginal M - Spleen          ACCGCCGACAGTTGGGACATCTTCTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTC
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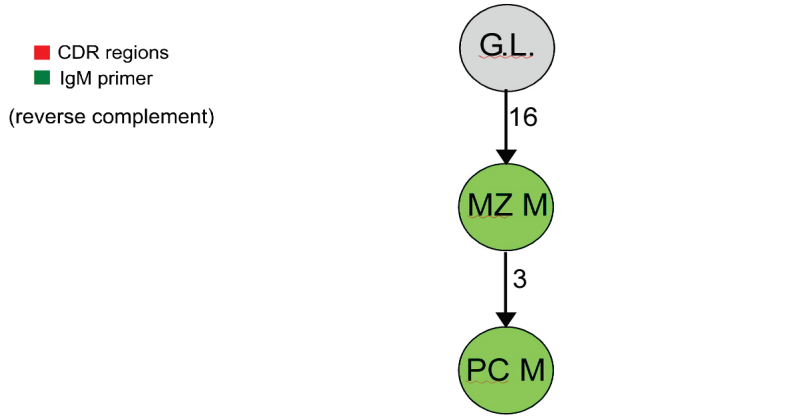

C

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Plasma Cell M - Spleen    ------GCAGCCTCTGGATTCACCTTTAGTAGCTATTGGATGAGCTGGGTCCGCCAGGCT
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Memory A - Spleen         GTGGACTCTGTGAAGGGCCGATTCACCATCTCCAGAGACAACGCCGAGAACTCACTGTAT
Plasma Cell M - Spleen    GTGGACTCTGTGAAGGGCCGATTCACCATCTCCAGAGACAACGCCGAGAACTCACTGTAT
                          ********************************************* * ************

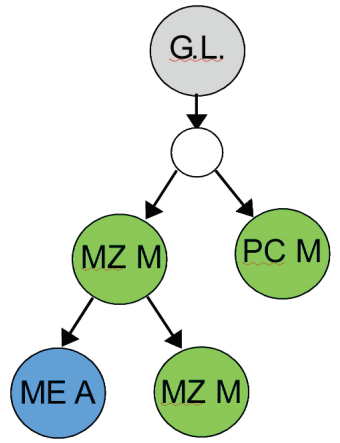
GERMLINE IGHV3-7 IGHJ4    CTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCGAGNNNNNNN
Marginal M - Spleen       CTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCGAGCCAGAGG
Marginal M - Spleen       CTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCGAGCCAGAGG
Memory A - Spleen         CTGCAAATGAACAGCCTGAGAGCCGACGACACGGCCGTGTATTACTGTGCGAGCCAGAGG
Plasma Cell M - Spleen    CTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCGAGCCAGAGG
                          ************************** ************************

GERMLINE IGHV3-7 IGHJ4    NNNNNNNNNNNNNNTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAG-----
Marginal M - Spleen       ACAAACGGGGCCTCTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAGGGAGT
Marginal M - Spleen       ACAAACGGGGCCTCTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAGGGAGT
Memory A - Spleen         ACAAACGGGGCCTCTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAGCATCC
Plasma Cell M - Spleen    ACAAGCGGGGCCTCTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAGGGAGT
                                  ****************************************

GERMLINE IGHV3-7 IGHJ4    -----------------------------------
Marginal M - Spleen       GCATCCGCCCCAACCCTTTTCCCCCTCGTCTCCTG
Marginal M - Spleen       GCATCCGCCCCAACCCTTTTCCCCCTCGTCTCCTG
Memory A - Spleen         CCGACCAGCCCCAAGGTCTTC--------------
Plasma Cell M - Spleen    GCATCCGCCCCAACCCTTTTCCCCCTCGTCTCCTG
```

CDR regions
IgM primer
IgA primer

(reverse complement)



D

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CLUSTAL O(1.2.1) multiple sequence alignment


GERMLINE IGHV3-23 IGHJ6    TCCTGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCT
Marginal M - Spleen        ----------CCTCTGGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCT
Memory G - Spleen          ------GCAGCCTCTGGGTTCACCTTCAGTGAATCTACTATGCACTGGGTCCGCCAGGCT
                                  ******* ******** **    * * * ***  ****************

GERMLINE IGHV3-23 IGHJ6    CCAGGGAAGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTAGCACATACTAC
Marginal M - Spleen        CCAGGGAAGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTAGCACATACTAC
Memory G - Spleen          TCCGGGAAGGGGCTGGAGTGGGTCTCAGTTGTTAGTGCTAGTAGTGGTAGCATATTTTAC
                            * ************************* * ****** **** ********* **  ***

GERMLINE IGHV3-23 IGHJ6    GCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTAT
Marginal M - Spleen        GCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTAT
Memory G - Spleen          GCAGACTCCGTGAAGGGCCGATTCACCATCTCCAGAGACAATTCCAAAAACACGCTGTAT
                           ******************** ************************** ************

GERMLINE IGHV3-23 IGHJ6    CTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAGNNNNN
Marginal M - Spleen        CTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAGGCGAT
Memory G - Spleen          CTGCAAATGAACAGCCTGAGAGCTGAGGACACGGCTGTGTATTACTGTGCGAAAGTGTAT
                           ********************** *********** ** ****************

GERMLINE IGHV3-23 IGHJ6    NNNNNNNNNNNNNNNNNNTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTC
Marginal M - Spleen        AGCAGTGGCTGGAACTACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTC
Memory G - Spleen          AGCAGTGGCTGGGCCTGGTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTC
                                             ********************************************

GERMLINE IGHV3-23 IGHJ6    ACCGTCTCCTCA-----------------------------------------
Marginal M - Spleen        ACCGTCTCCTCAGGGAGTGCATCCGCCCCAACCCTTTTCCCCCTCGTCTCCTG
Memory G - Spleen          GCCGTCTCCTCAGCCTCCACCAAGGGCCCATCGG-------------------
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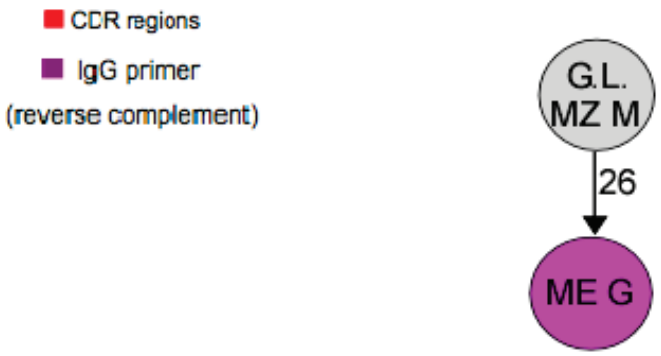

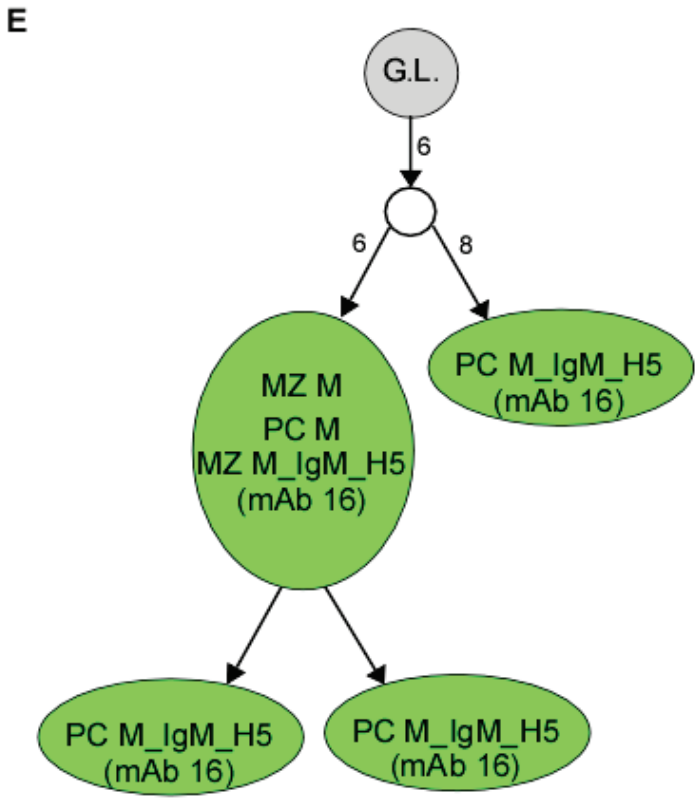

Figure 8. Human splenic MZ B cells clonally differentiate into PBs and PCs expressing IgM or class-switched IgG and IgA antibodies through GC-independent or GC-dependent pathways. Sequence alignment and Ig lineage trees of clonal families generated by MZ B cells that differentiate into PBs-PCs and/or ME B cells after emerging from putative GC-independent pathways with little or no SHM and with or without CSR (A, C) or from GC-dependent (B, D, E) pathways with abundant SHM and with or without CSR. Edges and numbers designate number of accumulated mutations along the lineage tree. CDR regions and primers are highlighted and color-coded. White small circles represent putative intermediate differentiation stages. GL, germline; IgM_H5, human recombinant mAb 16.

Remarkably, we also identified a clonal family that included two splenic MZ B cells, one IgM^+ PB-PC and one IgA^+ ME B cell harboring virtually no Ig VH gene mutations **(Fig. 8C)**. This example suggests a clonally organized MZ B cell response involving induction of CSR as well as ME B cell and PC differentiation in the absence of a typical SHM-inducing GC reaction. An additional clonal family included Ig VH sequences from splenic MZ B cells that had clonally differentiated into IgG^+ ME B cells following accumulation of 26 VH gene mutations **(Fig. 8D)**. This case points to a MZ B cell differentiation pathway involving clonal induction of both ME B cells and PBs-PCs through a typical GC reaction inducing SHM and CSR.

Donor-matched NGS data further permitted the clonal characterization of 15 of the 21 human recombinant mAbs isolated from single-sorted splenic IgM^+ PBs-PCs. One of these antibodies, mAb 16, belonged to a family of IgM^+ MZ B cells clonally affiliated with IgM^+ PBs-PCs that harbored several Ig VH gene mutations **(Fig. 8E)** and specifically recognized *Escherichia coli, Staphylococcus aureus* and *Lactobacillus* **(see earlier Fig. 3B)**. This example likely reflects a commensal-specific IgM response generated by PBs-PCs clonally emerging from a GC reaction possibly involving MZ B cells.

In summary, our findings indicate that human splenic MZ B cells can differentiate into PBs-PCs expressing IgM or class-switched IgG or IgA by following either a dominant GC-dependent pathway involving SHM or an alternative GC-independent pathway mostly uncoupled from SHM. Both pathways can induce CSR, but not in a consistent manner.

Human Splenic PBs and PCs Can Switch from IgM to IgG or IgA

A surprising finding emerging from the analysis of Ig VH gene trees was that some IgM^+ PBs-PCs were clonally related with IgG or IgA class-switched PBs-PCs. One possibility is that the immaturity of recently generated IgM^+ PBs-PCs permits them to retain CSR potential. In particular, we found an IgM^+ PB-PC presumably emerging form GC/ME B cell precursors harboring 12 Ig VH gene mutations that was clonally affiliated with an IgG^+ PB-PC that also contained 12 Ig VH mutations **(Fig. 9A)**. While CSR at an early phase of PB differentiation is plausible, it is also possible that our analysis missed an IgG^+ ME B cell intermediate. Alternatively, the two identical IgM^+ and IgG^+ PB-PC clonotypes may have co-emerged from identical GC precursors.

A

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CLUSTAL O(1.2.1) multiple sequence alignment

GERMLINE IGH1-2 IGHJ4       TCCTGCAAGGCTTCTGGATACACCTTCACCGGCTACTATATGCACTGGGTGCGACAGGCC
Plasma Cell G - Spleen      TCCTGCAAGGCTTCTGGATATACCTTTACCGGCTACTATATGCACTGGGTGCGACAGGCC
Plasma Cell M - Spleen      TCCTGCAAGGCTTCTGGATATACCTTTACCGGCTACTATATGCACTGGGTGCGACAGGCC
                            ********************* ***** ***********************************

GERMLINE IGH1-2 IGHJ4       CCTGGACAAGGGCTTGAGTGGATGGGATGGATCAACCCTAACAGTGGTGGCACAAACTAT
Plasma Cell G - Spleen      CCTGGACAAGGGCTTGAGTGGATGGGATGGATCTACCCTTACAGTGGTGCCACAAACTCT
Plasma Cell M - Spleen      CCTGGACAAGGGCTTGAGTGGATGGGATGGATCTACCCTTACAGTGGTGCCACAAACTCT
                            ********************************** ***** ********* ******** *

GERMLINE IGH1-2 IGHJ4       GCACAGAAGTTTCAGGGCAGGGTCACCATGACCAGGGACACGTCCATCAGCACAGCCTAC
Plasma Cell G - Spleen      GCACCGAAATTTCAGGGAAGGCTCACCATGACCAGGGACACGTCCATCAGCACAACCTAC
Plasma Cell M - Spleen      GCACCGAAATTTCAGGGAAGGCTCACCATGACCAGGGACACGTCCATCAGCACAGCCTAC
                            **** *** ******** *** ********************************** *****

GERMLINE IGH1-2 IGHJ4       ATGGAGCTGAGCAGGCTGAGATCTGACGACACGGCCGTGTATTACTGTGCNNNNNNNNNN
Plasma Cell G - Spleen      ATGGAGCTGAGCAGCCTGAGATCTGACGACACGGCCGTATATTACTGTGCCAATCAGGGA
Plasma Cell M - Spleen      ATGGAGCTGAGCAGCCTGAGATCTGACGACACGGCCGTATATTACTGTGCCAATCAGGGA
                            ************** ********************** ***********

GERMLINE IGH1-2 IGHJ4       NNNNNNNNNNNNNACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAG--------
Plasma Cell G - Spleen      GTGGCTGGTACTAACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAGCCTCCACC
Plasma Cell M - Spleen      GTGGCTGGTACTAACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAGGGAGTGCA
                                        ****************************************

GERMLINE IGH1-2 IGHJ4       --------------------------------
Plasma Cell G - Spleen      AAGGGCCCATCGG-------------------
Plasma Cell M - Spleen      TCCGCCCCAACCCTTTTCCCCCTCGTCTCCTG
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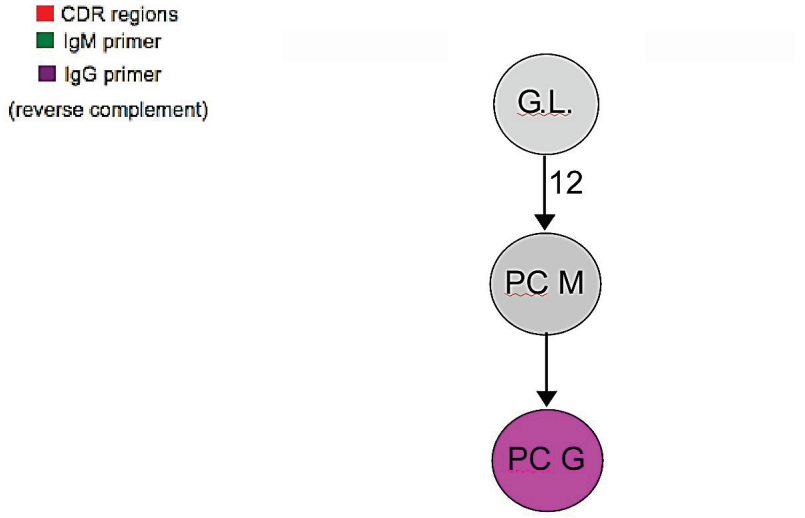
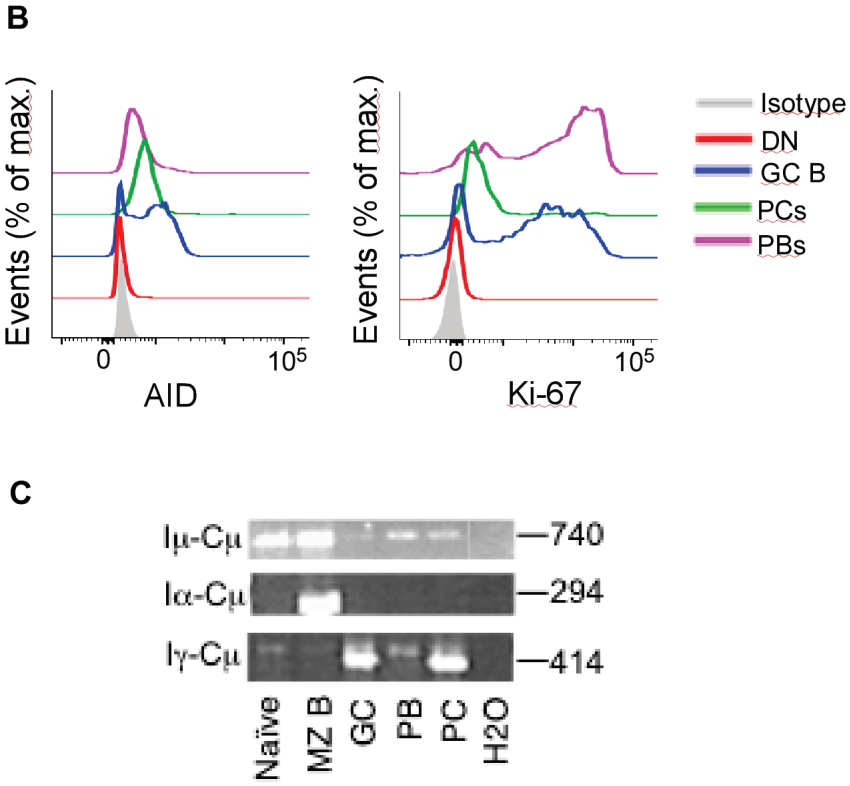



B
Events (% of max.)
0
10^5
AID
Events (% of max.)
0
10^5
Ki-67
Isotype
DN
GC B
PCs
PBs
C
Iμ-Cμ
Iα-Cμ
Iγ-Cμ
740
294
414
Naïve
MZ B
GC
PB
PC
H2O

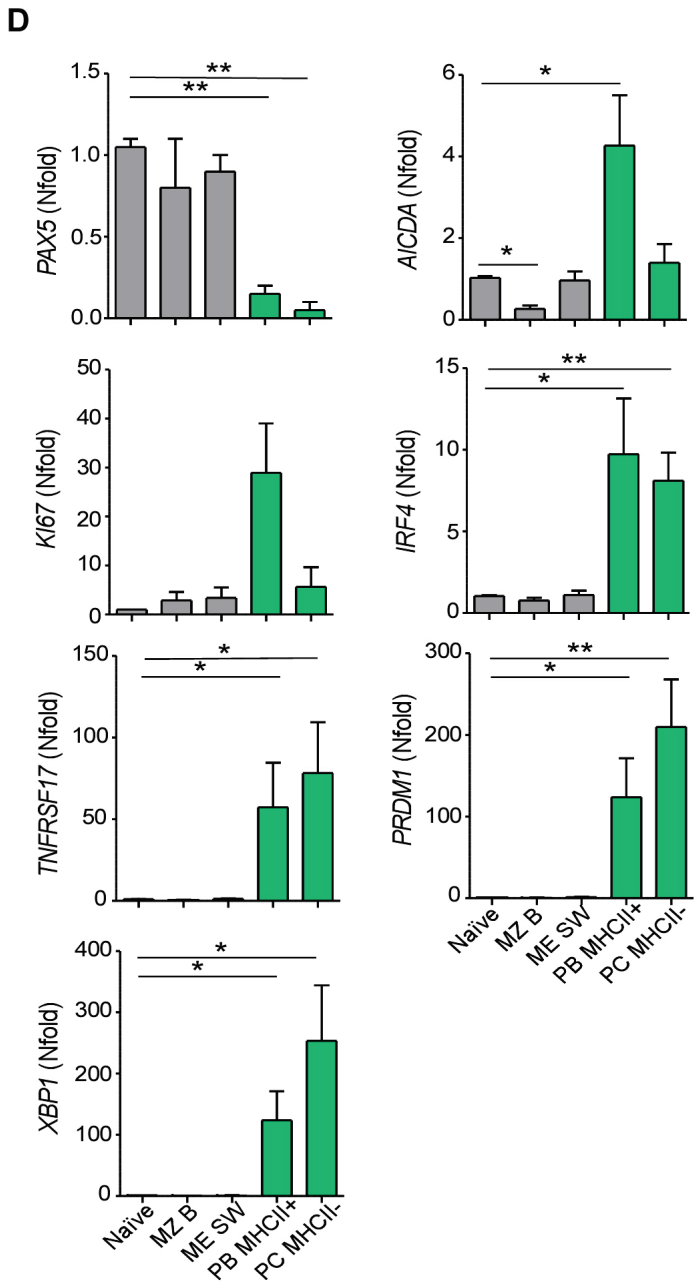


Figure 9. Human splenic IgM⁺ PBs and PCs may locally switch to IgG or IgA. (A) Multiple sequence alignment and Ig lineage tree of a clonal family including IgM⁺ PBs-PCs and IgG⁺ PBs-PCs. Edges and numbers designate number of accumulated mutations along the lineage tree. CDR regions and primers are

highlighted and color-coded according to the legend. (B) FC of AID and Ki-67 expression in mature naïve, MZ and ME B cells (DN, double negative for CD10 and CD38), GC B cells, MHC-II$^+$ PBs and MHC-II$^-$ PCs. Isotype control (grey) is also shown. (C) RT-PCR of Iμ-Cμ germline transcripts as well as Iα-Cμ and Iγ-Cμ switch circle transcripts in cells shown in (A). (D) QRT-PCR analysis of mRNAs encoding BCMA (*TNFRSF17*), BLIMP-1 (*PRDM1*), XBP1 (*XBP1*), IRF4 (*IRF4*), PAX-5 (*PAX5*), Ki-67 (*KI67*) and AID (*AICDA*) in sorted splenic naïve B cells, ME B cells, GC B cells, PBs and PCs. Results are normalized to those of mRNA encoding β-actin (*ACTB*) and are presented as relative expression (RE) compared with that of naïve B cells. GL, germline sequence. Error bars represent s.e.m. from five independent donors; *P<0.05, **P<0.01 (two-tailed unpaired Student's t-test). One experiment represents three with equivalent results (C and D).

To further clarify these aspects, we took advantage of FC to determine PB-PC expression of classical hallmarks of ongoing proliferation, SHM and CSR, including Ki-67 and AID. As expected, AID and Ki-67 were strongly expressed by splenic CD19$^+$CD10$^+$CD38$^+$ GC B cells (Fig. 9B). Albeit at lower levels, AID was also expressed by CD19$^+$CD10$^-$CD27^{hi}CD38^{hi}MHC-II$^+$ PBs (which typically express MHC-II) and some CD19$^+$CD10$^-$CD27^{hi}CD38^{hi}MHC-II$^-$ PCs **(Fig. 9B)**. Similar to GC B cells, PBs but not PCs also expressed Ki-67 **(Fig. 9B)**.

To validate these findings, additional molecular indicators of ongoing CSR were RT-PCR amplified from FACSorted MHC-II$^+$ PBs or MHC-II$^-$ PCs. Similar to GC B cells, PBs and to a larger extent PCs contained Iγ-Cμ circle transcripts, a hallmark of IgM-to-IgG CSR, whereas naïve and MZ B cells did not **(Fig. 9C)**. In contrast, PBs and PCs lacked Iα-Cμ circle transcripts, which instead were detected in MZ B cells but not GC or naïve B cells **(Fig. 9C)**. Finally, naïve and

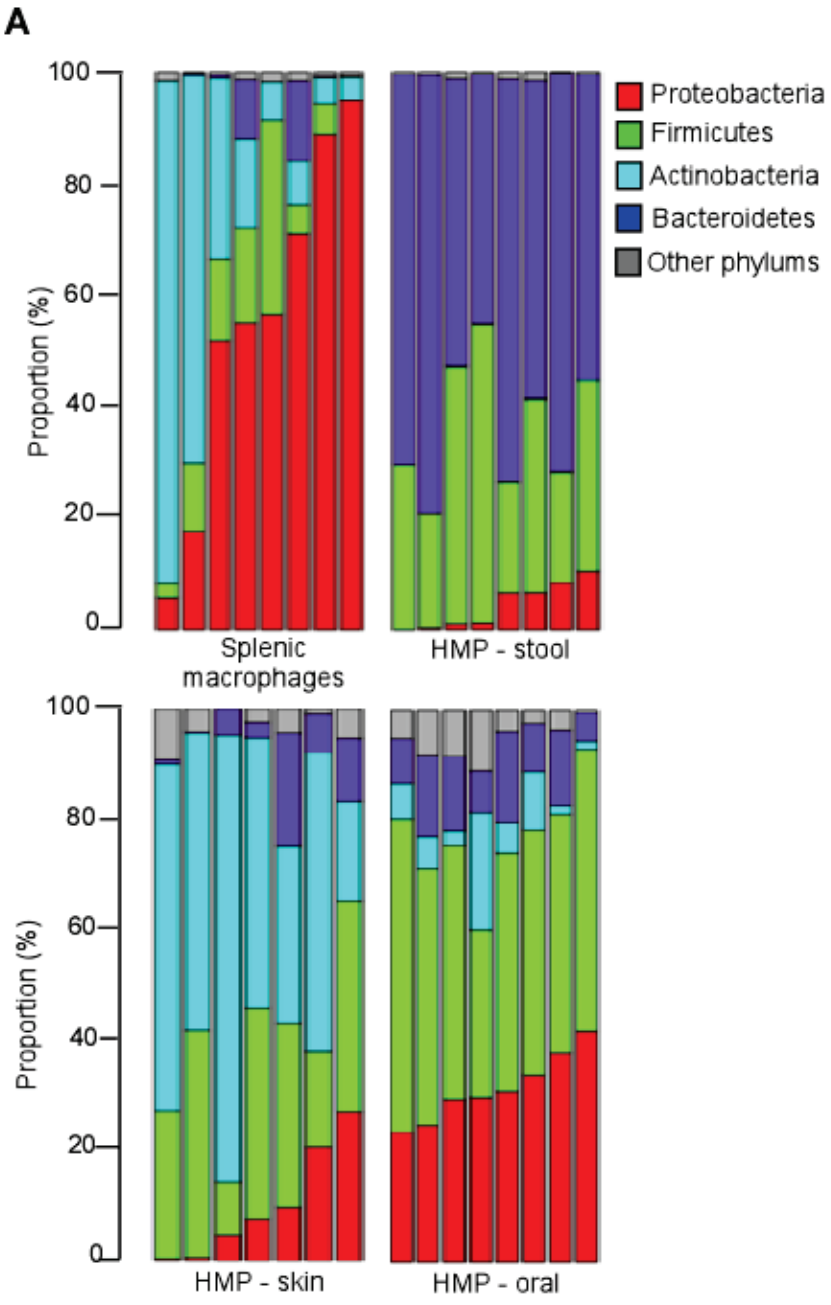
MZ B cells contained abundant Iμ-Cμ germline transcripts, which instead were scant in GC B cells, PBs and PCs **(Fig. 9C)**. These results showed some variability among different donors, but overall suggest that CSR is not confined to GC B cells, but may also occur in some MZ B cells and, more surprisingly, in a fraction of non-terminally differentiated PCs, including PBs.

To delve deeper into this issue, FACSorted splenic PBs and PCs were further transcriptionally profiled by measuring typical B cell-identity and PC-identity markers through QRT-PCR. Consistent with their expression of proliferation and CSR-related transcripts usually detected in activated B cells, splenic PBs showed a transcriptional signature intermediate between that of mature B cells (either naïve, MZ or ME) and that of PCs. Indeed, compared to naïve and MZ B cells, PBs contained not only more transcripts for AID and Ki-67, but also more transcripts for the PC-related factors BLIMP-1, XBP-1, IRF-4 and BCMA **(Fig. 9D)**. Yet, these PC-related transcripts were less abundant in PBs compared to PCs **(Fig. 9D)**. Compared to mature B cells, both PBs and PCs expressed fewer transcripts for the B cell-identity transcription factor PAX5 **(Fig. 9D)**, further corroborating their affiliation to the PB-PC lineage. Collectively, these experiments suggest that a fraction of human splenic MZ B cells as well as a fraction of non-terminally differentiated PBs and PCs may undergo CSR from IgM to IgA or IgG. This process may occur in the context of clonally organized GC-dependent or GC-independent responses to commensal antigens.

The Human Spleen Captures Microbial Commensal Products

Having shown that human splenic MZ B cells mount clonally organized PB and PC responses that generate IgM antibodies to commensal microbes, we wondered whether the spleen contained traces of microbial material. To ascertain this hypothesis, we took advantage of a 16S rRNA microarray PhyloChip approach to identify bacterial sequences in MACSorted $CD14^+$ macrophages, which represent the major antigen-capturing phagocyte subset in the MZ of the spleen, at least in mice. Macrophages purified under sterile conditions contained multiple bacterial phyla whose relative composition showed a predictable variability among donors (Fig. 10A). Despite this variability, Gram-negative Proteobacteria consistently represented the predominant phylum in most splenic samples, except two, which predominantly contained Gram-positive Actinobacteria **(Fig. 10A)**. Most samples also contained some Gram-positive Firmicutes, whereas Gram-negative Bacteroidetes were poorly or no represented **(Fig. 10A)**.

Bacterial sequences emerging from splenic macrophages were further compared with sequences obtained from the Human Microbiome Project (HMP) database. This approach suggested that the human spleen could capture bacteria originating from multiple compartments interposed between the host and the environment, including gut, oral cavity and skin **(Fig. 10A)**. Finally, when splenic 16S rRNA sequences were analyzed at a family level, the most abundant families corresponded to Pseudomonadaceae and Micrococcaceae **(Fig. 10B)**.


A
100
80
60
40
20
0
Proportion (%)
Splenic macrophages
HMP - stool
HMP - skin
HMP - oral
Proteobacteria
Firmicutes
Actinobacteria
Bacteroidetes
Other phylums

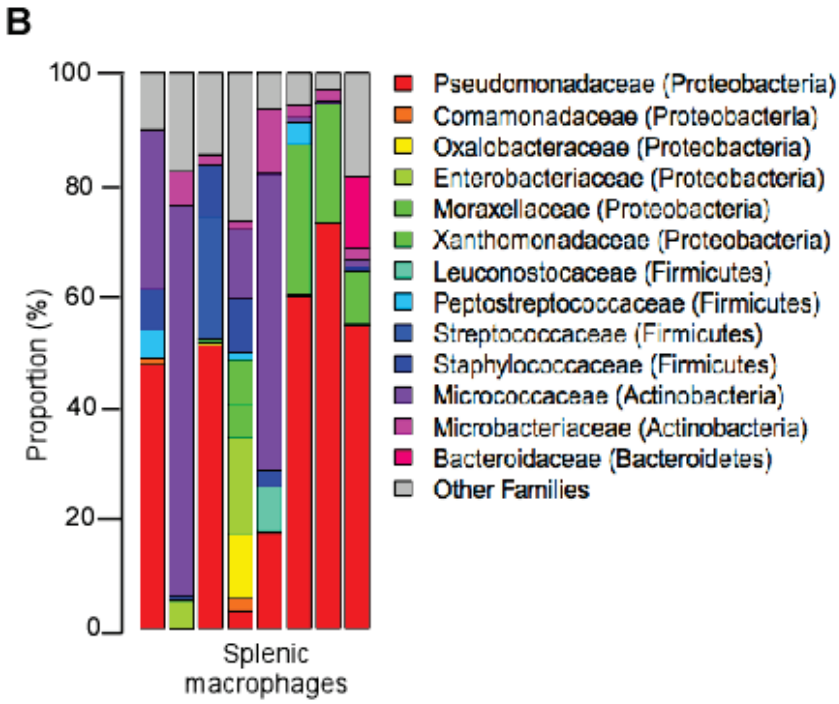


Figure 10. Human splenic macrophages capture commensal microbial products. (A) 16S DNA microarray analysis of bacterial phyla from 8 biological replicates of human splenic macrophages analyzed through PhyloChip array. Phyla from human spleen are compared with phyla from human stool, skin and oral cavity by taking advantage of archived data from the Human Microbiome Project. (B) Bacteria families from human splenic macrophages obtained through PhyloChip array.

By showing bacterial sequences of possible mucosal and skin origin in the spleen, these data suggest that splenic MZ B cells mount clonally organized PB-PC responses possibly instigated by local commensal determinants of possible mucosal origins. The mechanism whereby these commensal antigens enter the splenic MZ and get presented to commensal-specific MZ B cells remain to be determined.

The Human Splenic MZ Contains Abundant Stromal Cells

The MZ of the spleen serves as a filter for blood-borne antigens. In mice, these antigens are cleared by MOMA-1$^+$ metallophilic macrophages strategically positioned along the marginal sinus and by MARCO$^+$ macrophages located within the MZ. Besides supporting the structure of the MZ, these macrophages interact with MZ B cells to present captured antigens and initiate IgM or class-switched IgG and IgA responses[156,157,301]. In humans, published studies suggest that macrophages are rare inside the MZ, raising the possibility that the initiation of MZ B cell responses involves distinct antigen-capturing cell types.

In mice, IFA showed that the splenic MZ was segregated from B220$^+$ B cell follicles by a thin ring of MOMA-1$^+$ macrophages proximal to and partly overlapping with MAdCAM-1$^+$ MRCs **(Fig. 11)**. In humans, CD163$^+$ macrophages mostly inhabited the outer MZ as well as PFZ and RP areas, but were scarce in the proper MZ, which instead contained abundant MAdCAM-1$^+$ MRCs **(Fig. 11)**. These MRCs formed a complex reticular network throughout the MZ but were rare in other splenic compartments, except the PALS surrounding the central arteriole **(Fig. 11)**.

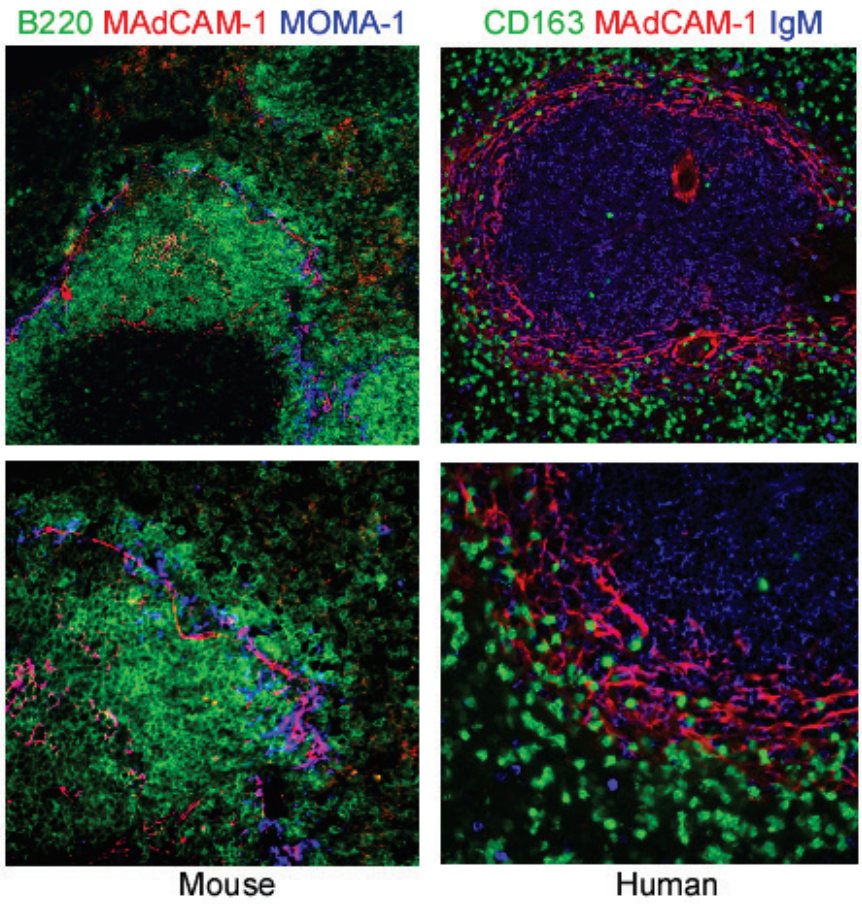


Figure 11. Human MRCs outnumber macrophages in the splenic MZ. IFA of mouse (left) or human (right) spleen sections stained for B220 (green), CD163 (green), MAdCAM-1 (red), MOMA-1 (blue) and/or IgM (blue). Original magnification, ×20 (top image) and ×40 (bottom image). B220, B cell isoform of 220 kDa; MAdCAM-1, mucosal vascular adressin cell adhesion molecule-1.

Given the scarcity of macrophages and abundance of MRCs in the human splenic MZ and considering that blood-borne antigens need to transit through the MZ to activate MZ B cells, human splenic MRCs could deploy some of the canonical functions of mouse splenic macrophages, including antigen capture and activation of MZ B cells.

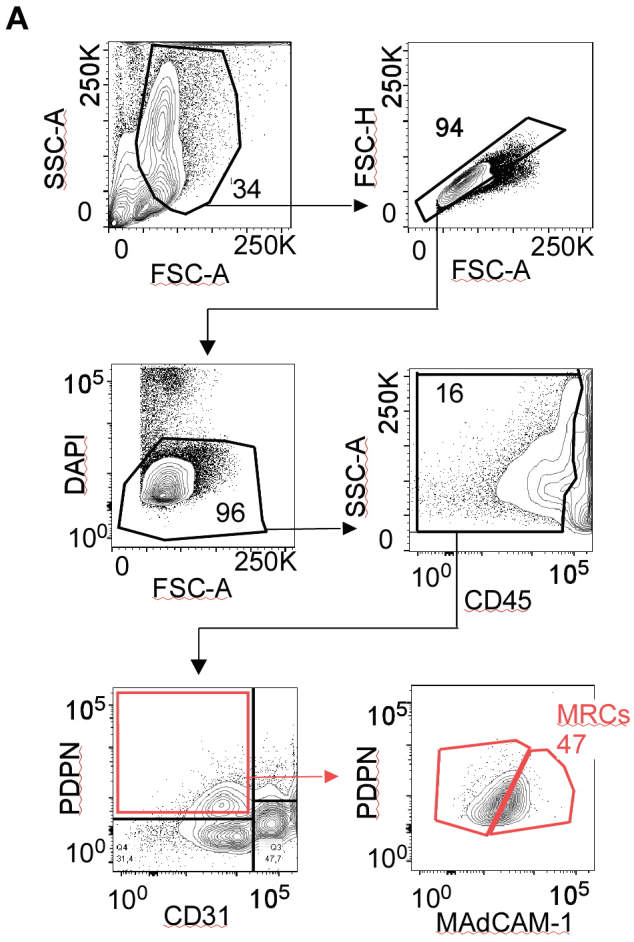
Should this be the case, MRCs may function as stromal innate response activator cells actively participating in the local induction of homeostatic IgM responses to commensal antigens.

Human Splenic MRCs Capture Commensal Products

In mice, a critical function of MZ macrophages is to capture blood-borne antigens. To explore whether human splenic MRCs capture commensal antigens, we first had to phenotypically define these elusive cells in order to implement their isolation through FACSorting. Human splenic MRCs were identified and isolated as $DAPI^-CD45^-CD31^-PDPN^+MAdCAM\text{-}1^+$ stromal cells **(Fig. 12A)** according to criteria recently published in mice[278]. At the same time, splenic $DAPI^-CD14^+$ monocytes-macrophages, $DAPI^-\ CD45^+CD14^-CD3^-CD11c^+$ DCs and $DAPI^-CD45^+CD3^+CD11c^-CD19^-$ T cells were FACSorted and used as internal controls **(Fig. 12B)**.

Using universal Illumina primers recognizing hypervariable regions of bacterial 16S rRNA, we specifically PCR amplified bacterial 16S rRNA from macrophages, which presumably capture microbial products in the PFZ and RP **(Fig. 12C)**. Together with DCs, also MRCs contained traces of bacterial 16S rRNA, whereas T cells did not **(Fig. 12C)**. Water and purified *E. coli* samples were used as negative and positive controls, respectively **(Fig. 12C)**. Overall, these data indicate that multiple splenic innate immune populations capture bacteria in the spleen, perhaps at different anatomical sites. While macrophages and DCs may mostly phagocytose antigenic material in the PFZ and RP of the spleen, MRCs may do so in the splenic MZ, presumably in collaboration with some DCs and PFZ macrophages. Should this be

the case, splenic MRCs could express macrophage-like phenotypic, transcriptional and functional profiles.



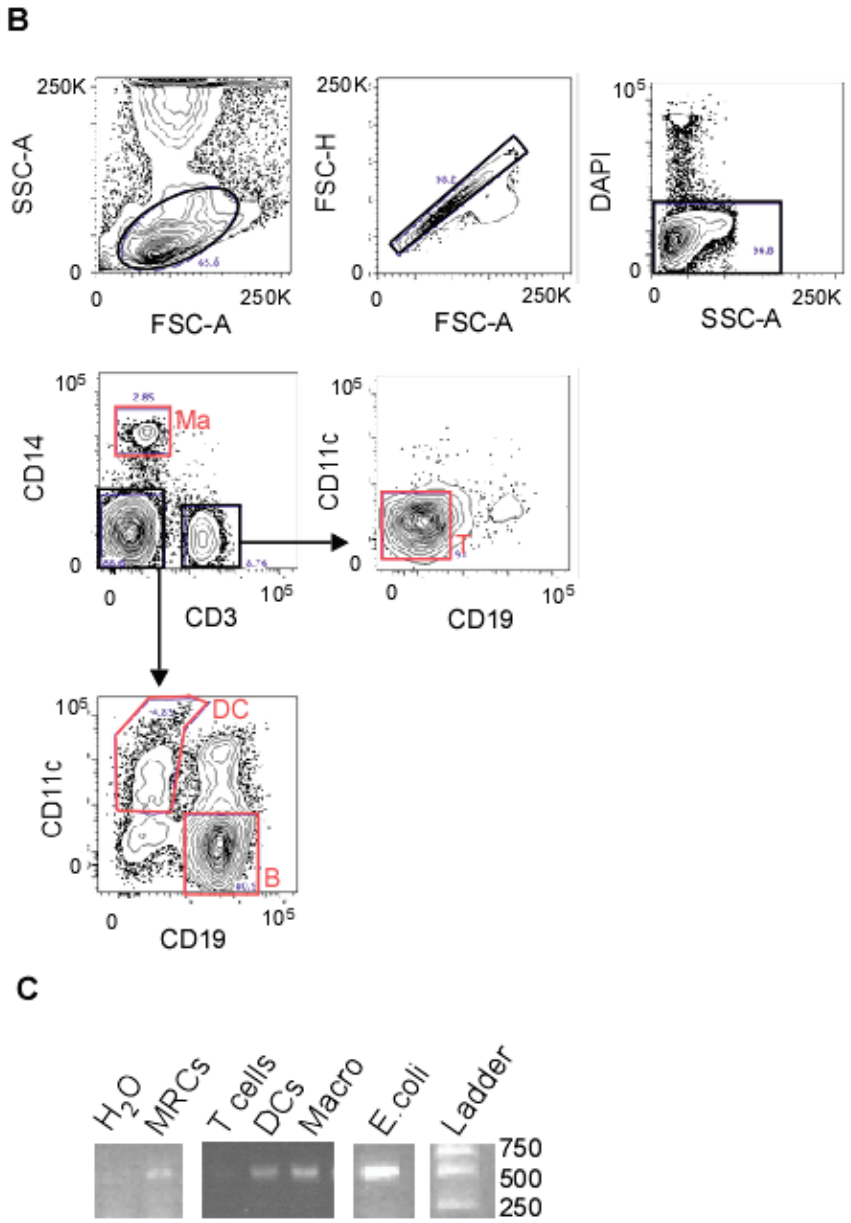


Figure 12. Human splenic MRCs capture microbial products (A) Gating strategy to FACSort human splenic DAPI$^-$CD45$^-$CD31$^-$Pdpn$^+$MAdCAM-1$^+$ MRCs. (B) Gating strategy followed to sort macrophages, DCs, B cells and T cells. (C) RT-PCR of bacterial 16S rRNA in FACSorted splenic MRCs, T cells, DCs and macrophages using Illumina universal primers. *Escherichia coli:* positive control; H_2O: negative control; Ma, macrophages.

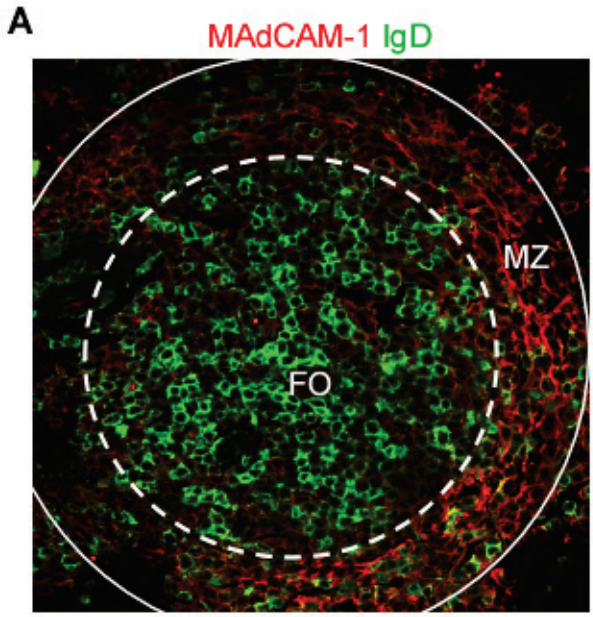
Human Splenic Stromal Cells Closely Interact With MZ B cells

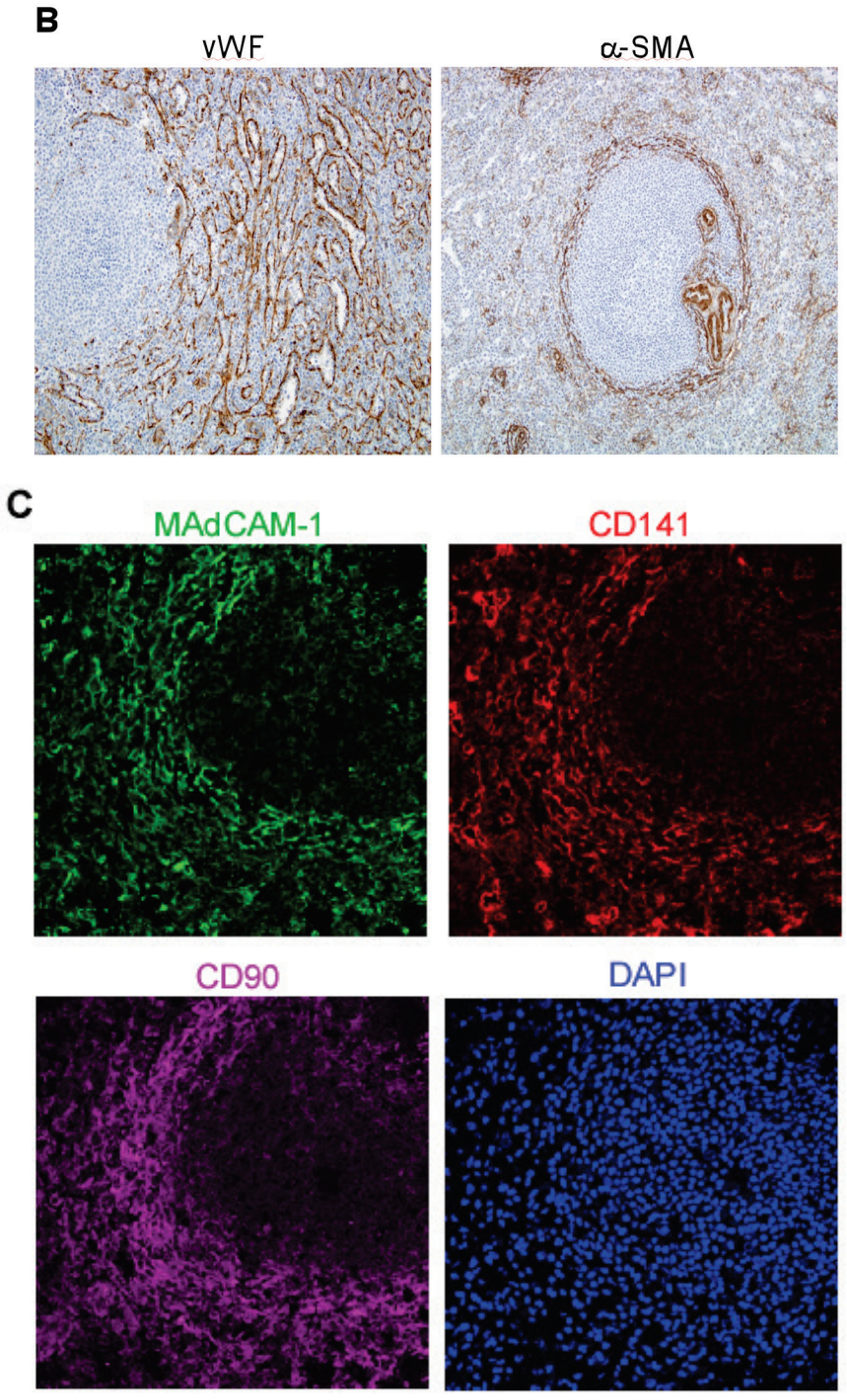
Having shown that MRCs occupied the entire splenic human MZ and were capable to internalize bacterial products, we set out to evaluate the geographical relationship of MRCs with B cells. IFA showed that human splenic MRCs were proximal to IgD^{low} MZ B cells, which were easily distinguishable from IgD^{high} naïve B cells forming Primary follicles **(Fig. 13A and Annex II, Fig. 2)**. Of note, a few MRCs were also proximal to IgD^{high} B cells positioned at the edge of the MZ, which perhaps represent naïve B cells transiting through the MZ to enter the sinusoidal circulation **(Fig. 13A)**. In general, these IFA studies provide a proof of principle that MRCs may functionally interact with B cells, including MZ B cells.

We next evaluated the stromal nature of splenic human MRCs. IFA showed that MRCs from both MZ and PALS expressed the stromal molecules α-smooth muscle actin (α-SMA), CD141 (thrombomodulin) and CD90 (Thy-1) but lacked the endothelial molecule von Willebrand factor (vWF) **(Fig. 13B-C and Annex II, Fig. 2 and Suppl. Fig. 3)**. Moreover, IFA determined that MRCs highly expressed the CXCR5 ligand CXCL13, a chemokine typically expressed by FDCs that may contribute to the recruitment of $CXCR5^{+}$ B cells to the MZ and/or to their retention in this area **(Fig. 13D)**. Compared to FDCs, which highly express the complement receptor CD21, MRCs were negative for this protein **(Fig. 13D)**.

Remarkably, MAdCAM-1 and CXCL13 expression patterns indicated that MRCs represented a heterogeneous population of stromal cells,

because MAdCAM-1^{+}CXCL13^{-} MRCs predominantly inhabited the outer ring of the MZ, whereas MAdCAM-1^{+}CXCL13^{+} cells mostly inhabited the inner part of the MZ **(Fig. 13E)**. Along the same lines, only MRCs positioned in the outer ring of the MZ expressed the stromal molecule collagen IV **(Fig. 13F)**. These findings indicate that human splenic MRCs form a phenotypically and possibly functionally heterogeneous network of stromal cells that closely interact with MZ B cells.




B
vWF
α-SMA
C
MAdCAM-1
CD141
CD90
DAPI

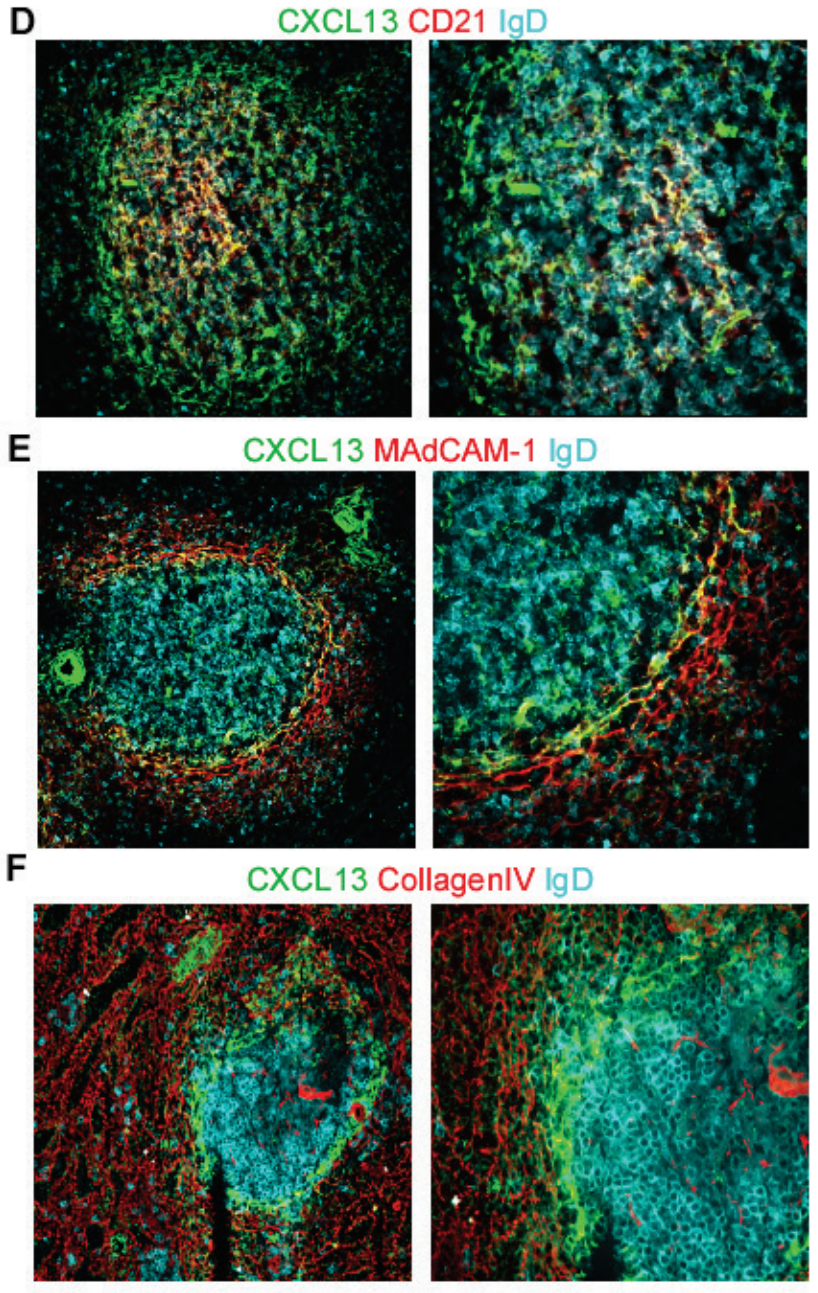
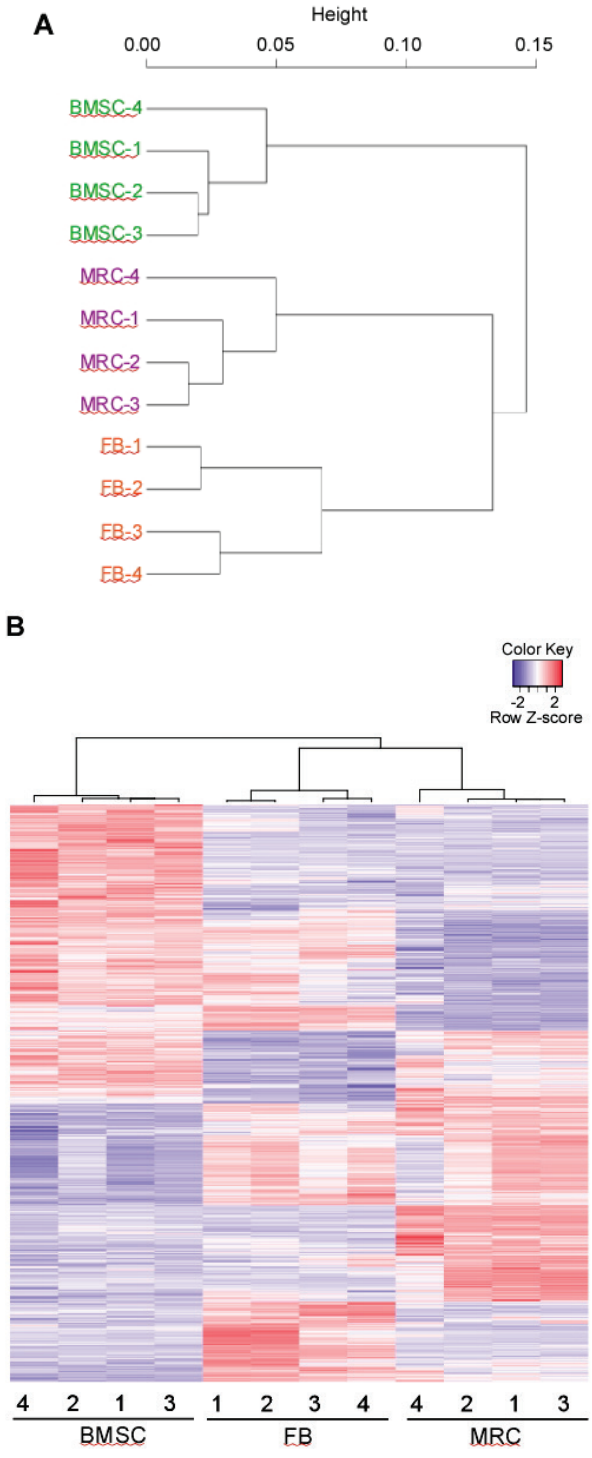


Figure 13. Human MRCs express canonical stromal molecules. (A) IFA of human spleen stained for MAdCAM-1 and IgD. Original magnification, ×20. (B) IHC of human spleen stained for vWF and α-SMA. Original magnification, ×10. (C)

IFA of human spleen stained for MAdCAM-1, CD141, CD90 and DAPI. Original magnification, ×40. (D) IFA of human spleen stained for CXCL13, CD21 and IgD. (E) IFA of human spleen stained for CXCL13, MAdCAM-1 and IgD. (F) IFA of human spleen stained for CXCL13, Collagen IV and IgD. Original magnification, ×20 (left) and ×40 (right) (D,E,F). MAdCAM-1, mucosal vascular adressin cell adhesion molecule-1; vWF, von Willebrand Factor; α-SMA, alpha-smooth muscle actin; CXCL13, chemokine (C-X-C motif) ligand 13.

Human Splenic MRCs Express a Transcriptome With Prominent Stromal, Macrophage-Like and Immune-Related Signatures

To further characterize the nature and function of human splenic MRCs, we performed a global transcriptome analysis of FACSorted MRCs and compared these results with those obtained from the analysis of dermal FBs and bone-marrow stromal cells (BMSCs). The choice of these cells was motivated by the fact that FBs have a reticular morphology similar to that of MRCs, whereas BMSCs are considered *bona fide* lymphoid organ-derived stromal cells. Unsupervised and supervised hierarchical clustering analysis of global gene expression profiles showed that MRCs clustered together with both dermal FBs and BMSCs, but at the same time clearly segregated from them **(Fig. 14)**. In general, MRCs were slightly more similar to FBs than to BMSCs **(Fig. 14A)**.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0.05
0.10
0.15
BMSC-4
BMSC-1
BMSC-2
BMSC-3
MRC-4
MRC-1
MRC-2
MRC-3
FB-1
FB-2
FB-3
FB-4
B
Color Key
-2
2
Row Z-score
4 2 1 3 1 2 3 4 4 2 1 3
BMSC
FB
MRC

Figure 14. Human splenic MRCs show a tissue-specific transcriptome. (A) Unsupervised hierarchical clustering analysis of global gene expression profiles of FACSorted splenic MRCs, dermal FBs and BMSCs using correlation distance and Ward's linkage method. Dendrogram height parameter indicates dissimilarity between clusters. (B) Heat map diagram displaying differentially expressed genes (adjusted P value <0.05) between splenic MRCs and FBs ($|\log_2 FC| > 1$) compared to BMSCs. The color bar depicts normalized intensity values performed on a row basis. From left to right, the x-axis of the heat map depicts BMSCs, FBs and MRCs. Data represents four biological replicates from each group. MRC, marginal reticular cells; FB, fibroblasts; BMSC, bone-marrow stromal cells.

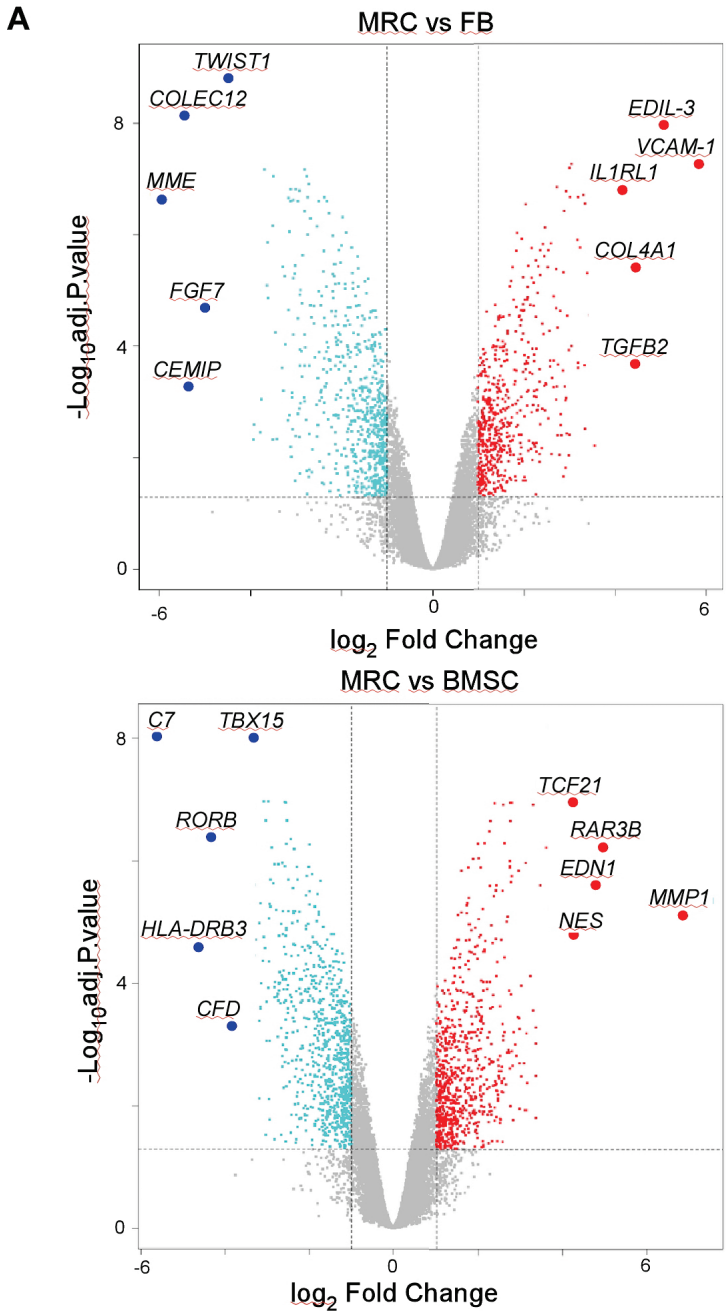
Volcano plot analysis showed that, among thousand of genes differentially expressed by human splenic MRCs, ten up-regulated genes stood out in comparisons with FBs or BMSCs **(Fig. 15A)**. These genes included *TGFB2*, *COL4A1*, *VCAM1*, *IL1RL* and *EDIL3* **(Fig. 15A)**, which are related to angiogenesis and vessel wall remodeling in addition to leukocyte adhesion and immunity[260,353]. Compared to BMSCs, splenic MRCs were also enriched in *TCF21*, which regulates splenic extramedullary haematopoiesis[354], *RAR3B*, which binds the immune regulator retinoic acid (RA), F3 (Factor III), which promotes blood coagulation, as well as *NES* (nestin) and *MMP1* (matrix metalloproteainase 1), which enhance tissue remodeling **(Fig. 15BA)**. Overall, this gene expression pattern clearly reflects canonical stromal properties.

Heat-map plot analysis confirmed the stromal nature of MRCs. Indeed, compared to FBs or BMSCs, splenic MRCs were enriched in *THBD*, which encodes the coagulation-regulating receptor thrombomodulin, *BST1*, which encodes a bone marrow stromal antigen 1 stimulating early B cell progenitors[355], *VCAM1* and *ICAM1*, which encode VCAM-1 and ICAM-1 adhesion molecules critically

involved in leukocyte extravasation and immunity, as well as genes encoding various collagen, laminin, fibronectin, filamin and nectin proteins, which shape the extracellular matrix **(Fig. 15B, C)**.

Remarkably, among the gene products enriched in MRCs, we identified several immune-related genes typically expressed by myeloid phagocytic cells, including macrophages and DCs. These MRC-enriched genes encoded the CD4 receptor along with microbial receptors such as C-type lectin receptors, scavenger receptors, NOD-like receptors and Toll-like receptors (TLRs) such as *CLEC4A, NLRP10, SCARF1, SCARB1*, *CD36 TLR3*, *TLR4, TLR6* and *TLR9* **(Fig. 15B)**. Furthermore, MRCs were enriched in myeloid molecules such as metalloproteinases (*ADAM19*), immune regulators (*PD-L1*) and immune enhancers (*IL18*) **(Fig. 15B, C)**.

The overall implication of MRCs in immune responses was further suggested by the expression of genes encoding proteins implicated in cell differentiation and immune regulation via TNF, NOTCH and RA signaling, genes encoding integrins and other adhesion molecules involved in leukocyte retention and extravasation, genes encoding IL-15, IL-18 and TGF-β2 cytokines mediating lymphocyte differentiation, and genes encoding multiple chemokines such as CCL13, CCL20 and CCL28, which promote leukocyte recruitment **(Fig. 15C)**.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MRC vs FB
TWIST1
COLEC12
MME
FGF7
CEMIP
EDIL-3
VCAM-1
IL1RL1
COL4A1
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-Log10adj.P.value
log2 Fold Change
MRC vs BMSC
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TCF21
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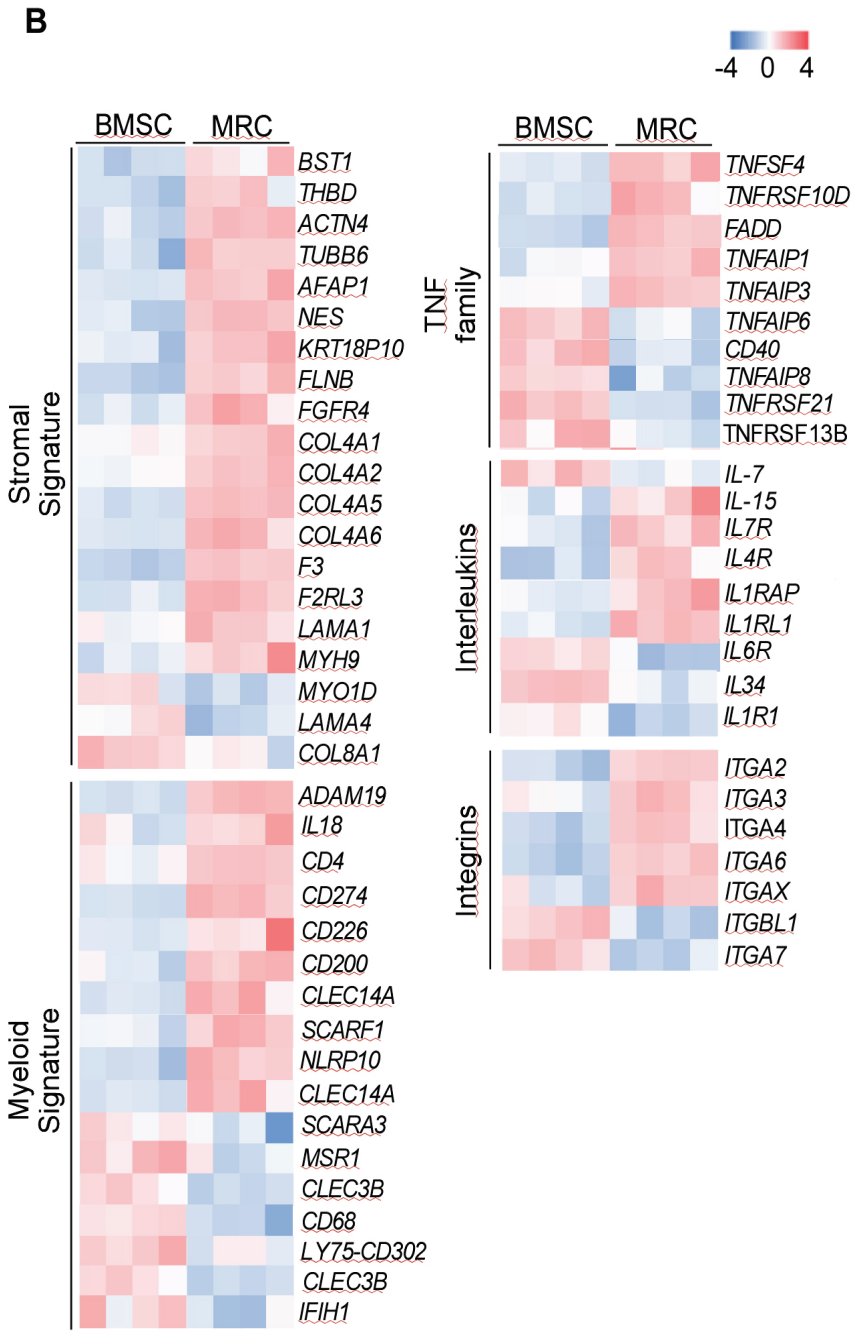
log2 Fold Change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MRC
Stromal Signature
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TUBB6
AFAP1
NES
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COL4A1
COL4A2
COL4A5
COL4A6
F3
F2RL3
LAMA1
MYH9
MYO1D
LAMA4
COL8A1
Myeloid Signature
ADAM19
IL18
CD4
CD274
CD226
CD200
CLEC14A
SCARF1
NLRP10
CLEC14A
SCARA3
MSR1
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CD68
LY75-CD302
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FADD
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TNFAIP3
TNFAIP6
CD40
TNFAIP8
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TNFRSF13B
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IL-15
IL7R
IL4R
IL1RAP
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ITGA4
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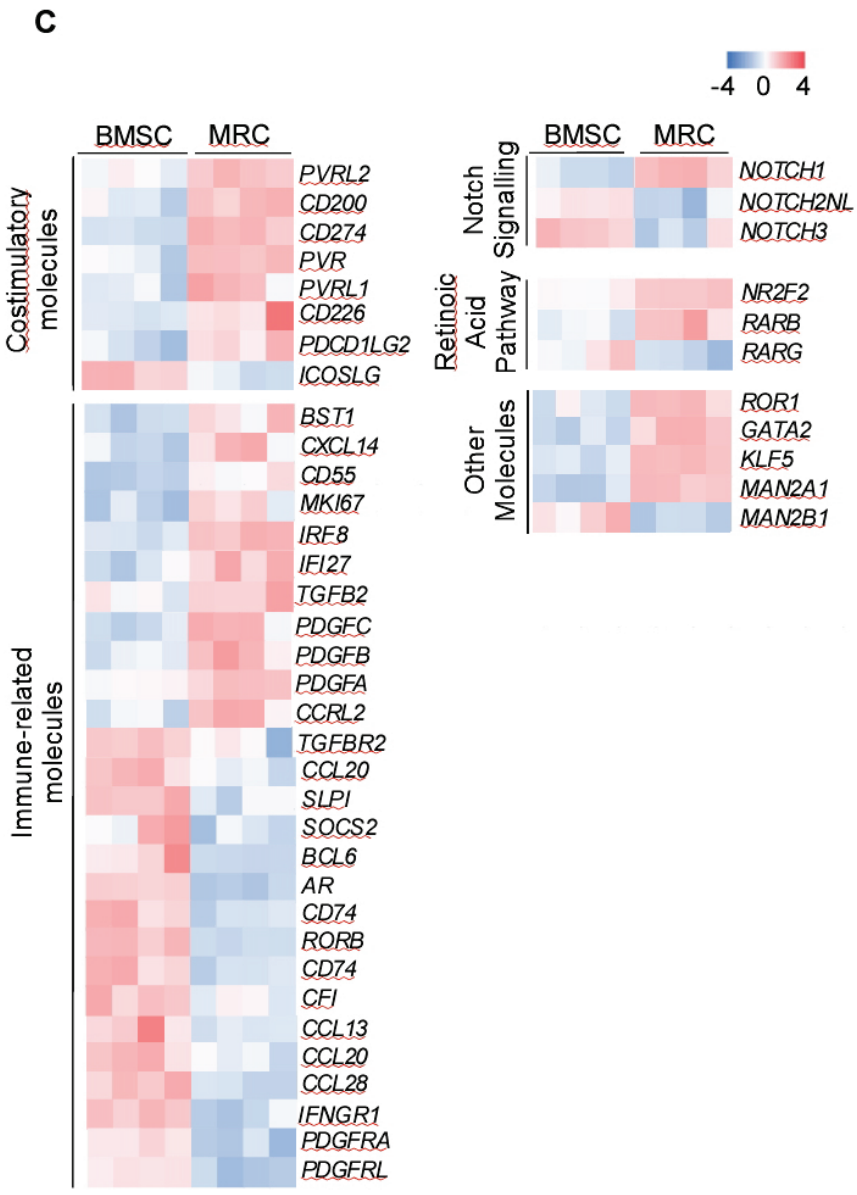
ITGAX
ITGBL1
ITGA7


C
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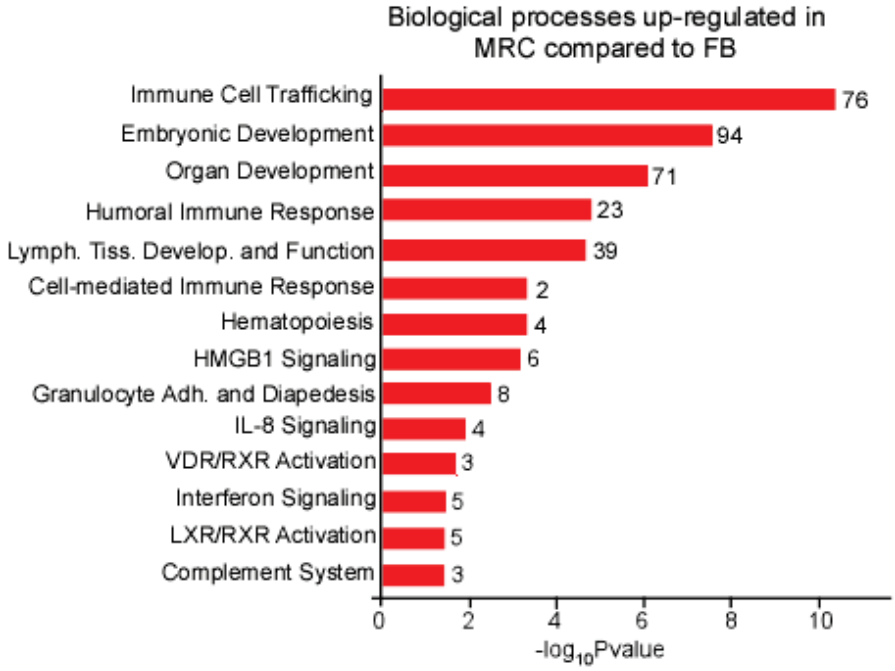
BMSC
MRC
Costimulatory molecules
PVRL2
CD200
CD274
PVR
PVRL1
CD226
PDCD1LG2
ICOSLG
Immune-related molecules
BST1
CXCL14
CD55
MKI67
IRF8
IFI27
TGFB2
PDGFC
PDGFB
PDGFA
CCRL2
TGFBR2
CCL20
SLPI
SOCS2
BCL6
AR
CD74
RORB
CD74
CFI
CCL13
CCL20
CCL28
IFNGR1
PDGFRA
PDGFRL
BMSC
MRC
Notch Signalling
NOTCH1
NOTCH2NL
NOTCH3
Retinoic Acid Pathway
NR2F2
RARB
RARG
Other Molecules
ROR1
GATA2
KLF5
MAN2A1
MAN2B1

D


Biological processes up-regulated in MRC compared to FB
Immune Cell Trafficking
Embryonic Development
Organ Development
Humoral Immune Response
Lymph. Tiss. Develop. and Function
Cell-mediated Immune Response
Hematopoiesis
HMGB1 Signaling
Granulocyte Adh. and Diapedesis
IL-8 Signaling
VDR/RXR Activation
Interferon Signaling
LXR/RXR Activation
Complement System
76
94
71
23
39
2
4
6
8
4
3
5
5
3
0
2
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6
8
10
-log10Pvalue

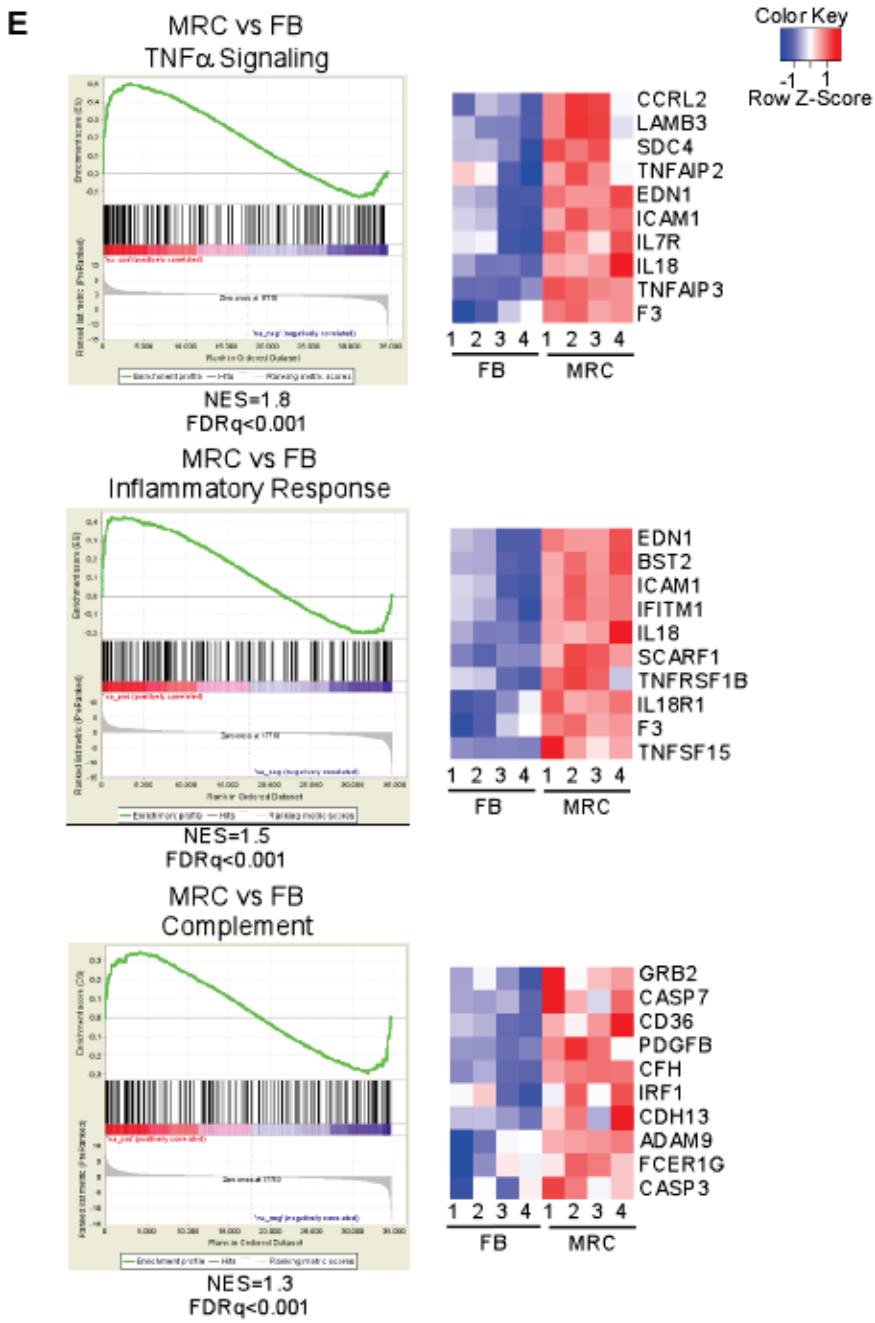


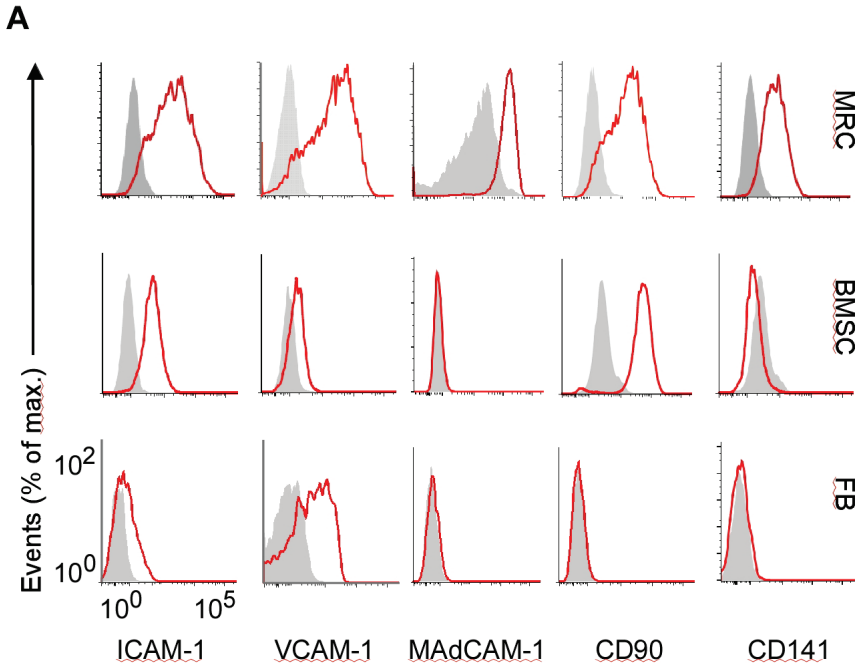
Figure 15. Human splenic MRCs show prominent myeloid and immune-activation gene signatures. (A) Volcano plots highlighting a total of 10 up-regulated and down-regulated genes per comparison. Each plot represents the list of

the differentiated expressed genes in MRC vs FB (top) and MRC vs BMSC (bottom), in base of the adjusted P value < 0.05 and the $|\log_2 FC| > 1$. (B) Heat map based on $\log_2 FC$ of genes belonging to stromal signature, myeloid signature, TNF family, interleukins and integrins in MRC compared to BMSC. (C) Heat map based on $\log_2 FC$ of genes encoding costimulatory molecules, immune-related molecules, Notch signalling, retinoic acid pathway and other molecules. (D) Gene enrichment analysis showing top 14 biological processes up-regulated (red) in human MRCs compared to FBs. Numbers indicate genes. (E) GSEA analysis illustrating TNFα signalling, inflammatory response and complement pathway gene sets enriched in MRC compared to FB. Heat maps highlighting 10 of the top genes with the greatest differences in expression associated with each pathway (red, high; blue, low) in MRC vs FB. Data represents four donors within each group.

In agreement with the possible involvement of human splenic MRCs in antigen capture and MZ B cell retention and activation, gene ontology (GO) analysis indicated that the top 14 biological processes enriched in MRCs compared to FBs belonged to functional categories related to immune cell trafficking, humoral immunity, cell-mediated immunity, IL-8 signaling, complement activation and various signaling events **(Fig. 15D)**. Moreover, gene set enrichment analysis (GSEA) of MRCs compared to FBs showed that TNFα and complement signaling cascades were highly enriched in MRCs along with gene products involved in inflammatory responses **(Fig. 15E)**.

Finally, we validated and further developed gene expression studies with FC studies determining expression of stromal and immune proteins by human splenic MRCs **(Fig. 16A)**. Despite being morphologically similar to BMSCs and dermal FBs, splenic MRCs expressed more ICAM-1 and VCAM-1 and were positive for MAdCAM-1, whereas BMSC and dermal FBs were not **(Fig. 16A and Annex II, Fig.2 and Suppl. Fig. 3)**. As expected from IFA and

transcriptome studies, MRCs expressed surface myeloid immune-inducing molecules such as B cell-activating factor of the TNF family (BAFF), CD36 and CD4 in addition to paradigmatic stromal molecules such as CD90, CD141, podoplanin (PDPN), RANK-L and BP-3 **(Fig. 16A, B and Annex II, Fig.2 and Suppl. Fig. 3)**.



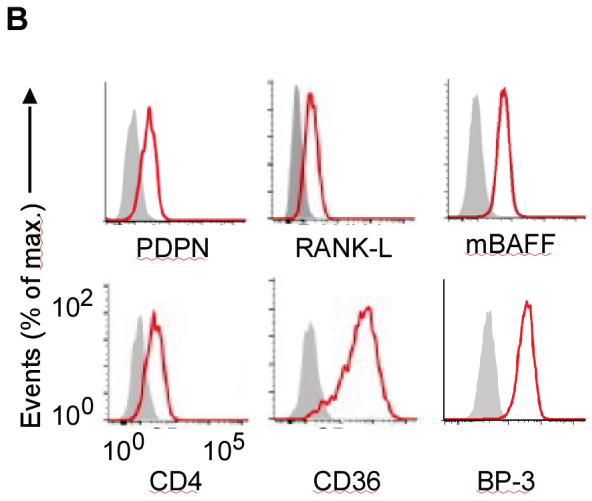


Figure 16. Human splenic MRCs express a macrophage-like surface phenotype. (A) FC showing ICAM-1, VCAM-1, MAdCAM-1, CD90 and CD141 expression on MRCs, BMSCs and FBs. (B) FC showing mBAFF, PDPN, RANK-L, CD4, CD36 and BP-3 expression on MRCs. ICAM-1, Intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; MAdCAM-1, mucosal vascular adressin cell adhesion molecule-1; mBAFF, membrane-bound B cell activating factor; PDPN, podoplanin; RANK-L, receptor activator for nuclear factor κB ligand. One experiment representative of four with similar results.

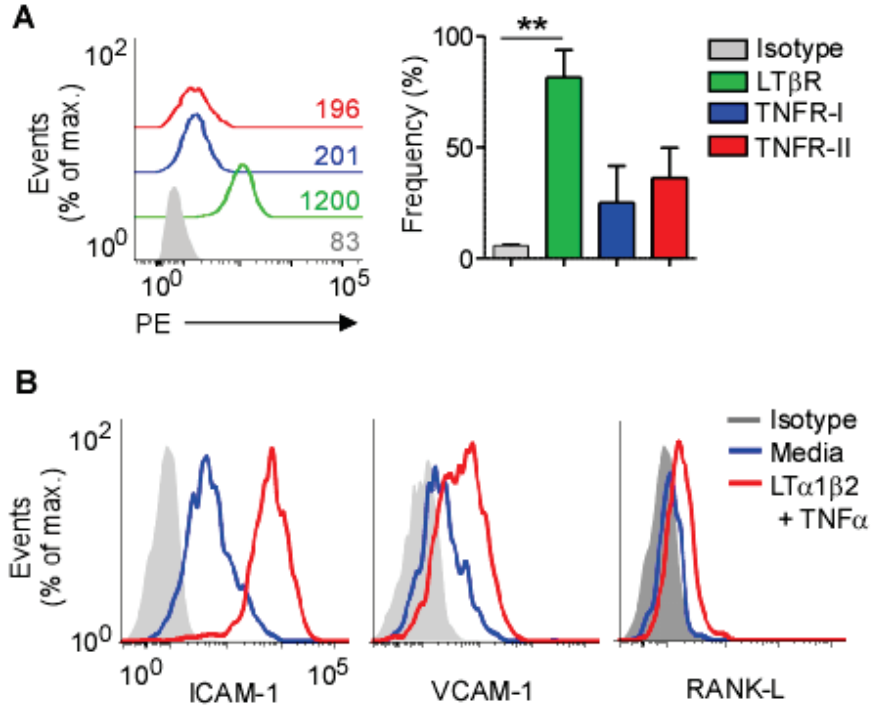
In summary, these data indicate that human splenic MRCs express phenotypic and transcriptional properties typical of both stromal and myeloid cells. These properties may include macrophage-like functions, including MZ B cell retention, antigen capture and MZ B cell activation.

Human Splenic MRCs Produce B Cell- and PC-Stimulating Factors in Response to Stromal Signals

Given that human splenic MRCs combine stromal with myeloid features, internalize microbial products and show immune activation-

related properties that correlate with extensive interaction with MZ B cells, we wondered whether canonical stromal signals could induce MRC production of MZ B cell-stimulation factors. These signals include the cytokines LTα1β2 and TNFα, which are homeostatically expressed by group 3 innate lymphoid cells (ILC3) inhabiting MZ as well as PFZ and RP areas of human spleens **(Annex II, Fig.2 and Suppl. Fig. 3)**.

As shown by FC, MRCs expressed functional LTα1β2 and TNFα receptors, including LTβR as well as TNFR-I and TNFR-II, respectively **(Fig. 17A)**. Similar to lymphoid tissue organizing (LTo) cells[279], MRCs robustly enhanced the expression of ICAM-1, VCAM-1 and RANK-L following exposure to LTα1β2 and TNFα for 24 h **(Fig. 17B and Annex II, Fig.2 and Suppl. Fig. 3)**.



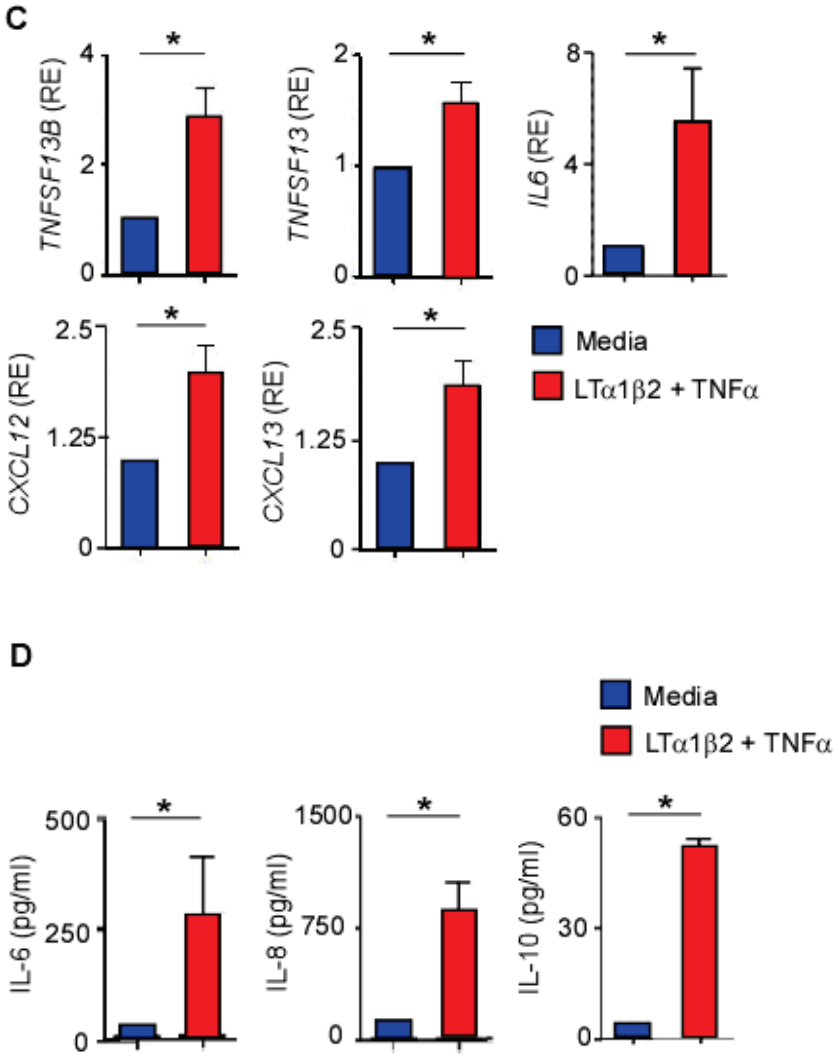


Figure 17. Human splenic MRCs up-regulate B cell-stimulating factors in response to stromal signals. (A) FC analysis of LTβR, TNFR-I and TNFR-II on MRCs. Numbers show frequency (%) of positive cells (B) FC analysis of ICAM-1, VCAM-1 and RANK-L on MRCs stimulated with LTα1β2 and TNFα for 24 h. (C) QRT-PCR of mRNA encoding BAFF (*TNFSF13B*), APRIL (*TNFSF13*), IL-6 (*IL6*), CXCL12 (*CXCL12*) and CXCL13 (*CXCL13*) in MRCs stimulated as in (B) for 24 h. (D) ELISA of IL-6, IL-8 and IL-10 released by MRCs stimulated as in (B) for 48 h.

Isotype control antibody (grey) (A, B). Data represents four different experiments (A, C and D); *P<0.05, **P<0.01 (two-tailed unpaired Student's t-test).

LTα1β2 and TNFα also induce MRC production of B cell-stimulating factors. Indeed, QRT-PCR and ELISA showed that MRCs up-regulated the B cell-stimulating cytokines BAFF, APRIL, IL-6 and IL-10, the B cell-recruiting/retaining chemokines CXCL12 and CXCL13, and the PC-inducing cytokine IL-6 upon exposure to LTα1β2 and TNFα **(Fig. 17C, D)**. Finally, activated MRCs induced the neutrophil-recruiting chemokine IL-8 **(Fig. 17D)**, which may guide perifollicular recruitment of a splenic subtype of MZ B cell-stimulating neutrophils termed N_{BH} cells. In conclusion, these data provide proof of principle that MRCs express B cell and PC-stimulating cytokines upon exposure to stromal signals.

Human Splenic MRCs Produce B Cell- and PC-Stimulating Factors in Response to Microbial Signals

Considering that human splenic MRCs capture microbial products and express a number of microbe-sensing receptors, we further determined whether MRC induced B cell and PC-stimulating factors in response to microbial signals. In agreement with earlier microarray data, FC detected the surface LPS receptor TLR4 as well as the intracellular CpG DNA receptor TLR9 in MRCs **(Fig. 18A and Annex II, Fig. 2)**. Of note, these receptors triggered non-redundant responses in MRCs. For instance, while LPS increased MRCs expression of ICAM-1 and VCAM-1, CpG DNA did not **(Fig. 18B and Annex II, Suppl. Fig. 3)**.

In contrast, CpG DNA was more efficient than LPS in increasing MRC expression of transcripts for BAFF **(Fig. 18C)**. On the other hand, CpG DNA but not LPS increased MRC expression of transcripts for APRIL **(Fig. 18C)**. Finally, LPS but not CpG DNA increased MRC production of IL-6 **(Fig. 18D)**. One possibility is that MRCs need stimulation of multiple microbial sensors to initiate full-fledged MZ B cell responses to microbial antigens, including commensals.

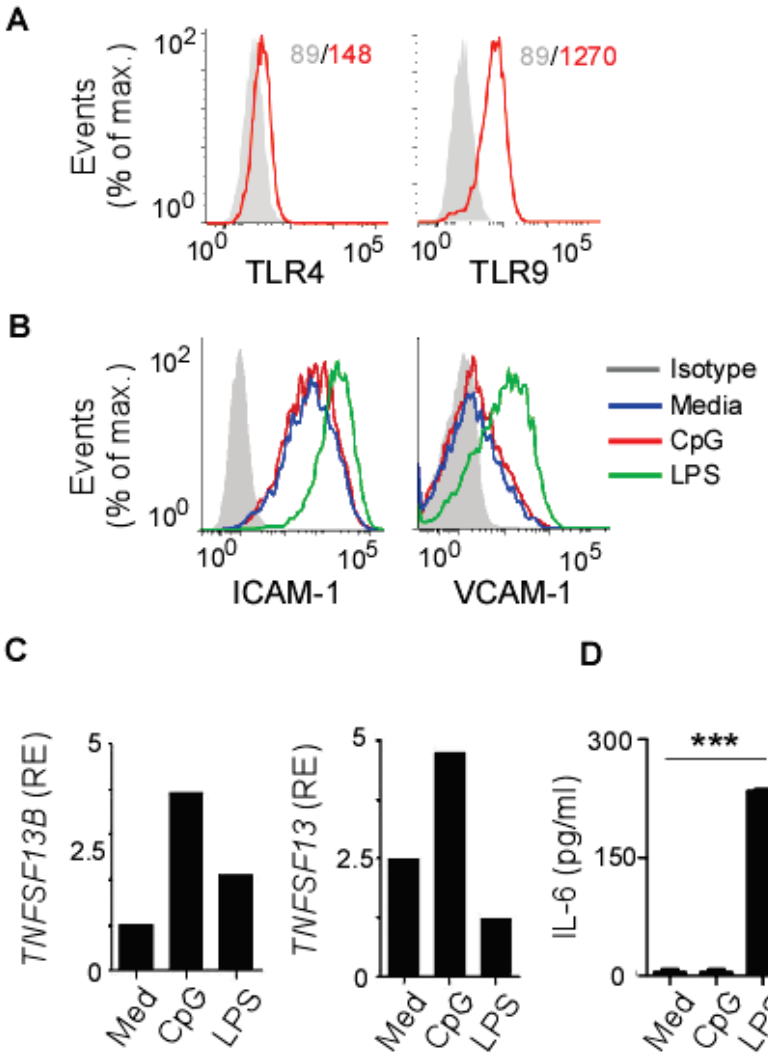


Figure 18. Human splenic MRCs produce B cell-stimulating factors in response to microbial signals. (A) FC of surface TLR4 and intracellular TLR9 in MRCs. Numbers indicate MFI. Grey profile shows isotype control antibody. (B) FC of ICAM-1 and VCAM-1 on MRCs incubated with CpG or LPS for 24 h. (C) BAFF and APRIL expression by QRT-PCR following MRC stimulation as in (B). (D) ELISA of IL-6 from MRCs stimulated as in (B) for 24 h. TLR, toll-like receptor; LPS, lipopolysaccharide. Data represents four different experiments (D); ***P<0.001 (two-tailed unpaired Student's t-test).

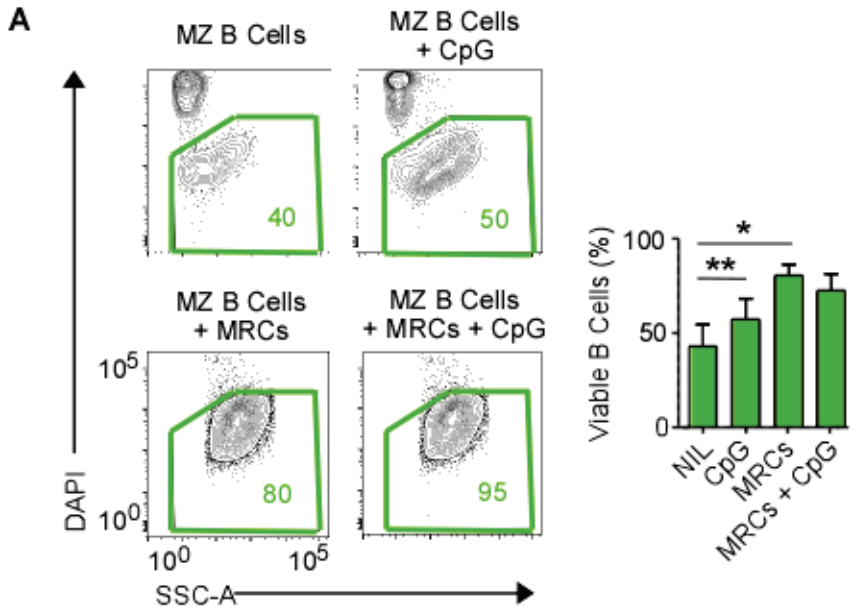
Human Splenic MRCs Stimulate Robust MZ B Cell Responses in Response to Microbial Signals

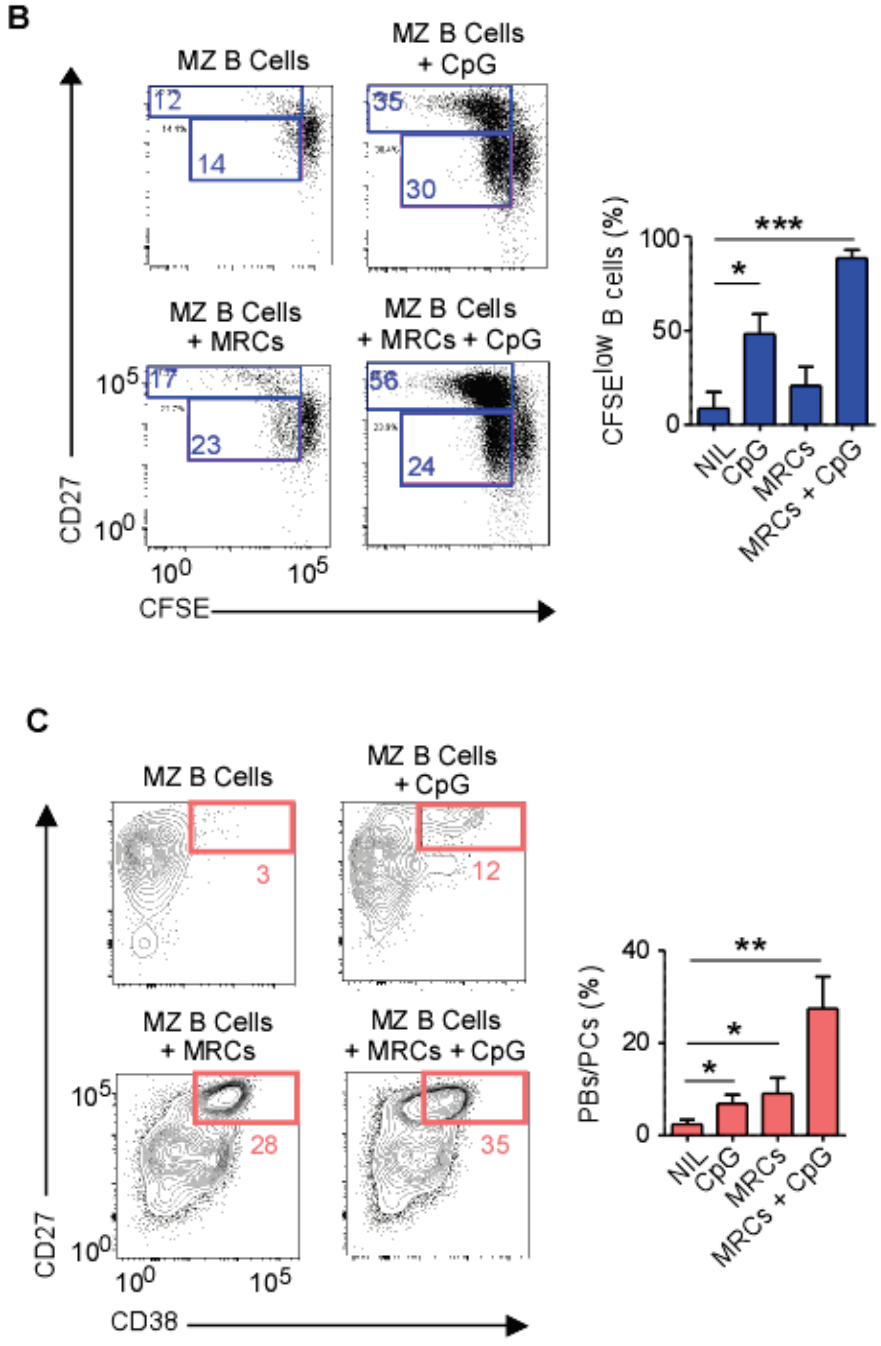
Given that MRCs respond to microbial stimulation by inducing B cell-stimulating factors and closely interact with MZ B cells, we determined whether MRCs enhance MZ B cell responses in the presence of microbial signals.

First, we anlaysed whether MRCs could increase MZ B cell survival, as shown by FC analysis of viable DAPI-negative cells, MRCs increased the frequency of MZ B cells by two-fold in a 5-day co-culture, irrespective of the presence or absence of CpG DNA **(Fig. 19A)**. Additional FC-based CFSE dilution assays were combined with CD27 staining to distinguish proliferating $CD27^{lo}CFSE^{lo}$ MZ B cells and $CD27^{hi}CFSE^{lo}$ PBs emerging at the end of the co-culture. We found that, compared to MRCs or CpG DNA alone, a combination of MRCs and CpG DNA induced more effective MZ B cell proliferation, including PC differentiation **(Fig. 19B and Annex II, Fig. 3)**.

Next, we determined whether MRCs could elicit MZ B cell differentiation into $CD27^{hi}CD38^{hi}$ PBs. FC showed that MRCs alone induced as much PC differentiation as CpG DNA alone **(Fig. 19C)**.

However, compared to MRCs or CpG DNA alone, a combination of MRCs and CpG DNA induced more robust PC differentiation **(Fig. 19C and Annex II, Fig. 3)**. Consistent with the PB-inducing function of MRCs, ELISA showed that MRCs elicited significant MZ B cell secretion of IgM as well as class-switched IgG and IgA **(Fig. 19D and Annex II, Fig. 3)**. This induction further increased in the presence of CpG DNA, though this microbial product was already effective at inducing antibody production even in the absence of MRCs **(Fig. 19D)**.




B
MZ B Cells
MZ B Cells + CpG
MZ B Cells + MRCs
MZ B Cells + MRCs + CpG
12
14
35
30
17
23
56
24
CD27
CFSE
CFSE^low B cells (%)
NIL
CpG
MRCs
MRCs + CpG
C
3
12
28
35
CD38
PBs/PCs (%)

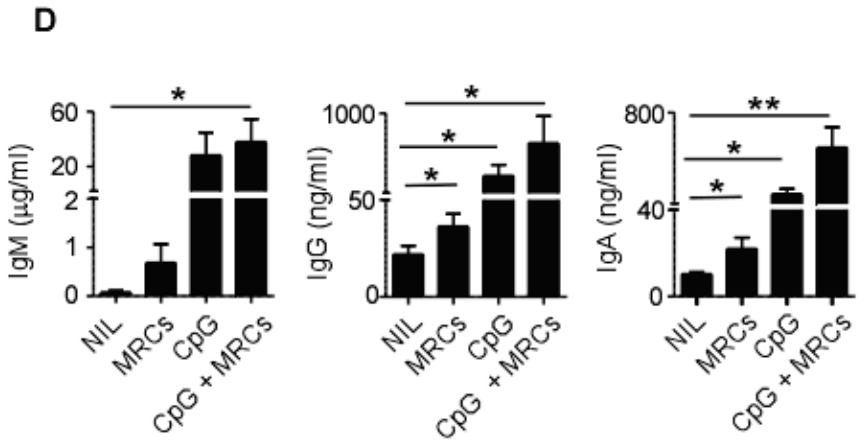


Figure 19. Human splenic MRCs promote MZ B cell survival, proliferation, PC differentiation, and antibody production. (A) FC of viable $DAPI^-$ MZ B cells following incubation with medium alone (NIL), MRCs, CpG DNA or MRCs plus CpG for 5 d. (B) FC of proliferation-induced CFSE dilution in MZ B cells cultured as in (A). (C) FC of $CD27^{hi}CD38^{hi}$ PBs-PCs generated by MZ B cells cultured as in (A). (D) ELISA of IgM, IgG and IgA secreted by MZ B cells cultured as in (A). Data represents at least 9 different experiments; *P<0.05, **P<0.01, ***P<0.001 (two-tailed unpaired Student's t-test).

Human Splenic MRCs Stimulate MZ B Cells Via Contact-Dependent MAdCAM-1 Signals

Having shown that human splenic MRCs elicit very effective MZ B cell responses, we wondered about the mechanisms underlying this induction. Preliminary data suggested that MRCs stimulated MZ B cell activation more effectively than MRC-derived conditioned medium (data not shown). This is consistent with the close proximity of MRCs and B cells in the MZ. We therefore wondered about the nature of MRC-bound receptors that could deliver contact-dependent MZ B cell helper signals. Given that MAdCAM-1 is abundantly expressed by

MRCs and interacts with α4β7 and VLA-4 receptors known for their ability to deliver B cell co-stimulatory signals, we determined α4β7 and VLA-4 expression on B cells inhabiting or surrounding the MZ.

FC showed that all of splenic naïve B cells as well as the vast majority of splenic MZ and ME B cells lacked α4β7 **(Fig. 20A)**. Nevertheless, this MAdCAM-1 receptor was visible on a variable but always small fraction of splenic MZ and ME B cells **(Fig. 20A)**. Compared to splenic mature B cell subsets, splenic PBs-PCs showed higher expression of α4β7 **(Fig. 20A)**.

Next, we took advantage of FC to ascertain whether a blocking antibody to MAdCAM-1 could attenuate MRC-induced activation of MZ B cells in a 5-d co-culture system. Compared to a control antibody, anti-MAdCAM-1 did not affect MRC-induced MZ B cell survival (data not shown), but significantly decreased MRC-induced differentiation of MZ B cells into PBs-PCs **(Fig. 20B)**. This inhibitory effect was evident irrespective of the presence of CpG DNA co-signals **(Fig. 20B)**.

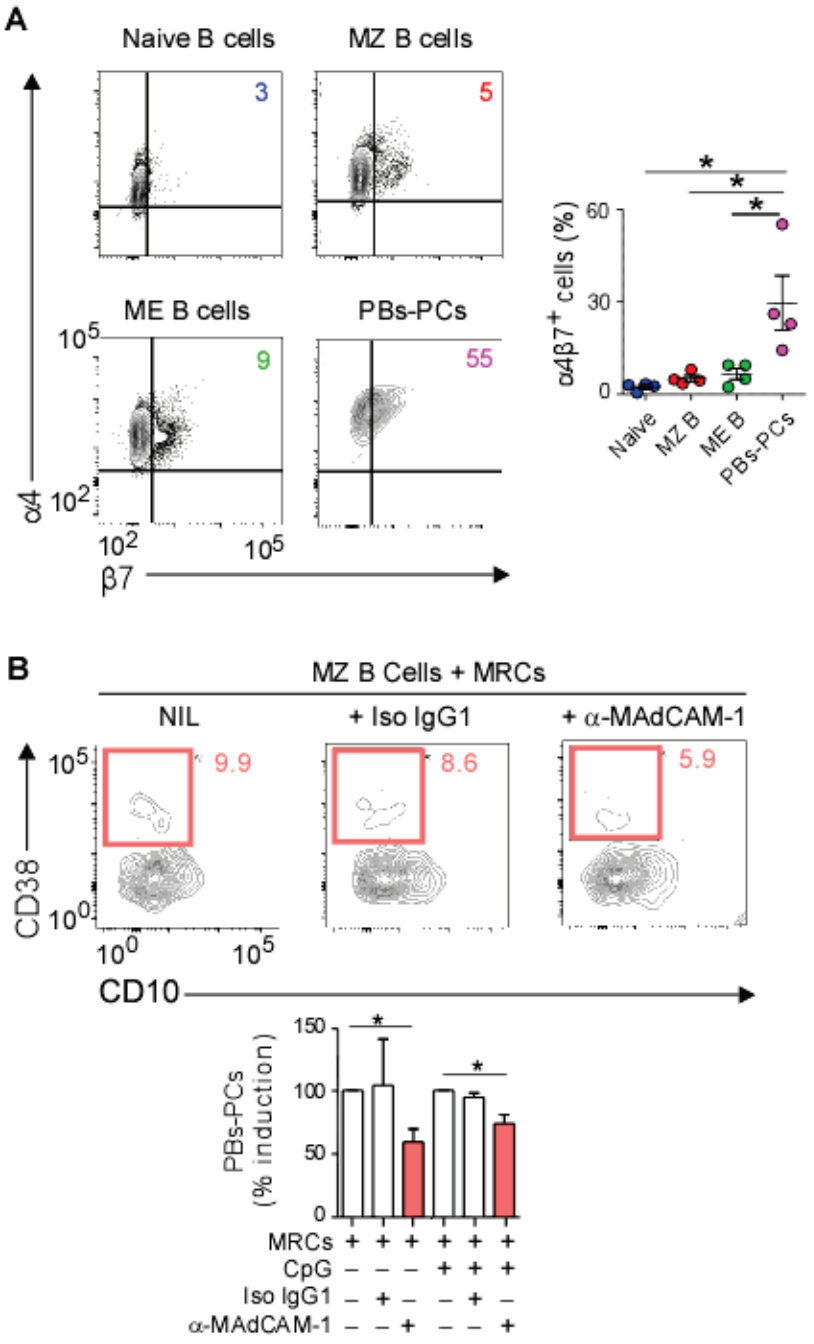


Figure 20. Human splenic MRCs stimulate MZ B cell differentiation into PBs through contact-dependent MAdCAM-1 signals. (A) FC analysis of α4β7 on

splenic naïve B cells, MZ B cells, ME B cells and PBs-PCs. (B) FC analysis of $CD27^{hi}CD38^{hi}$ PBs emerging from 5-d MRC-MZ B cell co-cultures in the presence of absence of CpG DNA and with or without a blocking anti-MAdCAM-1 antibody or an isotype-matched antibody with irrelevant reactivity. Data include four different donors (A) and three independent experiments (B). *P<0.05 (two-tailed unpaired Student's t-test).

These data indicate that human splenic MRCs deliver contact-dependent MZ B cell-activation signals that enhance PB formation through a mechanism involving MAdCAM-1-$\alpha4\beta7$ interactions.

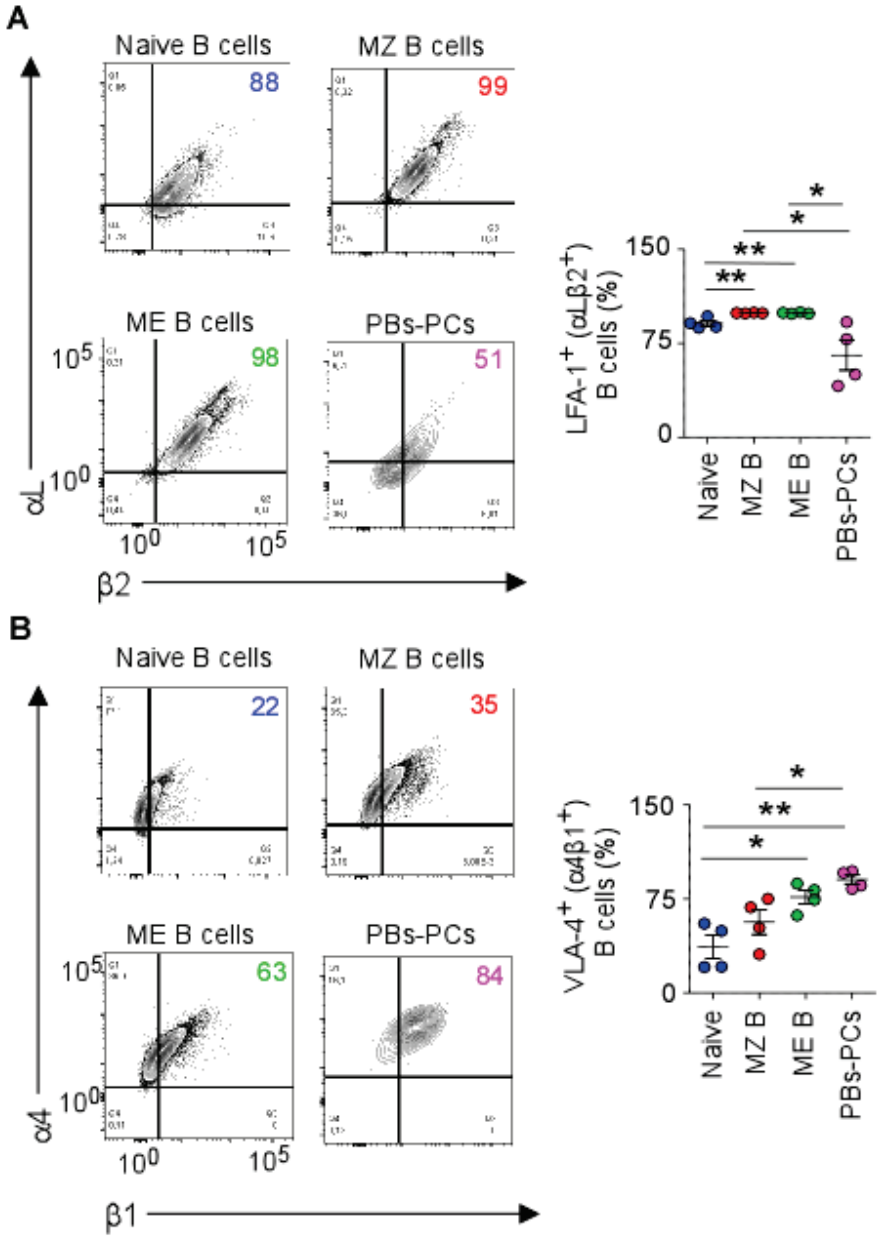
Human Splenic MRCs Stimulate MZ B Cells Via Additional Contact-Dependent VCAM-1 Signals

In addition to MAdCAM-1, human splenic MRCs strongly express the adhesion molecules VCAM-1 and ICAM-1, which bind VLA-4 and LFA-1 receptors, respectively. FC showed elevated levels of LFA-1 on splenic naïve, MZ and ME B cells as well as PBs-PCs, albeit LFA-1 expression was comparatively weaker on PBs-PC **(Fig. 21A)**. Also VLA-4 was highly expressed by splenic naïve, MZ and ME B cells as well as PBs-PCs, but its intensity pattern was progressively stronger on these B cell subsets and reached maximum levels on PBs-PCs **(Fig. 21B)**.

Next, we verified whether blocking antibodies to VCAM-1 and ICAM-1 could mitigate MRC-induced activation of MZ B cells in a 5-d co-culture system inclusive of CpG DNA. FC determined that, compared to an isotype-matched control antibody, anti-VCAM-1 or a combination of anti-VCAM-1 and anti-ICAM-1 but not anti-ICAM-1 alone decreased MRC-induced differentiation of MZ B cells into PBs-

PCs **(Fig. 21C)**. Of note, blockade of VCAM-1 diminished MRC-induced MZ B cell production of IgM and IgG but not IgA but did not affect MRC-induced MZ B cell survival (data not shown).

These data indicate that, besides MAdCAM-1-$\alpha4\beta7$ interaction, VCAM-1-VLA-4 but not ICAM-1-LFA-1 interaction generates contact-dependent activation signals in human splenic MZ B cells exposed to MRCs. Whether MAdCAM-1 and VCAM-1 adhesion molecules enhance MZ B cell responses to microbial antigens *in vivo* is being tested by ongoing experiments.


A
Naive B cells
88
MZ B cells
99
ME B cells
98
PBs-PCs
51
αL
β2
LFA-1+ (αLβ2+) B cells (%)
150
75
0
Naive
MZ B
ME B
PBs-PCs
B
Naive B cells
22
MZ B cells
35
ME B cells
63
PBs-PCs
84
α4
β1
VLA-4+ (α4β1+) B cells (%)

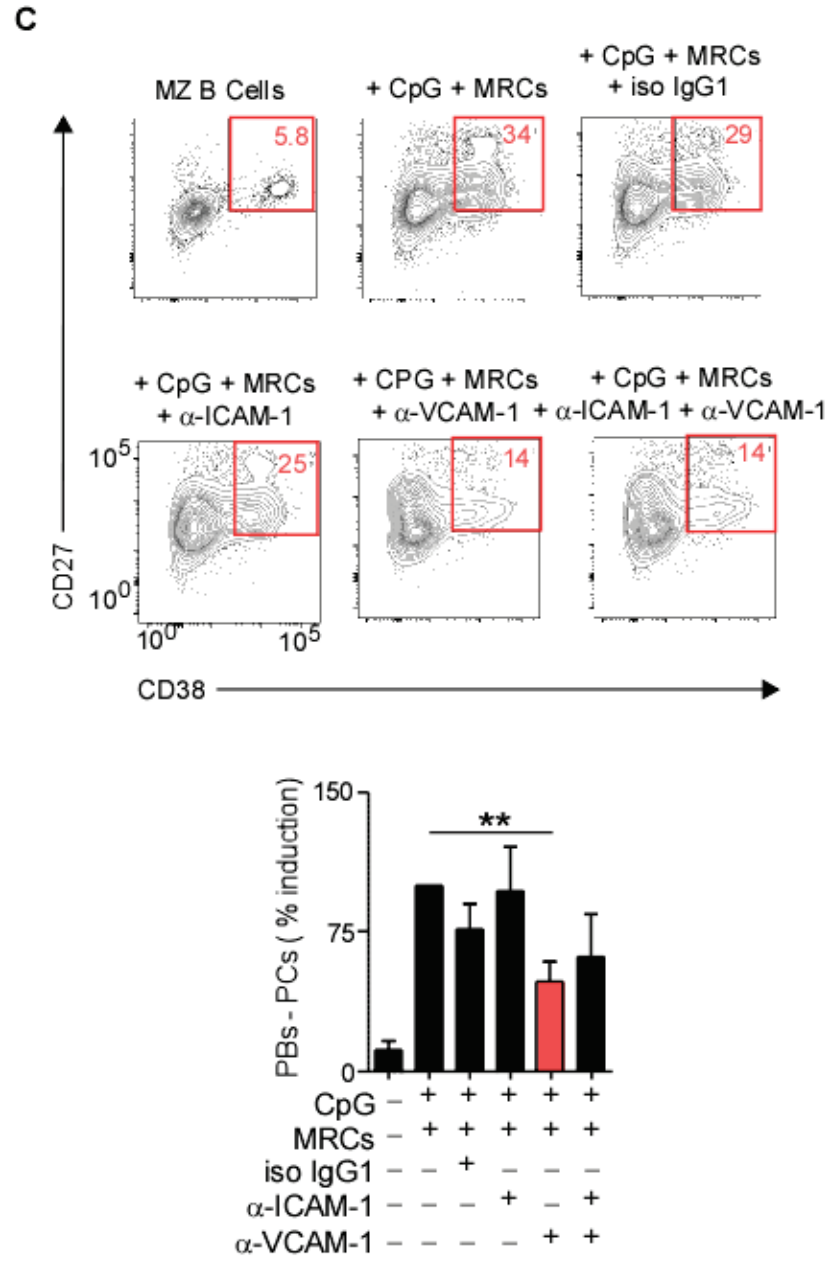


Figure 21. Human splenic MRCs stimulate MZ B cell differentiation into PBs through contact-dependent VCAM-1 but not ICAM-1 signals. (A) FC of LFA-1 on splenic naïve B cells, MZ B cells, ME B cells and PBs-PCs. (B) FC of VLA-4

expression on splenic cells as in (A). (C) FC analysis of $CD27^{hi}CD38^{hi}$ PBs-PCs emerging from MZ B cells co-cultured for 5 d with medium alone or MRCs in the presence or absence of CpG DNA and with or without blocking antibodies to ICAM-1 and/or VCAM-1. Data represent 4 different donors (A, B) and 3 independent experiments (C); *P<0.05, **P<0.01 (two-tailed unpaired Student's t-test).

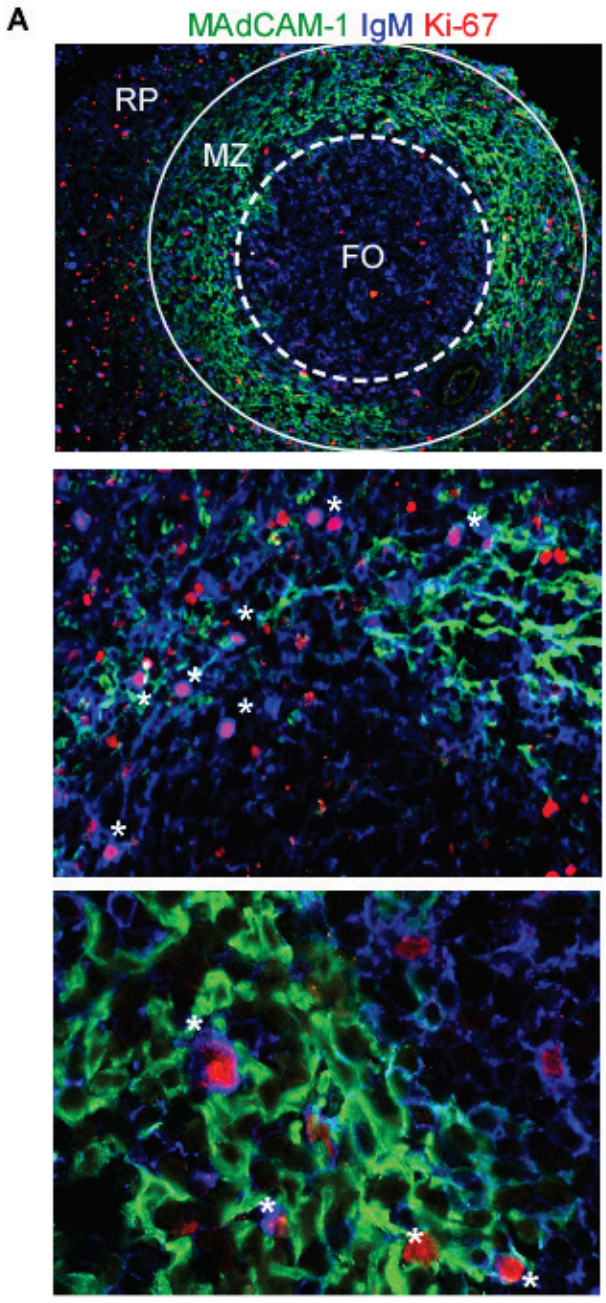
Human Splenic MRCs Extensively Interact With MZ B Cells and PBs

Given that a prominent subset of commensal-reactive PBs-PCs derive from MZ B cells in adult spleens, we wondered whether at least some PBs-PCs interact with MRCs in MZ and/or peri-MZ areas. Considering that MRCs capture commensal antigens and deliver robust MZ B cell helper signals, MRC interaction with PBs-PCs may further enhance homeostatic IgM responses to commensals.

IFA of splenic tissue sections showed $IgM^+Ki\text{-}67^+$ PBs containing abundant intracellular IgM **(Fig 22A)**. These splenic $IgM^+Ki\text{-}67^+$ PBs often surrounded GC-less ($Ki\text{-}67^-$) lymphoid follicles and inhabited MZ and peri-MZ areas containing extensive MRC networks. Together with NGS data, these observations raise the possibility that some $IgM^+Ki\text{-}67^+$ PBs emerge from extrafollicular pathways involving rapid activation of MZ B cells by antigenic signals involving MRCs. This possibility would be consistent with the detection of commensal products in MRCs.

To provide additional evidence that some IgM^+ PBs may emerge from GC-independent pathways, we performed IFA in fetal human spleens containing neither mature follicles nor GCs. Surprisingly; IgM^+ PBs-PCs were detected not only in adult spleens, but also in fetal spleens

(Fig. 22A, B). In both cases, IgM^+ PBs-PCs occupied extrafollicular areas proximal to α-SMA^+ stromal cells **(Fig. 22A, B)**. In fetal spleens, IgM^+ PBs-PCs were also proximal to α-SMA^+ vascular structures. Given that GC reactions are absent during fetal life, these findings corroborate the idea that some IgM^+ PBs-PCs emerge from primordial GC-independent maturation programs possibly elicited by TI antigens. The nature of these antigens remains unclear, but they might include maternally derived commensal antigens undergoing transplacental migration to initiate the "education" of the fetal immune system.


A
MAdCAM-1 IgM Ki-67
RP
MZ
FO

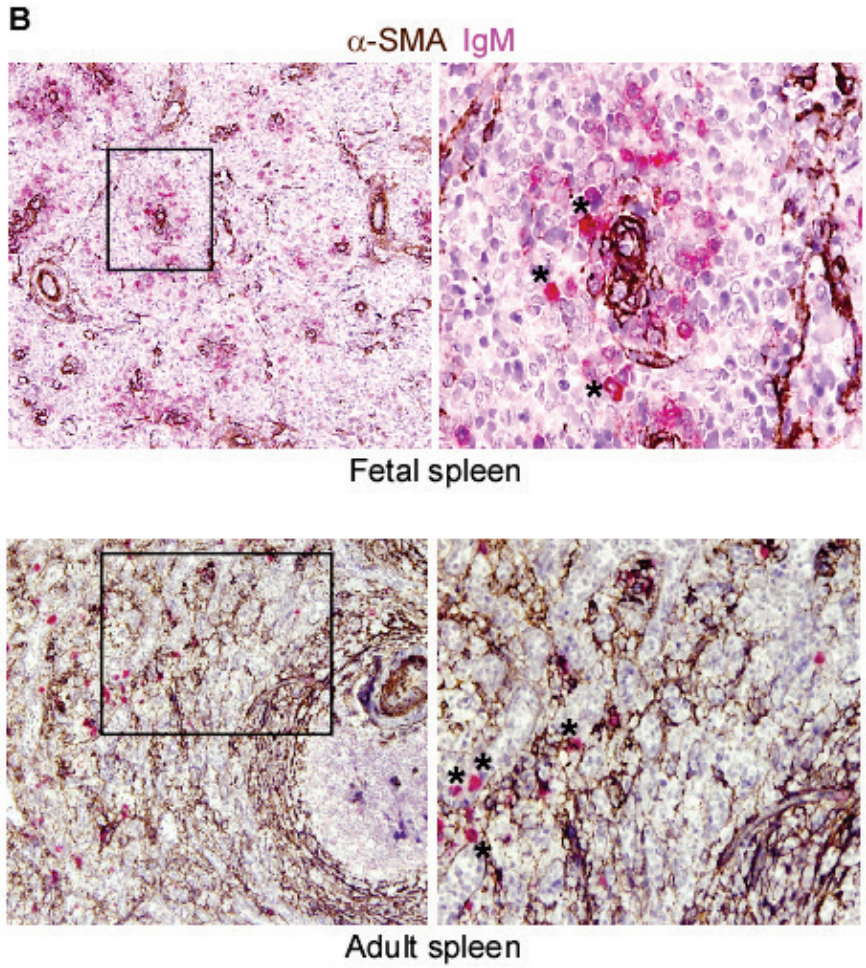


Figure 22. Human splenic MRCs may interact with PB emerging from extrafollicular GC-independent pathways. (A) IFA of adult spleens stained for MAdCAM-1 (green), IgM (blue) and Ki-67 (red). Asterisks indicate $IgM^+Ki\text{-}67^+$ PBs proximal to $MAdCAM\text{-}1^+$ MRCs. Original magnification, ×20 (top), ×40 (centre) and ×63 (bottom). (B) IHC of human fetal (top) and adult (bottom) spleens stained for α-SMA (brown) and IgM (red). Asterisks indicate IgM^+ PBs-PCs proximal to $\alpha\text{-}SMA^+$ stromal cells, including MRCs. Original magnification, ×10 (left) and ×40 (right).

MRCs Deliver Survival Signals to PBs and PCs Via BAFF and APRIL

Given the extensive interactions of splenic PBs-PCs with stromal cells, including MRCs, we wondered whether MRCs could provide survival signals to MZ B cell-derived PBs-PCs in addition to delivering activation and differentiation signals to MZ B cells. FC showed that the viability of FACSorted $CD27^{hi}CD38^{hi}$ PBs-PCs increased by two fold following co-culture with MRCs for 2 d **(Fig. 23A)**.

Having shown that MRCs produced B cell/PC survival factors such as BAFF and APRIL in response to stromal or microbial stimuli, we also tested whether BAFF and APRIL accounted for MRC-induced PBs-PCs survival. Compared to MRC-derived conditioned medium, MRCs provided stronger PB-PC survival signals **(Fig. 23B)**. This finding could be attributed to our earlier observation that MRCs expressed not only soluble but also membrane-bound BAFF **(Fig. 16B)**. Indeed, when we added the BAFF-blocking BAFF-R-Ig decoy receptor to the MRC-PC-PC co-culture system the survival is slightly decreased **(Fig. 23B)**.

This finding suggests that contact-independent BAFF and APRIL survival signals play an ancillary role compared to contact-dependent survival signals from MRCs. BAFFR-Ig and TACI-Ig significantly decreased the survival of PBs-PCs in an MRC-less culture containing MRC-derived supernatant alone **(Fig. 23B)**, indicating that soluble BAFF and APRIL play some role in the preservation of the splenic PB-PC repertoire by MRCs.

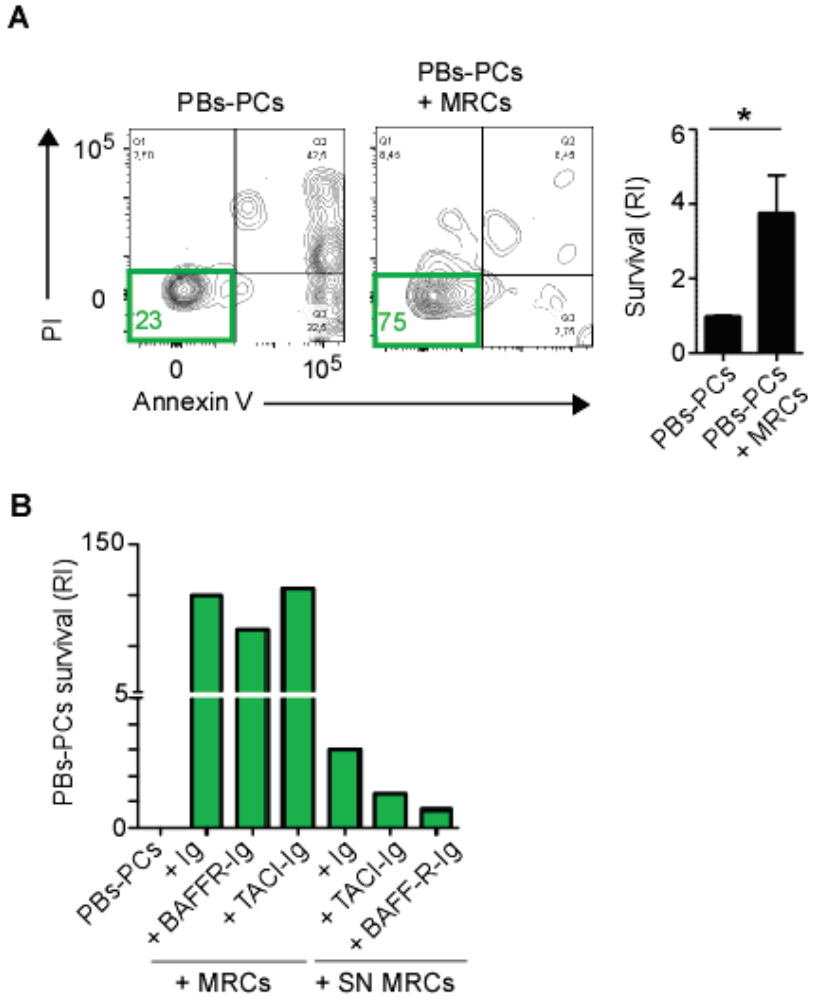


Figure 23. Human splenic MRCs deliver survival signals to PBs and PCs through BAFF and APRIL. (A) FC of viable propidium iodide (PI)$^-$Annexin-V$^-$ PBs-PCs following FACSorting and cultured in the presence or absence of MRCs for 48 h. Data include three different experiments; *P<0.05 (two-tailed unpaired Student's t-test). (B) FC of viable PBs-PCs cultured with MRCs or MRC-derived supernatant (SN) in the presence or absence of control Ig antibody, BAFFR-Ig or TACI-Ig. Data include one experiment.

CHAPTER 5
DISCUSSION

Here we have shown that the human spleen homeostatically produced antibodies against commensal microbes, including highly conserved molecular patterns from bacteria and fungi. This humoral response correlated with splenic capture of commensal antigens and local differentiation of MZ B cells into clonally affiliated PBs and PCs that produced commensal-reactive IgM and, to a lesser extent, class-switched IgG and IgA. Similar to macrophages from PFZ and RP areas, MRCs from the MZ internalized commensal antigens, presumably due to macrophage-like expression of multiple receptors mediating antigen sensing and clearance, including TLRs and scavenger receptors. Consistent with their strategic location in the MZ, MRCs elicited robust MZ B cell proliferation, CSR, PC differentiation and antibody production following exposure to microbial ligands and canonical MRC-stimulating cytokines such as LTα1β2 and TNFα. Mechanistically, these MZ B cell responses required contact-dependent and contact-independent signals from MRC-derived ligands and cytokines such as MAdCAM-1 and VCAM-1 as well as IL-6 and BAFF, respectively. Thus, splenic MRCs may function as innate response activator cells that integrate antibody-inducing signals from other splenic MZ B cell-helper cells to generate a second line of humoral defence against commensal antigens.

The Human Spleen Mounts Homeostatic IgM Responses to Commensal Antigens through Active PB and PC Induction

The spleen is a SLO that evolved throughout evolution to mount protective TI and TD immune responses against blood-borne

antigens, including pathogens. While splenic antibody responses to pathogens have been extensively studied in mice, less is known about splenic antibody responses unfolding in the absence of infection or immunization. In rodents, these homeostatic responses are comprised of IgM and IgG3 antibodies that react against highly conserved molecular determinants often shared by self-antigens and microbes, including commensal antigens from mucosal surfaces.

The mechanisms whereby splenic B cells come in contact with mucosal antigens remain unknown, but published studies suggest two alternative and non-mutually exclusive possibilities[163,164]. The first hypothesis postulates controlled translocation of "free" mucosal antigens to the spleen from the systemic circulation. In this scenario, opsonizing agents abundantly present in the blood, including antibodies and complement, would limit the induction of systemic inflammation by translocated antigens. The second hypothesis posits translocation of phagocytosed and/or liposome-enveloped mucosal antigens to the spleen from the systemic circulation. In this situation, endosomes and phagosomes from mucosa-derived DCs and neutrophils or lipids from gut-derived chylomicrons would attenuate systemic induction of inflammation by translocated antigens[211,212].

In agreement with the first hypothesis, both mouse and human studies show that tiny amounts of commensal products such as LPS and peptidoglycan physiologically translocate from the intestinal lumen to the circulation and thereafter reach systemic lymphoid organs with antigen-filtering fucntion, including the spleen and BM[205,208]. The second hypothesis stems from several works showing that chylomicrons incorporate LPS from intestinal Gram-negative bacteria,

enter the systemic circulation via the lymphatic system and undergo later clearance by splenic, hepatic and vascular macrophages following further processing into chylomicrons remnants[211,212]. Both hypotheses are consistent with the notion that gut antigens with immunostimulating properties are pivotal to optimize the development of systemic lymphoid organs and the magnitude and quality of systemic immune responses, including vaccine-induced antibody production.

Our results indicated that the human spleen contributed to IgM responses to *Escherichia coli,* which is an abundant Gram-negative commensal from the intestine, *Staphylococcus aureus*, which is a Gram-positive commensal from both skin and intestine, as well as *Lactobacillus*, which is a Gram-positive commensal from the intestine[356-358]. Splenic IgM responses additionally recognized the Gram-negative pathogen *Salmonella typhi,* perhaps due to the polyreactivity of some these IgM antibodies[359]. In addition to whole commensal bacteria, splenic IgM responses targeted LTA and CPS, which are soluble TI-1 and TI-2 products from Gram-positive or Gram-negative bacteria, respectively, as well as chitin from commensal fungi[110,347].

Compared to healthy donors, commensal-specific IgM antibodies were significantly reduced in splenectomized individuals, suggesting that the spleen actively mounts homeostatic IgM responses to commensals through the induction of short-lived PBs or supports secretory memory to commensals by sustaining long-lived PCs induced earlier on either locally or at distal inductive sites. Though reduced, commensal-specific IgM to commensals remained detectable in splenectomized individuals, indicating that additional PCs located in

the BM contribute to this homeostatic humoral response. Consistent with our human data, recently published mouse studies show that the spleen homeostatically induces systemic IgG responses predominantly targeting intestinal Gram-negative commensals[215]. Of note, these IgG antibodies derive from multiple B cell subsets, including MZ B cells, and confer protection against certain pathogens by targeting shared epitopic determinants. The possibility of a functional connection between gut and spleen is further supported by works showing impaired IgM and IgG responses to various microbial and non-microbial carbohydrates in GF mice lacking the gut microbiota[206,207].

Of note, we performed additional measurements that revealed a tendency toward decreased commensal-reactive IgA and IgG antibodies in the serum of splenectomized individuals (not shown), lending support to the notion that the human spleen supports not only systemic IgM but also some systemic IgG and IgA production to the commensal microbiota. In principle, these antibodies may emerge from recent vaccinations or infections, but this possibility seems unlikely, because the spleens used in these studies were obtained from adults and contained few or no secondary follicles and GC B cells, a hallmark of TD immunization. Yet, these spleens contained actively proliferating PBs in addition to terminally differentiated PCs, raising the possibility that at least a fraction of splenic antibody responses to commensals homeostatically emerge in response to ongoing stimulation by TI antigens.

Similar to PCs from the BM[360], both PBs and PCs from spleen down-regulated the expression of mature B cell-associated molecules such as CD20, CD21 and CD24, which is a hallmark of ASC differentiation.

Besides exhibiting a canonical $CD27^{hi}CD38^{hi}$ phenotype, splenic ASCs showed a bimodal CD138 expression pattern that suggested co-existence of $CD138^{-}$ PBs and $CD138^{+}$ PCs. In agreement with this possibility, splenic ASCs additionally showed bimodal expression of CD86 and MHC-II, two molecules characterizing recently activated and actively proliferating PBs, respectively[360-362]. Again, these findings suggest that ongoing splenic antibody production to commensals involves PBs generated in response to active antigenic exposure.

Remarkably, both splenic PBs and PCs evidenced increased BCMA expression along with decreased BAFF-R and TACI expression, suggesting that BAFF and APRIL support splenic ASC survival and antibody production via BCMA as they do in the BM[363]. Besides BAFF and APRIL, IL-6 may deliver additional survival signals to splenic PBs and PCs[28,360], which indeed expressed low but significant levels of IL-6R. Consistent with this phenotype, initial experiments indicated that neutralization of BAFF and IL-6 from splenic MRCs attenuated ASC survival (data not shown).

In general, PCs emerging from LN or spleen follicles move to the BM or RP, respectively, through a mechanism involving up-regulation of CXCR4, a chemokine receptor that promotes PC retention in stromal niches expressing CXCL12. Furthermore, PCs down-regulate the chemokine receptors CCR7 and CXCR5, which are chemokine receptors retaining PCs in follicular areas expressing CCL19/CCL21 and CXCL13, respectively[100,143,360]. Finally, up-regulation of β-7 and CCR9 permits PC migration to gut tissues expressing MAdCAM-1 and CCL25 ligands, respectively[364].

Remarakbly, splenic PBs and PCs showed an up-regulation of β7 and CCR9 in addition to a down-regulation of CXCR5 and an up-regulation of CXCR4. Collectively, these and earlier data indicate that human splenic PBs and PCs physiologically emerge from splenic follicular areas expressing CCR7 and CXCR5 ligands to colonize splenic MZ, PFZ and RP areas expressing CXCR4 ligands. By actively generating IgM as well as class-switched IgG and IgA in response to ongoing stimulation, these ASCs may clear commensal antigen and perhaps enhance protection against blood-borne pathogens sharing antigenic determinants with commensal bacteria.

Human IgM to Commensal Antigens Derive from Splenic MZ B Cells As Well As PBs and PCs

Havig shown that the spleen contributes to systemic IgM responses to commensal antigens and contains recently induced PBs, we wondered whether these PBs released commensal-reactive IgM and locally emerged from MZ B cells, a splenic innate-like lymphocyte subset ideally positioned to encounter blood-borne antigens. We found that human recombinant IgM from single-sorted splenic cells belonging to pooled PBs and PCs (PBs-PCs) recognized *Escherichia coli, Staphylococcus aureus* and *Lactobacillus*. Ongoing studies are further characterizing the reactivity of these IgM antibodies as well as human recombinant IgG and IgA to determine their reactivity to both microbial and autologous antigens, a feature common to IgM as well as many class-switched antibodies from memory B cells.

Consistent with the reactivity of PBs-PCs to commensal bacteria, we further determined that IgM from MZ B cells recognized CPS and

LPS, two major antigenic components of Gram-positive and Gram-negative bacteria[365]. Surprisingly, IgM from MZ B cells also recognized β-glucan, mannan and chitin, which are carbohydrates expressed by commensal fungi[349,366]. Of note, MZ B cell-derived IgM additionally recognized PCh, an antigen expressed by both autologous apoptotic cells and some encapsulated bacteria[367-370]. Though a more comprehensive reactivity screening comparing MZ B cells with PBs-PCs will be needed, these data strongly suggest that splenic MZ B cells represent the precursors of splenic PBs-PCs homeostatically secreting IgM to commensal bacteria.

Human Splenic PCs and PCs Expressing IgM to Commensal Antigens Clonally Expand from Locally Activated MZ B Cells

Though often engaged in TI antibody responses against microbial polysaccharides and lipids, including CPS, LPS and PhC[176,189], MZ B cells can also enter follicles to develop TD antibody responses to protein antigens[371-373]. These latter responses involve the acquisition of molecular fingerprints of GC selection, including extensive SHM, raising the possibility that MZ B cells are equivalent to ME B cells exiting the GC before undergoing CSR[374]. However, additional studies show that MZ B cells develop during fetal life in the absence of GCs and undergo SHM by following a TI pathway that does not require exogenous antigenic stimulation[180,375,376]. Moreover, immunodeficient patients lacking GCs due to the loss of CD40L show a profound depletion of canonical class-switched and hypermutated ME B cells, but retain MZ-like B cells in the circulation[84,375]. Importantly, these

cells express fewer mutations than ME B cells do and these mutations remain detectable in GC-less patients lacking CD40L[84,375]. Thus, one possibility is that the population of B cells that we phenotypically define as splenic MZ B cells is ontogenetically heterogeneous and includes effector innate-like subsets without GC history as well as effector memory subsets emerging from GC reactions.

We found that, besides showing similar Ig VH gene usage and mutational loads, splenic MZ B cells had a very extensive clonal affiliation with IgM^+ PBs-PCs, which clearly indicates that IgM^+ PBs-PCs originate from MZ B cells locally activated by antigen. Though expressing a broad spectrum of SHM levels, MZ B cells as well as IgM^+ PBs-PCs were less mutated than class-switched IgA^+ or IgG^+ ME B cells, lending support to some published studies proposing that MZ B cells are distinct from canonical ME B cells[176,189]. Nonetheless, we also identified some MZ B cell clonotypes that were clonally related to class-switched ME B cells and shared their Ig VH gene usage. This finding indicates that some MZ B cells can participate in GC reactions that yield a hypermutated progeny hardly distinguishable from canonical ME B cells. Thus, besides following TI extrafollicular pathways inducing poorly mutated IgM and some class-switched antibodies, MZ B cells may enter classical TD pathways giving rise to highly mutated IgM and large amounts of class-switched antibodies. This latter fate may indicate iterative stimulation of MZ B cells by common proteins and glycoprotein antigens, including protein-associated polysaccharides. Indeed, all our recombinant IgM antibodies to commensals belonged to GC-derived clonal families harboring an elevated number of Ig VH gene mutations.

Of note, MZ B cells were strongly selected against IGHV1-18 and IGHV4-34 genes when compared to naïve B cells. IGHV4-34 is often expressed by polyreactive autoantibodies emerging during autoimmune disorders[84,192,194,377,378] and can be also detected in polyreactive antibodies targeting conserved neutralizing HIV-1 epitopes[379]. In contrast, IGHV1-18 is often expressed by poly/autoreactive IgG antibodies induced by dengue virus presumably through a polyclonal TI pathway[380]. Thus, we conclude that MZ B cells engaged in commensal-reactive IgM responses do not use Ig VH genes enriched in autoreactivity.

In contrast, MZ B cells and IgM^+ PBs-PCs along with their class-switched counterparts were strongly enriched in IGHV3-7 and IGHV3-74 genes, which may be important to recognize common commensal antigens, including specificities shared by both foreign and autologous antigens[381]. For instance, IGHV3-7 is associated with the recognition of glucan from yeast and filamentous fungi, but can be also detected in mutated B cells from chronic lymphocytic leukemia patients, which may recognize self-antigens[382]. Clearly, additional studies involving antigenically arrayed commensal and autologous antigens will be needed to better dissect the reactivity of splenic MZ B cells and their plasmacellular progenies. However, our finding that MZ B cells are clonally organized around PB and PC responses strongly support the contention that the spleen generates commensal-reactive IgM antibodies through a locally induced antigen-driven pathway.

Human Splenic PBs and PCs Can Switch from IgM to IgG or IgA

Consistent with the notion that the spleen is continuously receiving antigenic signals, we found that MZ and peri-MZ areas next to primary follicles contained short-lived and actively proliferating Ki-67$^+$ PBs, which are generally thought to originate from MZ B cells locally engaged in TI antibody responses[161,383]. These PBs and a smaller fraction of presumably immature PCs expressed the CSR/SHM-inducing enzyme AID, a finding that echoes recent mouse studies showing that splenic PBs emerging from *Salmonella typhy*-induced IgG responses undergo CSR as well as some degree of SHM[187]. In humans, AID$^+$ PBs similar to those detected in the spleen inhabit extrafollicular areas of tonsils[73]. Remarkably, high-throughput Ig VH gene sequencing studies confirmed that indeed some splenic IgM$^+$ PBs-PCs had undergone class switching to IgG, thereby generating clonally affiliated IgG$^+$ PBs-PCs with identical Ig VH genes. In agreement with these results, PBs-PCs contained molecular byproducts of ongoing IgM-to-IgG CSR in addition to AID. Notably, MZ B cells contained molecular byproducts of ongoing IgM-to-IgA CSR, lending further support to the notion of a local and actively ongoing antigenic activation that may contribute to local or distal pools of IgA$^+$ PBs-PCs, including gut-homing IgA$^+$ PBs-PCs expressing β7 anf CCR9. However, due to ethical and logistical limitations in obtaining splenic and gut samples from the same donor, we cannot formally prove this last hypothesis.

A tenet of PC responses is that induction of the transcription factor BLIMP-1 suppresses PAX5-controlled B cell-identity genes, including AID, to up-regulate a PC differentiation program involving antibody secretion through the unfolded protein response pathway, which incudes the BLIMP-1-induced gene XBP-1[64,89,384]. However, recent findings show that PBs and immature PCs from the spleen express less BLIMP-1 than terminally differentiated PCs from the BM and thus do not completely shut off their B cell-identity program, including AID expression[92,360]. Consistent with these studies, we found a distinct transcriptional profile in MHC-II$^+$ PBs and MHC-II$^-$ PCs, the former expressing a more pronounced B cell-identity gene program than the latter. Indeed, PBs expressed somewhat less BLIMP-1 (though the difference was not statistically significant), but more *AICDA*, *KI67* and somewhat more *PAX5* (though, again, the difference was not statistically significant) than PCs did. Yet, both PBs and PCs expressed PC-related gene products such as *IRF4*, *TNFRSF17* (BCMA) and *XBP1*, through the latter was reduced in PBs compared to PCs. Thus, the less differentiated state of PBs and some PCs may allow them to undergo local AID-dependent CSR.

The elevated expression of transcripts for BCMA in splenic PBs and PCs reflects earlier phenotypic data showing robust surface BCMA expression in these cells and confirms that these splenic ASCs may be strongly dependent on signals from BAFF and APRIL for their survival[385]. Consistent with this possibility, N_{BH} cells, macrophages, DCs and ILC3s located in splenic MZ, peri-MZ, PFZ and RP areas produce large amounts of BAFF and APRIL, particularly upon sensing microbial products through TLRs[78,106,163]. Thus, splenic capture

of commensal antigens may not only induce local TI and TD antibody response, but also maximize niches for PB and PC survival.

The Human Spleen Captures Commensal Bacteria, Thereby Providing Antigenic Signals for Homeostatic MZ B Cell Responses

Having shown that the human spleen generates commensal specific antibodies through local MZ B cell responses, we wondered whether there was any trace of commensal antigens in canonical antigen-clearing phagocytes from splenic tissue, including macrophages. In mice, specialized MMMs and MZMs capture blood-borne antigens through various PRRs, including scavenger receptors, and thereafter activate antigen-reactive MZ B cells[131,182,220]. Additional blood-borne antigens reach the MZ through circulating DCs and granulocytes entering the MS[163,164].

In humans, together with N_{BH} cells and perhaps sinusoidal lining cells (SLCs), splenic macrophages from PFZ and RP areas exert powerful antigen-capturing functions in addition to MZ B cell-helper functions[78,134,223,246,386]. Thus, we initially looked for microbial material in sorted macrophages from human spleen. These studies led to the discovery that human splenic macrophages but not control non-phagocytic T cells contained bacterial 16S rDNA from different phyla, though the Proteobacteria phylum was usually more predominant. Of note, splenic commensals included both Gram-negative and Gram-positive bacteria and had a heterogeneous family composition that suggested their possible origin from multiple anatomical districts at the

interface between host and environment, including skin, gut and oral cavitiy[343,344,387-391].

The detection of commensal material in the spleen correlates with and may account for homoestatic MZ B cell responses leading to the clonal expansion of PBs and PCs secreting IgM as well as class-switched antibodies to commensal antigens. Indeed, human recombinant IgM mAbs from splenic PBs-PCs recognized members of both Proteobacteria (*Escherichia coli* and *Salmonella sp.*) and Firmicutes (*Lactobacillus sp.* and *Staphyloccocus aureus*) phyla detected in splenic macrophages.

As discussed earlier, the mechanism whereby tiny amounts of commensal antigens translocate from mucosal surfaces to the spleen remain unclear, but multiple hypotheses are under consideration. It is also unclear whether distinct subsets of splenic phagocytes capture different sets of bacteria. In any case, our studies and those recently published in mice[215] suggest that capture of blood-borne commensal antigens by the spleen triggers homeostatic antibody responses that generate a secondary line of systemic (i.e., post-mucosal) defense against microbes evenetually breaching mucosal barriers. This process may also generate protective antibodies capable to cross-recognize conserved antigenic determinants from pathogens.

Human MRCs Combine Stromal with Macrophage-Like Features and Capture Commensal Antigens in the MZ

Unlike the mouse MZ, the human MZ is not surrounded by a MS and

lacks specific macrophages such as MMMs and MZMs, raising questions as to which cells capture antigens gaining access to the MZ. Scattered macrophages expressing CD68 and CD163 located in the MZ and RP areas, together with CD169 macrophages forming peri-capillary sheats in the PFZ may fit the bill and serve many of the functions currently ascribed to MMMs and MZMs in mice[132,134]. However, it seems unlikely that the human MZ, which is at least 30% larger than the mouse MZ, lacks specific antigen-capturing systems. Our studies confirm that few or no macrophages inhabit the inner and intermediate areas of the human MZ. Instead, these areas contain a very extensive and elaborate reticular network of MAdCAM-1$^+$ stromal cells potentially equivalent to mouse splenic MRCs, which form a thin stromal layer along the MS[279].

Human splenic MAdCAM-1$^+$ reticular cells expressed a core stromal gene signature largely shared with stromal cells from the BM but not with dermal FBs. The identity of these MAdCAM-1$^+$ reticular cells with stromal cells was further confirmed by their lack of CD45 and the endothelial molecule CD31, coupled with expression of canonical stromal surface molecules such as MAdCAM-1, CD90 (Thy-1), CD141, PDPN, nestin, SMA-α, RANK-L, BP3 and CXCL13 along with unusually elevated surface levels of ICAM-1 and VCAM-1, which further increased upon exposure to LTα1β2 and TNFα. These findings demonstrated that human splenic MAdCAM-1$^+$ reticular cells likely corresponded to MRCs.

Of note, neither the gene expression profile nor the phenotype of human splenic MRCs completely overlapped with those of stromal cells from the BM, which indeed expressed less ICAM-1 and VCAM-1

and lacked MAdCAM-1 and CD141. More importantly, splenic MRCs contained a unique macrophage-like gene signature, whereas stromal cells from the BM did not. This finding together with the abundance of MRCs but not macrophages in the human MZ prompted us to hypothesize that MRCs deploy macrophage-like immune functions that could contribute to the regulation of MZ B cell responses, including homeostatic production of antibodies to commensal antigens.

Growing evidence indicates that MRCs as well as other SC subsets such as FRCs play a pivotal role in the initition, regulation and duration of effector immune responses. In mice, MRCs from the SCS of LNs generate a conduit network specialized in the transportation of small soluble antigens from the outer to the inner follicle[284,299]. Whether splenic MRCs form a similar conduit network remains unknown, but our data show MRC expression of several ECM molecules required for conduit formation[392,393] and collagen fibers important to transport antigens, cytokines and chemokines. Should its existence be confirmed, this conduit system would be important to exclude large molecules and particulate antigens, which instead may be channeled into the splenic WP by phagocytes[284].

Though MRC-derived conduits express some ECM molecules, other ECM molecules such as laminin, collagen and fibronectin derive from the extracellular environment and serve as anchoring factors for chemokines as well as trafficking lymphocytes[394]. By showing that some splenic MRCs are proximal to macrophages positioned in the outer MZ and PFZ, our studies suggest that MRCs may facilitate the retention and function of peri-MZ macrophages, including the

delivery of particulate-antigens to MZ B cells[300,301]. Should this be the case, MRCs may functionally bridge MZ B cells with antigen-capturing macrophages located in peri-MZ areas of the human spleen. However, MRCs may also capture antigens independently of macrophages.

Consistent with this possibility, human splenic MRCs contained bacterial 16S rDNA as classical splenic phagocytes such as macrophages and DCs did[395]. This finding correlated with transcriptomic and phenotypic data showing that human splenic MRCs expressed macrophage-like gene signatures and phenotypes encompassing expression of various PRRs involved in antigen sensing, capture and internalization[395], including TLRs, CLRs, NLRs and scavenger receptors. In particular, highly purified human splenic MRCs expressed the surface CD4 co-receptor, surface TLR4 and endosomal TLR9 and the surface scavenger receptor CD36 together with transcripts for the C-type lectin receptor *CLECL14A* and the scavenger receptor *SCARF1*, a phenotype highly resembling that of macrophages. Altogether, these observations lend further support to our hypothesis that MRCs may serve as macrophage-like immune sentinels capable to capture, archive and possibly expose antigens penetrating in the human splenic MZ.

A similar notion has been recently brought forward by mouse studies showing that several types of LN stromal cells, including FRCs, archive soluble or particulate antigens and eventually present them in the context of MHC-I or MHC-II complexes together with the inhibitory molecule PD-L1 to regulate homeostatic or infection-induced T and B cell responses[396]. Human splenic MRCs may play a similar role and additionally retain opsonized antigens through Fc

receptors as FDCs do[268]. Regardless of the mechanism, these MRC-captured antigens might be exposed to MZ B cells for the initiation of specific antibody responses.

In general, it is becoming clear that multiple types of splenic innate immune cells capture antigen in different areas of the spleen. While macrophages, DCs and N_{BH} cells may mostly phagocytose antigenic material in the PFZ and RP, MRCs may do so in the splenic MZ, perhaps in cooperation with DCs and PFZ macrophages. Should this be the case, multiple cell types may cooperatively regulate distinct phases of MZ B cell responses to commensal antigens. A putative role of MRCs in both inductive and effector phases of MZ B cell responses is consistent with our finding that MRCs expressed multiple cytokines and chemokines known to have pivotal immunoregulatory functions. Besides facilitating the recruitment, activation and retention of antigen-engaged MZ B cells and their plasmablastic progeny, these soluble factors may promote the formation of specialized survival niches for MZ B cell-derived PBs and PCs.

Human Splenic MRCs Produce MZ B Cell-Stimulating Factors in Response to Microbial and Stromal Signals

Homing of lymphoid and myeloid cells to the splenic WP is an active process requiring migration across a layer of MRCs. These SCs delineate the boundaries between the WP and RP in addition to demarcating B and T cell zones[279]. Besides orchestrating cell migration, chemokines released by MRCs promote the formation of appropriate niches for lymphoid and myeloid cells residing within and in proximity

of the MZ, including B cells, ILCs, macrophages, DCs and N_{BH} cells[78,106].

Similar to mouse stromal cells, human splenic MRCs expressed B cell-activating cytokines as BAFF, APRIL and IL-6 as well as B cell-attracting chemokines such as CXCL12 and CXCL13, both under steady state conditions and following exposure to microbial TLR ligands, including LPS or CpG DNA, or canonical SC-activating signals, including LTα1β2 and TNFα[27,146,353]. This functional profile confirmed the possibility that MRCs may orchestrate local immune responses to commensal antigens in addition to deploying classical structural and lymphoid tissue-inducing functions as LTα1β2 and TNFα-responsive stromal cells do[279,303,305].

The stromal nature of human splenic MRCs was further confirmed by their expression of several TNF-related molecules usually espressed by LTo cells, including RANK-L, which is required for the development and homeostasis of SLOs[397]. Besides RANK-L, MRCs strongly up-regulated the expression of ICAM-1, VCAM-1 and CXCL13 in response to LTα1β2 and TNF. These findings echo published mouse data showing that MRCs regulate B cell trafficking in the spleen[279,398].

Consistent with their macrophage-like properties, human splenic MRCs also produced IL-8 (also known as CXCL8), a chemokine that may attract antigen-transporting and/or antigen-capturing N_{BH} cells into the outer MZ and PFZ to help the activation of splenic MZ B cells[78,106]. Chemotaxis and blocking assays are currently ongoing to further dissect this interesting aspect of human MRC biology.

In summary, besides showing classical stromal responses, human MRCs expressed a broad assortment of immunoregulatory cytokines and chemokines involved in the migration, retention and activaton of both lymphoid and myeloid cells. The production of some of these factors, including B cell-stimulating mediators, increased following MRC exposure to microbial TLR ligands, lending further support to our contention that MRCs provide critical help for the initition of MZ B cell responses.

MRCs Stimulate MZ B Cell Activation through Contact-Dependent MAdCAM-1 and VCAM-1 Signals

Consistent with their antigen-capture ability, close proximity to MZ B cells and homeostatic or signal-induced production of B cell-stimulating factors, MRCs increased MZ B cell survival, proliferation, PC differentiation and IgM as well as class-switched IgG and IgA production in response to CpG DNA, a microbial ligand associated with 16S rDNA and recognized by a TLR family member termed TLR9. This endosomal receptor is highly expressed by MZ B cells, but was also detected in MRCs and may thus elicit highly cooperative MRC-induced MZ B cell responses. Similar to MZ B cells, class-switched ME B cells express elevated levels of TLR9 and thus may mount MRC-driven antibody responses as MZ B cell do, at least *in vitro* (data not shown).

Despite producing powerful soluble B cell-stimulating factors such as BAFF and APRIL, MRCs predominantly stimulated MZ B cell responses, including increased survival and PC differentiation, through contact-dependent mechanisms. Thus, we hypothesized that MRC-

bound molecules usually involved in lymphocyte migration and retention, including MAdCAM-1, VCAM-1 and ICAM-1, may also drive contact-dependent activation signals to MZ B cells.

While ICAM-1 and VCAM-1 are crucial for the retention of B cells within the MZ[174,399-401], the function of splenic MAdCAM-1 remains unknown. In the gut LP, MAdCAM-1 is highly expressed by HEVs and guides intestinal homing of circulating IgA^+ PCs expressing $\alpha4\beta7$, a counter-receptor of MAdCAM-1[402-405]. In mice, MAdCAM-1 additionally regulates lymphocyte trafficking in PPs and MLNs, but does not seem to play an important role in lymphocyte trafficking in the spleen[399,405,406]. Thus, splenic MAdCAM-1 may have additional as yet unknown functions.

We found that human splenic MZ B cells expressed elevated surface levels of LFA-1 and VLA-4, the counter-receptors of ICAM-1 and VCAM-1, respectively. In addition, human splenic PBs expressed elevated surface levels of $\alpha4\beta7$, the counter-receptor of MAdCAM-1. Thus, we determined the impact of these molecules in MRC-MZ B cell co-cultures supplemented with CpG DNA and appropriate blocking antibodies. These assays demonstrated that interruption of MAdCAM-1-$\alpha4\beta7$/VLA-4 or VCAM-1-VLA-4 but not ICAM-1-LFA-1 interactions attenuated MZ B cell differentiation into PBs-PCs and antibody production, without affecting MZ B cell survival (data not shown). Consistent with these data, studies published earlier show that VCAM-1 but not ICAM-1 from non-specific human splenic stromal cells enhances B cell antibody production induced by CD40L and cytokines[407]. Thus, we propose that MAdCAM-1 and VCAM-1

from human splenic MRCs not only preserve the structural organization of the MZ, but also facilitate homeostatic MZ B cell responses to commensal antigens.

MRCs Stimulate PB Survival through Contact-Independent BAFF Signals

A large body of evidence shows that non-hematopoietic stromal cells cooperate with hematopoietic cells to form functionally flexible PC survival niches in the BM[408]. In these niches, stromal cells deliver contact-dependent PC survival signals involving VCAM-1-VLA-4 interaction[28,100,409,410], fibronectin/hyaluronic acid-CD44 interaction[100], and CD80/CD86-CD28 interaction[411].

In the spleen, published data indicate that stromal cells from the RP promote PC survival via ICAM-1-LFA-1 interaction[287]. Similar to these stromal cells and those inhabiting the BM, human MRCs are likely to generate contact-dependent PC survival niches in both MZ and peri-MZ areas. Consistent with this possibility, MRCs strongly expressed VCAM-1, ICAM-1 and fibronectin, which have appropriate counter-receptors on splenic PBs and PCs. Experiments aimed at demonstating the involvement of these interaction in the survival of splenic PBs and PCs are currently ongoing, but preliminary data show that human splenic MRCs predominantly deploy contact-dependent signals to support the survival of splenic PBs-PCs and that these signals may include mBAFF expressed on the surface of MRCs.

In addition to contact-dependent signals, stromal cells from the BM deliver contact-independent PC survival signals involving BAFF, APRIL, IL-6 and CXCL12[412,413].

By culturing human splenic PBs-PCs with MRC-conditioned medium supplemented with BAFF-Ig or TACI-Ig, two decoy receptors that block BAFF or both BAFF and APRIL, respectively, we generated preliminary data suggesting that MRCs use BAFF and to a lesser extent APRIL to enhance splenic PB and PC survival. In agreement with published studies[287,363,385,414], more data indicate that MRC-derived IL-6 cooperates with PB and PC survival signals from BAFF and APRIL (not shown).

Thus, besides contributing to the inductive phase of MZ B cell responses by capturing and potentially exposing antigen and delivering MAdCAM-1 and VCAM-1 signals; MRCs may enhance the survival of MZ B cell-derived PBs and PCs by releasing BAFF and APRIL signals.

Clinical Implications

The protective role of the spleen and MZ B cells against encapsulated bacteria has been amply documented in mice. In humans, individuals that lack the spleen or have a defective MZ B cell function are at higher risk of pneumonitis, meningitis and fulminant septic syndrome caused by encapsulated bacteria. These pathogens include antibiotic-resistant strains that will require the development of novel vaccines. Achieving this goal will require a better understanding of the biology of MZ B cell responses.

In this study, we have elucidated the reactivity, clonal architecture and regulation of homeostatic MZ B cell responses against commensal bacteria. Tiny amount of commensals and their products physiologically translocate from mucosal surfaces to the spleen via the

general circulation, which may help MZ B cells to build a ready-to-use repertoire of protective antibodies.

Our findings indicate that human MZ B cells mount commensal-specific antibody responses clonally organized around MRCs. This macrophage-like stromal cell type inhabits the MZ and stimulates the activation, differentiation and survival of MZ B cells and their progeny of PBs and PCs, by expressing BAFF, APRIL and MAdCAM-1 and VCAM-1 adhesion molecules.

Given the relative lack of macrophages in the human MZ, our studies suggest that MRCs need to be taken into account in the design and development of novel vaccine strategies against encapsulated bacteria. Moreover, our work suggests that MRC targeting agents may be beneficial for the disruption of splenic niches supporting the survival of pathogenic PCs emerging during autoimmune disorders.

In healthy individuals, the spleen usually generates short-lived PBs and PCs that migrate to the bone marrow, where they acquire long-term longevity. However, the spleen of patients with autoimmune disorders such as ITP includes abnormally long-lived PCs that secrete pathogenic antibodies. In these patients, classical B cell-depleting therapies with B cell-depleting agents targeting the CD20 molecule do not consistently work. This is due to the lack of CD20 on long-lived and terminally differentiated PCs inhabiting the spleen. Our data indicate that biologicals capable to disrupt MRC survival niches, including antibodies to MAdCAM-1 and VCAM-1, may integrate conventional anti-CD20 therapies by attenuating the generation and viability of pathogenic splenic PCs.

Concluding Remarks

Our studies indicate that the human spleen contributes to the production of IgM and class-switched IgG and IgA antibodies specific for commensal antigens. These antibodies emerge under homeostatic conditions from MZ B cell responses clonally organized around a subset of MZ-based stromal cells, termed MRCs. Consistent with the virtual lack of macrophages in the human splenic MZ, MRCs may function as stromal innate response activator cells involved in the coordination of antigen-specific MZ B cell responses. Indeed, MRCs capture commensal antigens and express macrophage-like properties that may facilitate the initiation of antigen-specific antibody production by MZ B cells. Accordingly, MRCs deliver contact-dependent helper signals to MZ B cells through a mechanism involving MAdCAM-1 and VCAM-1 adhesion molecules. These signals may contribute to the induction of commensal-specific PB and PC responses via GC-independent pathways or GC-dependent pathways. In addition to PBs and PCs, the latter pathways generate ME B cells clonally affiliated to MZ precursors. Finally, MRCs may provide a nursing niche to PBs- and PCs emerging from MZ and ME B cells by delivering contact-dependent and contact-independent survival signals, including BAFF and APRIL. This multi-pronged activity likely permits MRCs to sustain homeostatic IgM as well as class-switched IgG and IgA responses to microbial products physiologically translocating from mucosal and skin surfaces. The resulting "natural" antibody repertoire may form a secondary line of systemic humoral defense against mucosal microbes crossing epithelial barriers undergoing inflammatory damage under pathological

conditions. Furthermore, pre-existing "ready-to-use" specificities emerging from MZ B cells may recognize highly conserved epitopes expressed by classical invasive pathogens, thereby helping their clearance.

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CHAPTER 6
CONCLUSIONS

1. The human spleen contributes to the induction of systemic IgM responses that recognize commensal antigens under homeostatic conditions.

2. Human splenic MZ B cells and PCs express IgM antibodies that recognize lipid and carbohydrate antigens from commensal organisms.

3. Human splenic MZ B cells locally generate clonally-related PCs expressing IgM or class-switched IgG and IgA throught GC-dependent and independent pathways.

4. Human splenic MZ B cells interact with an extensive network of stromal MRCs that combine stromal and macrophage-like properties, including expression of innate receptors involved in the sensing and uptake of microbes.

5. Human splenic MRCs up-regulate the expression of factors implicated in MZ B cell and PC activation following exposure to innate lymphoid or microbial signals.

6. Human splenic MRCs induce MZ B cell differentiation into antibody-secreting PCs through contact-dependent MAdCAM-1 and VCAM-1 signals and may also create a PC survival niche by releasing cytokines such as BAFF and APRIL.

7. In summary, MZ B cells generate a ready-to-use repertoire of commensal-targeting antibodies through a mechanism involving MRCs. Besides generating a secondary line of defence against commensals breaching epithelial barriers, these antibodies may help the early clearance of invasive pathogens.

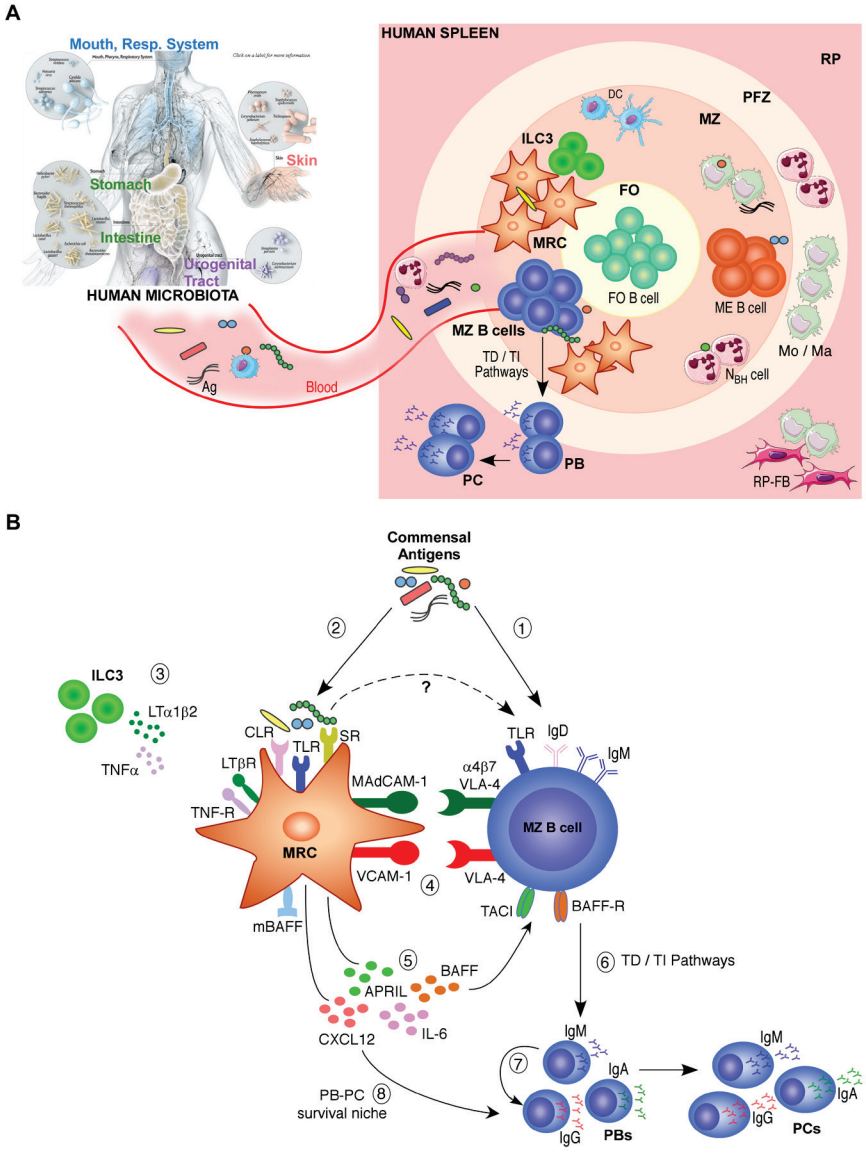

Figure 24. Human splenic MZ B cells mount PB-PC responses to commensal antigens clonally organized around MRCs. (A) The human spleen captures small amounts of commensal antigens that systemically translocate from mucosal and skin surfaces through elusive mechanisms. After traveling in the lymphatic and blood circulation as immunocomplexes, phagocytosed particles or chylomicron-embedded molecules, commensal antigens reach MZ and PFZ areas of the spleen inhabited by

MRCs, macrophages, N_{BH} cells and DCs. These stromal and myeloid cells may recycle captured antigen on the surface to engage specific BCRs expressed by MZ and ME B cells. Together with nonspecific signals from stromal, myeloid and lymphoid cells, including ILC3s, specific BCR signals stimulate MZ and ME B cell responses via intertwined TI and TD pathways. These responses generate clonally related PBs and PCs that secrete IgM as well as class-switched IgG and IgA to commensal antigens. In this manner, the spleen may mount a secondary line of systemic humoral defence against commensals breaching the mucosal barrier. These antibodies may also target highly conserved antigenic determinants expressed by invading pathogens. (B) Commensal antigens likely stimulate MZ B cells through the following direct and indirect mechanisms, which involve MRCs located in the MZ. (1) Antigen may engage BCRs, TLRs and complement receptors on MZ B cells. (2) Antigen may also engage TLRs, CLRs and SRs on MRCs, thereby promoting antigen sensing, capture and possibly presentation. (3) In MRCs, TLR ligands cooperate with ILC3-derived LTα1β2 and TNFα to increase MAdCAM-1 and VCAM-1 expression. (4) Engagement of α4β7 and VLA-4 by MAdCAM-1 and VCAM-1, respectively, activates MZ B cells locally primed by antigen, thereby inducing MZ B cell proliferation, PC differentiation and antibody production. (5) In addition to contact-dependent signals, activated MRCs deliver contact-independent activation signals, including BAFF and APRIL that engage BAFF-R and/or TACI on MZ B cells. (6) After receiving both antigen-specific and nonspecific signals, MZ (or ME) B cells enter TI and TD differentiation pathways that generate clonally affiliated PBs and PCs secreting commensal-specific IgM as well as class-switched IgG and IgA. (7) Besides occurring in MZ B cells, some class switching targets MZ B cell-derived PBs retaining the capacity to induce AID expression. (8) Finally, MRCs may generate a survival niche for MZ B cell-derived PBs and PCs through contact-independent and contact-dependent signals involving BAFF, APRIL, IL-6, CXCL12 and VCAM-1. Innate-lymphoid cells type 3, ILC3; marginal reticular cell, MRC; marginal zone B cell, MZ B cell; plasmablasts, PBs; plasma cells, PCs; Toll-like receptor, TLR; scavenger receptor, SR; C-type lectin receptor, CLR; mucosal-adressin cell adhesion molecule, MAdCAM-1; vascular-cell adhesion molecule, VCAM-1; lymphotoxin-beta receptor, LTβR; tumor necrosis factor-receptor, TNF-R; membrane-B-cell activating factor, mBAFF; B cell activating factor, BAFF; A proliferation inducing

ligand, APRIL; lymphotoxin-alpha1 beta2, LTα1β2; tumour-necrosis factor-alpha, TNFα; integrin alpha4 beta7, α4β7; very-late antigen-4, VLA-4; Transmembrane activator and CAML interactor, TACI; B-cell activating factor-receptor, BAFF-R; T-cell dependent, TD; T-cell independent, TI; Immunoglobulin, Ig.

ANNEX I

References

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ANNEX II

Publications

Jordi Sintes, Maurizio Gentile, Shuling Zhang, Yolanda Garcia-Carmona, Linda Cassis, Alessandra Ciociola, Giuliana Magri, Sabrina Bascones, Laura Comerma, Marc Pybus, David Lligé, Irene Puga, Emilie Grasset, Cindy Gutzeit, Bing He, Marta Crespo, Julio Pascual, Anna Mensa, Juan Ignacio Aróstegui, Manel Juan, Jordi Yagüe, Sergi Serrano, Josep Lloreta, Eric Meffre, Michael Hahne, Charlotte Cunningham-Rundles, Beverly A. Mock and Andrea Cerutti. The Metabolic Rheostat mTOR Drives Marginal Zone B Cell Responses by Interacting with the Antibody-Inducing TACI Receptor Via the Adaptor MyD88.

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Magri G, Miyajima M, Bascones S, Mortha A, Puga I, Cassis L, et al. Innate lymphoid cells integrate stromal and immunological signals to enhance antibody production by splenic marginal zone B cells. Nat Immunol. 2014 Apr 23;15(4):354–64. DOI: 10.1038/ni.2830