UNIVERSITY OF CALIFORNIA,
IRVINE

The Regulation of B Cells by N-glycosylation

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Sciences

by

Christie-Lynn Mortales

Dissertation Committee:
Professor Michael Demetriou, Chair
Professor Eric Pearlman
Professor Marian Waterman

2019

DEDICATION

To

my parents,

Junita and Michael Yoshimoto

my partner and best friend,

Nathan Aschenbach

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGMENTS

First and foremost, I would like to thank my thesis advisor, Dr. Michael Demetriou, for his expertise and guidance on this research and my scientific training. I greatly appreciate the high level of autonomy he granted me. The extensive knowledge and experiences I have gained is a testament of his mentorship.

I would like to thank my committee members, Dr. Marian Waterman and Dr. Eric Pearlman who have given time, effort, and especially support so willingly.

I would like to thank Barbara Newton. Without her, the lab would fall apart. She is always so attentive towards what we need so we could do our work efficiently and without complications.

I would like to thank my labmates throughout the years - Carey Li, Sung-Uk Lee, Michael Sy, Yanni Zhu, Raymond Zhou, Haik Mkhikian, Lindsey Araujo, and Ken Hayama. I am grateful to know such brilliant, kind, and humorous people. It has been a pleasure working with them all.

Thank you to the Department of Microbiology and Molecular Genetics in the School of Medicine for their guidance, support, and encouragement in navigating the graduate school process.

I am grateful for all the friends I have made at UCI over the years. Their comradery, empathy, and kindness means more to me than any words I can find to express my thanks. Thank you for all the wonderful memories.

I would especially like to thank my parents, Michael and Junita. Without their love and sacrifice, I would not have been able to accomplish so much.

And finally, I would like to thank my partner and best friend, Nathan Aschenbach. He has been my rock through the toughest times. I could not have earned this degree without his daily encouragement, understanding, and love.

Financial support was provided by the NIH Immunology Research Training Program at UCI (grant T32AI060573, 2014-2015), and the UCI Brython Davis Fellowship (2017).

Curriculum Vitae

Christie-Lynn L. Mortales

EDUCATION

2019 Ph.D. Biomedical Sciences
University of California, Irvine, CA

2010 B.S. Cellular, Molecular, and Developmental Biology
University of Washington, Seattle, WA

EMPLOYMENT

Sep 2010 – Sep 2012
Research Technician I, Program in Immunology, Clinical Research Division
Fred Hutchinson Cancer Research Center, Seattle, WA

PUBLICATIONS

Sung-Uk Lee, Carey Li, **Christie-Lynn Mortales**, Judy Pawling, James Dennis, Ani Grigorian, and Michael Demetriou. "Increasing cell permeability of N-acetylglucosamine via 6-acetylation enhances capacity to suppress T-helper 1 (TH1)/TH17 responses and autoimmunity." *PLOS ONE* 2019 March 26

Lindsey Araujo, Phillip Khim, Haik Mkhikian, **Christie-Lynn Mortales**, and Michael Demetriou. "Glycolysis and glutaminolysis cooperatively control T cell function by limiting metabolite supply to N-glycosylation." *eLife* 2017 January 6

Haik Mkhikian, **Christie-Lynn Mortales**, Raymond Zhou, Khachik Khachikyan, Gang Wu, Stuart M. Haslam, Patil Kavarian, Anne Dell, and Michael Demetriou. "Golgi self-correction generates bioequivalent glycans to preserve cellular homeostasis." *eLife* 2016 June 8

Jeffrey Chou, Matthew P Fitzgibbon, **Christie-Lynn L. Mortales**, Andrea M. H. Towlerton, Melissa P Upton, Raymond S Yeung, Martin W McIntoshm, and Edus H. Warren. "Phenotypic and transcriptional fidelity of patient-derived colon cancer xenografts in immune-deficient mice." *PLOS ONE* 2013 Nov 20;8(11):e79874

Jeffrey Chou, Lilien Voong, **Christie L. Mortales**, Andrea M. H. Towlerton, Seth M. Pollack, Xiaoji Chen, Cassian Yee, Paul F. Robbins, and Edus H. Warren. "Epigenetic modulation to enable antigen-specific T cell therapy of colorectal cancer." *Journal of Immunotherapy* 2012 Feb-Mar;35(2):131-41

HONORS & ACHIEVEMENTS

UCI Graduate Division "Grad Slam" Research Communication Competition, 3rd Place Winner – March 2018

UCI Brython Davis Fellowship – Spring 2017

UCI School of Medicine Professional Development Supplement for the Science Outside the Lab (SOtL) science policy immersion program in Washington D.C. – April 2017

UCI AGS Graduate Research Symposium Judge's Choice Award and Audience Choice Award – April 2017

American Association of Immunologist (AAI) Trainee Abstract Travel Award – March 2017

UCI School of Medicine Travel Award to attend the San Diego Glycobiology Symposium (February 3-4, 2017) – January 2017

UCI School of Medicine Travel Award to attend the American Association of Immunologist (AAI) Annual Meeting (May 13-17, 2016) – March 2016

NIH Immunology Research Training Program Fellow (grant T32AI060573) – Sep 2014-Aug 2015

NSF Graduate Research Fellowship Program, Honorable Mention – April 2014

CONFERENCE & RESEARCH PRESENTATIONS

Christie-Lynn Mortales and Michael Demetriou

"N-glycan branching inhibits pro-inflammatory B cell activity by controlling TLR4 and BCR signaling"

Immunology Fair, UCI. December 1, 2017 (Poster)

American Association of Immunologist (AAI) Annual Meeting, Washington D.C. May 12-16, 2017 (Podium & Poster)

San Diego Glycobiology Symposium, San Deigo. February 3-4, 2017 (Poster)

Christie-Lynn Mortales and Michael Demetriou

"N-glycosylation Regulates B Cell Peripheral Functions"

Immunology Fair, UCI. December 1-2, 2016 (Poster)

American Association of Immunologist (AAI) Annual Meeting, Seattle, WA. May 13-17, 2016 (Poster)

San Diego Glycobiology Symposium, San Deigo. March 25-26 2016 (Poster)

Immunology Fair, UCI. December 3-4, 2015 (Poster)

Immunology Fair, UCI. November 20-21, 2014 (Poster)

Jeffrey Chou, **Christie-Lynn L. Mortales**, Sheila Ojeaburu, Raymond S. W. Yeung, and Edus H. Warren

"Autologous T cell responses against patient-derived colorectal cancer xenografts"

American Association for Cancer Research Special Conference, Tumor Immunology: Multidisciplinary Science Driving Basic and Clinical Advances, Miami, FL. December 2-5 2012 (Poster)

Jeffrey Chou, Matthew Fitzgibbon, **Christie L. Mortales,** Martin McIntosh, and Edus H. Warren

"Evaluating the transcriptional fidelity of xenografted human colorectal cancers by RNA-seq"

20th Annual Cancer Research Institute Cancer Immunotherapy Symposium, New York, NY. October 1-3 2012 (Poster)

Jeffrey Chou, **Christie L. Mortales**, Raymond S. Yeung, and Edus H. Warren

"Phenotype and the T-cell receptor repertoire of colorectal cancer tumor infiltrating lymphocytes"

19th Annual Cancer Research Institute Cancer Immunotherapy Symposium, New York, NY. October 3-5 2011 (Poster)

Christie L. Mortales, Heon Park, Mark Tsang, and Brian Iritani

"Defining the Role of the Myc Oncoprotein in B Lymphocyte Development"

Undergraduate Research Symposium, UW. May 21, 2010 (Podium)

Northwest Louis Stokes Alliances for Minority Participation Program Conference, UW. February 26, 2010 (Poster)

Christie L. Mortales, Oscar Silva, and Carrie Miceli

"Ezrin Association to the i3 Domain of Dlgh1 in T cells"

HHMI Department of Biology Undergraduate Research Symposium, UW. October 16, 2009 (Poster)

UCR/CARE Summer Programs for Undergraduate and Graduate Research, UCLA. August 26, 2009 (Poster)

Christie L. Mortales, Heon Park, Mark Tsang, and Brian Iritani

"Analysis of the Role of the Myc Oncoprotein in Immunoglobulin E (IgE) Production"

Undergraduate Research Symposium, UW. May 15, 2009 (Poster)

Annual Biomedical Research Conference for Minority Students, Orlando, FL. November 5-8, 2008 (Poster)

UNDERGRADUATE RESEARCH EXPERIENCE

June – Aug 2009

Amgen Scholars Program under Dr. Carrie Miceli

Department of Microbiology, Immunology, and Molecular Genetics, UCLA

June 2008 – Sep 2010

NIH-NIGMS Initiative for Maximizing Student Diversity (IMSD) Program

Undergraduate Research Assistant under Dr. Brian Iritani

Department of Comparative Medicine, University of Washington

CO-CURRICULAR ACTIVITIES

Dr. Mai Khanh Tran for Congress 2018 Campaign – Political Consultant, Feb-April 2018

Conducted policy research and analysis to inform and advise the candidate

Field operations lead (recruiting, messaging/communication, etc.)

Arizona State University Consortium for Science, Policy & Outcomes (CSPO) Science Outside the Lab Program, June 2017

Networked with federal agencies, NGOs, think tanks, and the press in Washington D.C.

Strengthened science communication skills

UCI Public Policy Prep (P3) Program, 2017-2018

Identify, invite, and co-host speakers for workshops and panels; coordinate and participate in program sessions and events.

UCI Science Policy Group, 2016-Present

Social & Events Coordinator, 2017-2018
Member, 2016-Present

UCI Reach Out Teach Out, 2017-2018
Treasurer, 2017-Present

UCI Graduate Professional Success (GPS)-BIOMED program, 2014-2018
Participate in professional development courses and events, including science communication training and mentoring programs

Orange County Asian and Pacific Islander Community Alliance, October 2013-2014
Tutor high school students in science, math, and college application preparation

UW Filipino American Student Association est. 1917
Vice President, 2009-2010
Activities Chair, 2008-2009
Official Member, 2006-2008

ABSTRACT OF THE DISSERTATION

The Regulation of B Cells by N-glycosylation

By

Christie-Lynn Mortales

Doctor of Philosophy in Biomedical Sciences

University of California, Irvine, 2019

Professor Michael Demetriou, Chair

Eukaryotic cells employ vast and intricate molecular mechanisms to carry out biological functions that help them thrive. One such mechanism is the dynamic movement and reorganizing of the plasma membrane in order to facilitate inter- and intra-cellular events via receptor and/or ligand engagement. The plasma membrane is surrounded by a very dense layer of sugars, or glycans, which extend from glycolipids and glycoproteins, and can interact with galectins, a family of sugar-binding proteins. These interactions form molecular networks called galectin-glycoprotein lattices that function to control the localization, clustering, and retention of glycoproteins to globally regulate cell activation, growth, arrest, and differentiation.

Glycoproteins are built in the endoplasmic reticulum and the Golgi apparatus, in which proteins are folded and then extensively modified with glycans before transport to the cell surface. In the Golgi, the N-acetylglucosaminyl transferases (Mgat1, 2, 4, and 5) mediate the branching of Asparagine (N)-linked glycans in an “assembly line” manner to progressively increase the production of ligands for galectins. More N-glycan branching increases galectin avidity to maintain lattice integrity, cellular homeostasis, and

appropriate functional outcomes. Less N-glycan branching reduces galectin avidity, weakens the lattice, and promotes cellular dysfunction and disease states.

We have shown the galectin-glycoprotein lattice and the N-glycan branching pathway are critical regulators of T cells. An intact lattice on T cells promotes basal TCR signaling and appropriate activation states. Furthermore, the lattice inhibits pro-inflammatory T_H1/T_H17 differentiation, and promotes humoral/immunomodulatory T_H2 and anti-inflammatory iT_{REG} differentiation. When the lattice is weakened due to reduced N-glycan branching, T cells become hyperactive and more pro-inflammatory, exacerbating autoimmunity (i.e., EAE and MS).

How the galectin-glycoprotein lattice and N-glycan branching regulates B cells has not been shown before. We provide evidence N-glycan branching promotes B cell generation, differentially regulates CD19/BCR and TLR surface expression and signaling, and affects antigen presentation to $CD4^+$ T cells to influence T_H1, T_H17, and iT_{REG} differentiation. Furthermore, reduced N-glycan branching in B cells resulted in more severe EAE progression. These studies have begun to delineate how the galectin-glycoprotein lattice regulates B cell homeostasis, and may have implications in B cell targeting therapies.

Chapter 1

Introduction

Portions of this chapter are taken from:

Nemazee, D. Mechanisms of central tolerance for B cells. *Nat Rev Immunol* **17**, 281-294 (2017).

Yang, L., and Seki, E. Toll-Like Receptors in Liver Fibrosis: Cellular Crosstalk and Mechanisms. *Frontiers in Physiology* **3** (2012).

The immune system is our ultimate line of defense against harmful invaders (i.e., pathogens) like bacteria, viruses, fungi, and parasites. It is an army of various cell types that communicate and interact with each other, along with other tissues, as they survey the body for invaders, and launch concerted, defensive attacks when necessary in order to clear infections. Generally, the innate side of the immune system quickly delivers nonspecific defense to control an infection, while the adaptive side ramps up to execute a strong and precise response to eliminate the invaders. Unique among the immune cells, the B cell harbors both innate and adaptive immune functions, putting them at the interface of critical immune outcomes.

Increasing evidence demonstrates glycosylation as an indispensable regulator of cellular functions. Our group has extensively demonstrated how cell surface N-glycosylation, specifically N-glycan branching, and the galectin-glycoprotein lattice serve as essential mechanisms of regulating T cell basal signaling, activation, growth arrest, metabolism, and thymic development. However, almost nothing is known about the role of N-glycan branching and the galectin-glycoprotein lattice in B cell biology. Here, we have begun to elucidate the importance of N-glycan branching in B cell central tolerance (i.e., positive selection), peripheral functions, and autoimmunity. The focus of this dissertation is to share these discoveries and ideas for future research.

A Brief History

B cells are generally defined as a group of cells that can detect antigens via clonally diverse cell surface immunoglobulins (Ig) serving as B cell receptors (BCR). They originated more than 500 million years ago during the evolution of adaptive

immunity in jawed vertebrates.[1] In 1938, it was first the identification of antibodies (i.e., Ig) in serum that began the path towards the discovery of B cells, and ten years later, plasma cells were identified as the producers of antibodies.[2,3] B and T cells were discovered around the same time in 1965 with identification of functionally distinct lymphoid systems from chicken bursa and thymus (signifying their respective names), the former generating cells as antibody producers and the latter necessary for cell-mediated responses.[4,5] Transplantation models in mice showed cells derived from bone marrow became the generators of antibody responses.[6,7] In 1971, the developmental link between antibody production and B cells in humans was revealed when normal and leukemic B cells were distinguished using differential surface Ig expression.[8] Using cell surface markers to characterize malignant B and T cells from these studies began the classification of blood cancers.[8-10] Today, immunophenotyping methods remain as some of the most sophisticated and effective diagnostic and research tools.

Since their discovery, extensive research has been done to delineate the processes of B (and T) cell development in mice and humans, which has demonstrated somatic rearrangement of the Ig (and T cell receptor (TCR) α/β) loci by the recombination activating genes 1/2 (*RAG-1/2*) as the mechanism for generating B (and T) cell receptors.[11-14]

B Cell Development and Selection

Generating B Cell Central Tolerance

T and B cells undergo similar developmental events and checkpoints that involves the dynamic expression of transcription factors, response to extracellular

environmental cues such as cytokines, and the somatic rearrangement, expression, and screening of their antigen receptors in order to generate a functionally diverse, self-tolerant lymphocyte repertoire.[15-18] Abnormalities in lymphocyte development and selection give rise to certain congenital immunodeficiencies, leukemias/lymphomas, and autoimmune diseases.[1] In the bone marrow, B cell development begins when a common lymphoid progenitor (CLP) cell becomes a progenitor B (pro-B) cell which are committed to the B cell lineage.[19] With the expression of the RAG enzymes, rearrangement of the variable (V), diversity (D) and joining (J) gene segments in the μ heavy (H)-chain locus begins, where successful VDJ recombination drives H-chain protein expression. The H-chain proteins pair with the surrogate light (L)-chain which becomes expressed as the pre-BCR on the surface of what are now large pre-B cells.[19,20] Interleukin 7 receptor (IL-7R) signaling supports pro-B cell survival, the pro-B to large pre-B cell transition, and large pre-B cell proliferation, whereas attenuation of IL-7R signaling leads to cell cycle arrest and induction of RAG gene expression for L-chain rearrangement.[21-23] The pre-BCR does not signal on its own and must form complexes with Igα and Igβ (also known as cluster of differentiation (CD)79A and CD79B) transmembrane proteins, which have cytoplasmic tails containing tyrosine phosphorylation sites within immunoreceptor tyrosine-based activation motifs (ITAMs), for signal transduction.[24,25] Once large pre-B cells enter a low IL-7 niche and IL-7R signaling diminishes, proliferation stops and they become small pre-B cells where pre-BCR signaling induces V and J segment rearrangement from one of two L-chain loci, κ or λ.[21-23] Successful L-chain VJ recombination, expression, and pairing with μ H-chain in

small pre-B cells results in surface bound IgM expression and complex formation with Igα and Igβ to form the BCR on what are now immature B cells.[26-28]

At this point, surface bound IgM can bind antigens; with the likeliness of immature B cells encountering self-antigens in the bone marrow being very high, the B cell employs several regulatory processes to reduce self-reactivity and control central tolerance. Here, BCR ligation directs immature B cell fate – when the BCR is engaged by self-antigen, immature B cells die by clonal deletion (negative selection by apoptosis) or undergo receptor editing (continuous L-chain rearrangement) to reduce/eliminate autoreactivity.[29] Alternatively, immature B cells expressing a self-tolerant BCR continue through transitional developmental stages (T1 and T2) in the bone marrow and the spleen, but mostly continue maturation into follicular (FO) or marginal zone (MZ) B cells in the spleen and co-express surface bound IgM and IgD.[30] Evidence also suggests about 25% of developing B cells can mature into FO B cells in the bone marrow.[31] In the periphery, mechanisms such as anergy, receptor desensitization, or antigen co-engagement with sialic acid-binding immunoglobulin-like lectin (Siglecs) regulate peripheral tolerance of mature B cells.[32-35]

B Cell Positive Selection via Differential CD19/BCR Signaling Pathways

When it comes to thymic T cell development and selection, it has been thoroughly demonstrated double positive (DP) T cells require modest self-peptide-major histocompatibility complex (MHC) to TCR engagement to reach a threshold of tonic TCR signaling permissive for positive selection and transition into mature single positive (SP) $CD4^+$ or $CD8^+$ T cells.[36] However, BCR engagement for immature B cell positive

selection remains controversial with the most/strongest evidence suggesting positive selection of immature B cells requires an unengaged BCR[29]. Here, it is non-self-reactive BCR that imparts tonic signaling to drive developmental progression and survival of immature B cells in a phosphoinositide 3-kinase (PI3K) dependent manner.[25,29] BCR signaling is summarized in Figure 1.1. It has been suggested BCR engagement induces negative selection by impeding PI3K signaling while promoting the BLNK pathway.[29] This leads to BCR internalization and hindered AKT activity, which promotes transcription factor forkhead box protein O1 (FOXO1) nuclear translocation to drive continued RAG expression (for receptor editing) and developmental arrest of immature B cells. Alternatively, an innocuous BCR promotes basal tyrosine phosphorylation of the co-receptor CD19 by Src family kinases (SFKs), promoting PI3K/AKT activity, inhibiting FOXO1 nuclear translocation, turning off RAG expression and ceasing κ/λ L-chain recombination necessary for developmental progression; PI3K/AKT also activates the transcription factor, MYC, for cell survival.[29,37,38] For positive selection, CD19 has been shown to play a significant role.

CD19 is a transmembrane glycoprotein first expressed in pro-B cells in the bone marrow during B cell development.[11,39] It has been proven to be a critical BCR co-receptor as CD19 knockout mice have greatly reduced B cell numbers, impaired B cell function, and defective immune responses.[40,41] For immature B cells, lack of CD19 results in loss of basal PI3K signaling which continued RAG expression and L-chain receptor editing, thus inhibiting positive selection.[42] Phosphatase and tensin homologue (PTEN) opposes PI3K activity; subsequent studies demonstrated absence of PTEN activity reversed the effect of CD19 loss and promoted immature B cell positive

selection.[43,44] As CD19 is expressed in pre-B cells, it has been demonstrated to be required for pre-BCR mediated positive selection as well.[25,45] Holistically, CD19 augments BCR signaling at multiple points during B cell development, maturation, and differentiation.[45-47] CD19 also sustains tonic signaling necessary for the survival of naïve recirculating B cells, and can function as a signaling hub to crosstalk with multiple B cell receptors (e.g., TLRs, CD40, cytokine receptors) and influence B cell effector functions.[48-50]

BCR Signaling Strength in B Cell Selection and Maturation

Tonic BCR signaling is necessary for immature transitional T1 and T2 B cells in the bone marrow and spleen, whereas follicular (FO) and marginal zone (MZ) B cell maturation in the spleen is, in part, influenced by self-reactivity and BCR signaling strength.[30] Strong BCR signaling drives FO B cell maturation, while modest BCR signaling drives MZ B cell maturation. Most mature B cells are recirculating FO B cells (>75%), reside in follicles adjacent to T cell zones, mount robust T cell-dependent responses to protein antigen, and have antigen presenting cell (APC) capabilities.[30,51] FO B cells also recirculate through the bone marrow where they can mount T-independent responses to blood-borne pathogens.[52] MZ B cells are the minority of the splenic B cell population (~15%), reside and are retained in the outer white pulp of the spleen, and respond vigorously to blood-borne pathogens in a T-independent manner. MZ B cells have characteristics similar to memory B cells in that they have pre-activated qualities and can self-renew.[53]

A general model for how the spectrum of BCR signaling strength influences B cell development, selection, and maturation from low to high can be interpreted in this way: death by neglect (no signaling; loss of CD19/BCR/PI3K) < immature/T1/T2 (tonic CD19/BCR/PI3K signaling) < MZ (modest BCR self-reactivity) < FO (strong BCR self-reactivity) < receptor editing or anergy < death by negative selection (extreme BCR self-reactivity). Defining distinct boundaries between these BCR signaling requirements and their outcomes has been a challenge. However, our understanding is being improved as we assess how and when other extrinsic and intrinsic factors cooperate with BCR signaling to influence B cell homeostasis and activation.

B Cells in Immune Responses

Humoral Immunity

While not a focus of this dissertation, humoral immunity will be briefly described. B cells are champions of the humoral immune response as they are the sole creators of infection fighting antibodies. Upon infection or immunization, humoral immunity can be generated through the germinal center (GC) reaction in secondary lymphoid organs (i.e., spleen, lymph nodes, Peyer's patches).[54] Here, class switch recombination (CSR) and somatic hypermutation (SHM) of immunoglobulin genes result in the progressive selection of high-affinity diversified antibodies secreted by short-lived plasmablasts, long-lived plasma cells, and memory B cells.[54] The GC reaction is namely T cell-dependent (TD) and begins when resting B cells identify and extract antigen onto their BCRs from follicular dendritic cells (FDCs).[55] B cells internalize and process the antigen, migrate to the border between the B cell follicle and T cell zone, and present antigen

peptide-MHCII (pMHCII) to $CD4^+$ helper T (T_H) cells which then provide B cells CD40 co-stimulation via CD154.[56,57] This induces the activation and rapid proliferation of B cells, followed by an iterative process of CSR and SHM regulated by activation-induced cytidine deaminase (AID) to select for high-affinity BCRs and eliminate low-affinity ones.[54] B cells expressing a high-affinity receptor exit the GC to differentiate into memory B cells or terminally differentiated plasma cells which secrete large amounts of antibody.[57] Once antibody encounter their antigen on pathogens or infected cells, their functions include neutralization, inducing macrophage phagocytosis, antibody-dependent cellular cytotoxicity, and complement-mediated elimination.[58]

Toll-Like Receptor Activation of B Cells

B cell activation and humoral immunity is also induced in a T cell-independent (TI) manner. While TD antigens are typically soluble proteins, or haptens conjugated to proteins, TI antigens are diverse and often comprised of repetitive epitopes.[59] B cells sense TI antigens through a family of pattern-recognizing receptors (PRRs), the toll-like receptors (TLRs), which respond to various pathogen associated molecular patterns (PAMPs) like bacterial cell wall components, and bacterial or viral DNA and RNA.[60] TLR signaling is summarized in Figure 1.2. Mouse B cells express TLR1, 2, 3, 4, 6, 7 and 9 at various levels across different subsets.[61] Lipopolysaccharide (LPS) is a potent mouse B cell mitogen, inducing activation via TLR4[62], and has been a useful tool in studying B cell activation and functional outcomes including antibody and cytokine production. TLR expression in human naïve B cells is low, whereas activated and memory B cells express TLR1, 2, 6, 7, and 9 at various levels.[63-65] TLRs can also detect endogenous

damage-associated molecular patterns (DAMPs) released by damaged or dying cells during inflammation. For example, nuclear histone and high mobility group box 1 (HMGB1) proteins can trigger TLR2 and TLR4 on B cells, as well as myeloid cells.[66,67] Evidence implicates DAMPs like HMGB1 play a significant role in chronic inflammation and autoimmune diseases like rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and multiple sclerosis (MS).[68,69]

B Cells as Antigen Presenting Cells (APCs) and Cytokine Producers

While B cells are best known for their antibody producing functions, they also influence immune responses via their APC and cytokine secreting capabilities. B cell proliferation, germinal center antibody affinity maturation, and engagement with $CD4^+$ T_H cells is proportional to the volume of antigen acquired and presented on MHCII.[70,71] Furthermore, generation and survival of memory $CD4^+$ T_H cells is suggested to be reliant on B cell antigen presentation.[72] In order to present antigen as an APC, B cells must first acquire antigen, usually from a different APC (i.e., FDCs).[73] After antigen induced BCR clustering occurs, an immunological synapse forms with the APC in which B cells extract antigen for internalization via a combination of mechanical force and secretion of lysosomal proteases and lipases.[74-76] Endocytosed antigen are intracellularly trafficked to antigen-processing compartments for proteolytic degradation, yielding antigen peptide which is loaded onto MHCII; pMHCII carrying vesicles are then trafficked to the cell surface where they associate with TCRs on $CD4^+$ T_H cells.[77] Accompanying pMHCII-TCR engagement, B cells provide co-stimulation via CD80 and/or CD86. CD80 and CD86 are part of the B7 family of co-stimulatory ligands which

interact with CD28/CTLA-4 co-activating/inhibitory receptors on T cells.[78] On B cells, CD86 is induced early and rapidly, while CD80 is induced later and more slowly.[79,80]

B cells harbor both pro- and anti-inflammatory roles as cytokine secretors. They can produce IL-12, IL-6, tumor necrosis factor (TNF)α, and lymphotoxin (LT)α to drive T_H1/T_H17 cell-mediated responses, IL-4 to drive T_H2 humoral responses, and IL-10 and transforming growth factor (TGF)β to drive anti-inflammatory T_{REG} responses.[81] B cell derived cytokines can also influence dendritic cell and macrophage activation and function.[82] Overall, B cells play a pivotal and multifaceted role in influencing the dynamics of a range of immune responses, making them a popular target for development of immunomodulatory therapies.

B Cells in MS and EAE

MS is the most common autoimmune disease of the central nervous system, characterized by inflammatory demyelination and neurodegeneration[83]. Long-standing evidence from animal models of MS, particularly experimental autoimmune encephalomyelitis (EAE), implicate $CD4^+$ T cells and their differentiation into pro-inflammatory T_H1 and T_H17 cells as predominant contributors in inflammatory demyelination[84,85]. T_H1 and T_H17 responses are also observed in MS patients[86,87]. Genetic analysis in MS signifies immune system involvement in promoting disease development, including T cell growth factors (IL-2, IL-7 pathways) and their differentiation into pro-inflammatory T_H1 and T_H17 cells versus anti-inflammatory T regulatory cells.[88-92] The strongest genetic risk factor for MS are variants of the molecules that present antigen to T cells, namely the human leukocyte antigen (HLA)

complex.[88,93] Moreover, the dominant role of T cells in MS has been challenged by the powerful therapeutic activity of B cell depleting therapies in MS[94], with one of the earliest clinical trials demonstrating mouse-human chimeric anti-CD20 monoclonal antibody rituximab virtually eliminating brain inflammatory lesions in relapse-remitting (RR) MS patients.[95] More recently, the newer and humanized anti-CD20 monoclonal antibody ocrelizumab is shown to be superior than commonly prescribed high-dose interferon-β-1a (Rebif) at reducing relapse rates in RRMS, as well as improving prognosis in progressive MS.[96,97]

The therapeutic benefit of B cell depletion in MS has stirred up immense curiosity amongst immunologists, who are attempting to better define B cell mechanisms of action in the disease. B cell aggregates have been found in ectopic lymphoid structures (ELSs) in the meninges of MS patients, suggesting B cells help form and/or perpetuate localized inflammatory lesions in the CNS.[98,99] Aberrant humoral responses in MS have been implicated for decades, and the presence of cerebrospinal fluid (CSF) antibody oligoclonal bands is used as a diagnostic[100]. Neuronal myelin targeting autoantibodies in CSF have been identified, and antibody mediated complement activation associated with myelin membrane disintegration observed in inflammatory lesions.[101-103] B cell depleting anti-CD20 therapy, however, does not seem to affect antibody production, evidenced by unchanged IgG concentration, IgG index, IgG synthesis rate or oligoclonal band number in the CSF of treated MS patients.[95,104] Thus, it is B cell antigen presentation to $CD4^+$ T cells that is suggested to predominantly drive autoimmunity in MS, as anti-CD20 therapy reduces pro-inflammatory T_H1 and T_H17 responses as well as B and T cell numbers in CSF.[95,104,105] Results from EAE supports this conclusion,

with B cells promoting T_H1 and/or T_H17 responses as well as inflammatory demyelination via their APC and cytokine secreting function rather than through antibody production.[106-110] B cells from MS patients were demonstrated to produce more pro-inflammatory TNFα and LTα in response to TLR9 agonist or T_H1 derived IFNγ.[111] B cells may also play an important role in MS via TLR4 and/or TLR2 signaling mediated alterations in anti-inflammatory IL-10 production[112-114], a cytokine utilized by B cells to inhibit autoimmunity.[115]

The N-glycan Branching Pathway

Essentially all eukaryotic cell surfaces are surrounded by a thick (~100 nm) molecular network of sugars, or glycans, and sugar binding proteins.[116] This structure is called the glycocalyx, is ten times wider than the plasma membrane, and contains a dynamic mixture of glycolipids, glycoproteins, and proteoglycans. The glycan structures covalently attached to glycoproteins vary significantly in number, size, and complexity, yet in contrast to the genome and transcriptome, the glycome is not template driven.[117] Increased understanding of the influence glycans have in intra- and inter-cellular interactions is continuing to provide researchers insight on the unique role of the highly heterogenous glycome in biology and diseases.

The majority of key cell surface receptors, transporters, and ligands are co- and post-translationally modified with asparagine (N)-linked and serine/threonine (O)-linked glycans. Cell surface N-glycans serve as ligands for various multivalent carbohydrate binding protein families, including galectins, Siglecs, and selectins, all of which are important in immune cell responses. N-glycan biosynthesis begins in the endoplasmic

reticulum (ER) and continues through the Golgi apparatus where it's controlled by the activity of glycohydrolases and glycosyltransferases, as well as the availability of nutrients and supply of metabolic substrates.[118,119] N-glycan branching is depicted in Figure 1.3. The transfer of a pre-assembled glycan, $Glc_3Man_9GlcNAc_2$, to the asparagine residue of Asn-X-Ser/Thr (NXS/T, X≠P) motifs by oligosaccharyltransferase in the ER initiates the process. After trimming two glucose (Glc) units, calnexin and calreticulin bind to $Glc_1Man_9GlcNAc_2$, and the remaining Glc residue is recycled until folding is complete[120]. The glycan is then extensively modified by the removal of mannose (Man) residues and addition of N-acetylglucosamine (GlcNAc) units during transit through the Golgi. Here, the N-acetylglucosaminyltransferase enzymes (Mgat1, Mgat2, Mgat4a/b, and Mgat5) act in a sequential order to initiate branching by adding GlcNAc from uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) onto Man residues, forming N-glycan intermediates. GlcNAc branches can be extended with a galactose (Gal) by galactosyltransferase-3 (GalT3) to form N-acetyllactosamine (LacNAc) units, a disaccharide that is the binding ligand for galectins. Branches can be repeatedly extended to give poly-LacNAc via alternating action of β1,3-N-acetylglucosaminyltransferase-2 (β3GnT2, or "little I" iGnT) and GalT3 enzymes, terminally capped with sialic acid, or modified with fucose and/or sulfate.[118-122] Plant lectins are useful tools in gauging N-glycan levels. For example, phaseolus vulgaris leucoagglutinin (L-PHA), binds the product of Mgat5 (i.e., β1,6 branched N-glycans), and is used to measure degree of branching. Additionally, lycopersicon esculentum agglutinin (LEA) binds poly-LacNAc units (i.e., three linear LacNAcs) and is used to assess extension of branches.

The importance of the N-glycan branching pathway is exemplified in the manifestation of N-glycan deficiency outcomes. Notably, germline deletion of Mgat1 is embryonic lethal at day 9 in mice.[123] Humans with homozygous recessive mutations in the Mgat2 catalytic domain have what is named congenital disorder of glycosylation type IIa (CDG-IIa) that results in a childhood disease displaying dysmorphic facial features, feeding difficulties, and mental retardation.[124] Many groups continue to study N-glycan deficiencies in different biological contexts. Our group investigates how N-glycan branching abnormalities affect immune cell function and the outcome of autoimmune diseases. A primary focus of our work is on how altering N-glycan branching ultimately affects the integrity of the galectin-glycoprotein lattice.

The Galectin-Glycoprotein Lattice

Galectins are a ubiquitously expressed group of sugar binding proteins that are generated in the cytosol and secreted to the cell surface via a non-classical pathway.[125] Each galectin has a single carbohydrate recognition domain (CRD) which binds LacNAc units on glycans.[126] They form several quaternary protein structures – prototypical homodimers, tandem-repeat dimers, and chimeric type oligomers. Galectin-3, which is heavily studied in the context of immunity, is the only chimeric type galectin and usually exists as a pentamer.[127] Galectin-glycoprotein interactions lead to the formation of microdomains, or molecular “lattices”, on the cell surface.[125,127,128] This functions to regulate receptors via their membrane localization, lateral movement, clustering, and surface retention.[128-138] Thus, the galectin-glycoprotein lattice controls the steady-state, activation, growth arrest, and differentiation of cells by facilitating ligand-receptor

sensitivity in order to affect signaling strength. In immunity, mounting evidence has established galectins as important regulators of immune cell homeostasis, inflammation, cancer, and autoimmunity.[139-142]

The stability of the galectin-glycoprotein lattice is established by both genetic and metabolic factors yielding binding avidities between galectins and glycoproteins which is proportional to LacNAc quantity on the attached glycans. This is determined by the number of available and occupied N-glycan sites (i.e., genetically encoded NXS/T motifs, X≠P), the degree of N-glycan branching, and/or poly-LacNAc extended branching.[117,129,143] The degree of branching and poly-LacNAc extensions are dependent on the expression and efficiency of Golgi enzymes (i.e., Mgat1-5, β3GNT/GalT) and availability of their respective substrates (e.g., UDP-GlcNAc supplied by the hexosamine pathway). More branching/poly-LacNAc increases galectin binding avidity and the integrity of the galectin-glycoprotein lattice. Conversely, the lattice is weakened when fewer branches/poly-LacNAc are present, and this dysregulates receptor signaling due to enhanced clustering and/or altered surface retention which contribute to disease states.[117,125,127-131,135,144-148]

The role of both galectins and glycosylation have been focus of intense study. Considering galectins are a family of 15 proteins, and the number and variation of glycans is extremely vast, compensatory/redundant functions are expected among both. Nonetheless, when examining *in vivo* phenotypes, interpretations of results for studies on galectins are complicated by whether outcomes are cell intrinsic or extrinsic because galectins are secreted and free to diffuse in extracellular space. The advantage of studying N-glycosylation is that it is cell intrinsic as glycans are covalently attached to

the cell. While progress has been made in characterizing how specific glycoproteins interact with the lattice, determining the effects of N-glycan branching on every individual glycoprotein for every cell type would be a long and challenging endeavor. Therefore, we investigate, and are able to appreciate, how N-glycan branching holistically sets lattice thresholds to deliver concerted consequences for cellular outcomes.

N-glycan Branching Regulates T Cells

T cell activation, proliferation, and inhibition must be carefully controlled to maximize efficacy while preventing aberrant immune responses. TCR complex signaling above a certain threshold is determined by concurrent peptide-MHC (pMHC) and CD28 co-receptor engagement at the immune synapse.[149] We have reported on how the galectin-glycoprotein lattice regulates basal, activation, and arrest signaling to control T cell growth, block pro-inflammatory T_H1/T_H17 differentiation, and promote humoral/immunomodulatory T_H2 and anti-inflammatory iT_{REG} differentiation.[150] Multivalent galectin binding to the N-glycans of the TCR complex inhibits spontaneous clustering of TCRs to prevent unwarranted T cell activation and growth.[128,131,151] However, mice deficient in the Golgi N-glycan branching enzymes Mgat5, Mgat2, Mgat1, or in poly-LacNAc generating β3GnT2 have reduced thresholds to TCR clustering in proportion to their loss of LacNAc ligand, hyperactivating T cells and promoting autoimmune disease.[128,143,152] Similarly, removal of N-glycan encoded sites in the TCR results in increased TCR diffusion, clustering, and activation.[153] In this manner,

N-glycan branching proportionally defines the limits of affinity from which pMHC-TCR interactions trigger T cell activation.

Inhibitory regulators of the T cell response are critical for T cell growth arrest, differentiation and self-tolerance.[149] Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) is an inhibitory receptor that competes with CD28 for CD80/CD86 co-stimulatory ligand on antigen presenting cells (APCs) and is induced to the cell surface 4-5 days after T cell activation to initiate growth arrest.[154] Transcription levels and intracellular stores of CTLA-4 protein increase with TCR signaling during early T cell growth, but endosomal trafficking limits CTLA-4 surface expression and, therefore, it is predominantly located in endosomes. Galectin binding to branched N-glycans on CTLA-4 enhances surface retention by opposing endocytic loss of CTLA-4, increasing and sustaining growth arrest signaling.[129]

Increasing TCR signal strength promotes pro-inflammatory T_H1 and inhibits humoral/immunomodulatory T_H2 differentiation.[155,156] Under neutral conditions (i.e., absence of exogenous polarizing cytokines), N-glycan branching induces differentiation of $CD4^+$ T cells into T_H2 (IL-4 secreting) cells while suppressing T_H1 (IFNγ secreting) differentiation.[157] Furthermore, N-glycan branching is essential for the induction of immunosuppressive T_{REG} cells, with loss of branching (i.e., Mgat1 deletion) virtually eliminating the ability of $CD4^+$ T cells to differentiate into T_{REG} cells.[158] Here, N-glycan branching drives iT_{REG} differentiation by promoting IL-2Rα (or CD25) surface expression and signaling in $CD4^+$ T cell blasts. N-glycan branching is also a critical suppressor of pro-inflammatory T_H17 differentiation.[158] We found T_H17 inducing cytokines (TGFβ+IL-6+IL-23) markedly down-regulated branching in $CD4^+$ T cell blasts, consequently

lowering IL-2Rα surface expression and associated IL-2 signaling to drive T_H17 over iT_{REG} differentiation. Notably, reversing the lowered branching caused by T_H17 induction with GlcNAc supplementation not only blocks T_H17 differentiation, but induces a cell fate switch to iT_{REG} by restoring IL-2Rα (CD25) surface retention and signaling.[158] Consistent with being a critical negative regulator of pro-inflammatory T cell function, N-glycan branching deficiency promotes neuroinflammation in mouse models[128,146,147,159] and humans with multiple sclerosis[160-162].

N-glycan Branching in T Cell Positive Selection

As previously mentioned, T cells develop similarly to B cells – they undergo developmental checkpoints involving the rearrangement, pairing, and expression of the TCR α/β (and to a lesser extent, γ/δ) loci.[163] T cell development begins when common lymphoid progenitor (CLP) cells migrate to and seed the thymus. They continue through four $CD4^-CD8^-$ double negative (DN) stages (DN1-DN4) prior to becoming $CD4^+CD8^+$ double positive (DP) thymocytes that express surface TCR which are screened for reactivity against self-pMHC.[164] DP thymocytes either die by neglect when lacking/insufficient TCR engagement occurs, or negative selection when TCR engagement is too strong – thus, intermediate TCR signaling strength allows DP thymocytes to be positively selected and mature into single positive (SP) $CD4^+$ or $CD8^+$ T cells.[164]

Zhou et al. from our group demonstrated N-glycan branching regulates thymic positive selection by bi-directionally defining the boundaries of pMHC-TCR affinity.[151] Using a T cell specific (Lck driven cre) knockout of Mgat1, we observed reduced thymic

and splenic T cell numbers due to enhanced cell death. Mgat1 deletion enhanced thymocyte death by neglect resulted from decreased CD4/CD8 co-receptor surface retention (i.e., enhanced CD4/CD8 endocytosis) and reduced Lck induced Erk signaling, which are important for augmenting low affinity TCR engagement. N-glycan deficient thymocytes simultaneously exhibited increased death by negative selection due to excessive Ca^{2+} flux driven by enhanced TCR clustering. In summary, N-glycan branching provides a mechanism for decoupling CD4/CD8 co-receptor and TCR signaling to maintain the appropriate range of TCR complex signal intensity necessary for thymocyte positive selection and generation of functional circulating $CD4^+$ and $CD8^+$ T cells.

Regulation of B cells by Glycosylation and Galectins

Glycosylation and Galectins in B Cell Development, Tolerance, and Maturation

The sialic acid-binding immunoglobulin-type lectin (Siglec) and inhibitory co-receptor, CD22, has been shown to have a role in B cell maturation and tolerance. β-galactoside α-2,6-sialyltransferase 1 (ST6Gal1) adds sialic acid to terminal galactose residues on N-glycans, a ligand for CD22.[165] Ligand *cis* interactions mask CD22, but when its ligand is absent due to ST6Gal1 deficiency, CD22 downregulates BCR signaling by recruiting and activating Src homology region 2 domain-containing phosphatase-1 (SHP-1) to its immunoreceptor tyrosine-based inhibition motifs (ITIMs).[166] Removing CD22 enhances BCR signaling, reverses effects of ST6Gal1 loss, and alone can hyper-activate B cells.[166,167] CD22 is initially expressed at low levels on the surface of pre-B cells, however implications of expression at this stage are unclear

since early B cell development is normal in $CD22^{-/-}$ mice.[168,169] CD22 expression is upregulated to higher levels on immature/transitional B cells, and remains high on mature B cells with highest expression on MZ precursors.[170,171] Notably, $CD22^{-/-}$ mice are MZ B cell deficient, while FO B cells are unaffected, suggesting enhanced BCR signaling in $CD22^{-/-}$ B cells bypass the weaker signaling needed for MZ B cell maturation.[172] Siglec-G is another inhibitory coreceptor on B cells primarily expressed on innate-like B1 cells predominantly residing in the peritoneal cavity.[173] The importance of siglec regulation of B cell tolerance is demonstrated by $CD22^{-/-}$ x Siglec-$G^{-/-}$ mice that develop autoantibodies and spontaneous systemic autoimmunity.[174] Multimeric T-independent antigens coated with α2,6- and α2,3-linked sialic acid (CD22/Siglec-G ligands) lose immunogenicity and can induce tolerance to the same non-sialylated antigen, suggesting B cells use siglecs to screen for sialic acid ligands in *trans* as a mechanism to distinguish between self and nonself.[32] ST6Gal1 functions intrinsically in Golgi, but a significant amount is also found in extracellular spaces where it can extrinsically sialylate glycans.[175] A recent report suggests B cell intrinsic versus extrinsic ST6Gal1 mediated sialylation has differential effects on B cell development – intrinsic ST6Gal1 activity is necessary for MZ B cell maturation, whereas extrinsic activity promotes T1 B cell development in bone marrow and spleen.[176]

Other studies showed the importance of glycosylation in pre-BCR assembly, in which defects resulted in developmental arrest. The μ H-chain has five potential N-glycosylation sites, and one of them (N46) was shown to be required for pairing with the surrogate L-chain in order to induce pre-BCR signaling.[177] Core fucosylation of the N-glycans on the pre-BCR μ H-chain is also important for surrogate L-chain pairing. Pro-B

cells from mice deficient in α1,6-fucosyltransferase (Fut8), which attaches fucose to the innermost GlcNAc residue of hybrid/complex N-glycans via an α1,6-linkage, failed to join surrogate L-chain with μ H-chain for pre-BCR formation.[178]

Galectins also play a role in B cell development. Interestingly, galectin- (Gal-)1 has been proposed to regulate pre-BCR signaling through glycan dependent and independent binding.[179-183] In the bone marrow, small pre-B cells are localized in niches with Gal-1 secreting stromal cells.[182] At the pre-B cell to stromal cell interface, Gal-1 simultaneously binds to the surrogate L-chain of the pre-BCR via protein-protein interactions and to glycosylated integrins via protein-glycan interactions.[179-181] Through this mechanism, Gal-1 serves as a ligand to induce pre-BCR clustering and signaling for developmental progression.[183] Notably, Gal-1$^{-/-}$ mice only have slightly impaired B cell development. Only when bone marrow B cell development was disrupted was the slow reconstitution of small pre-B cells observed, suggesting other factors inherently compensate for Gal-1 loss.[183] Recent evidence suggests Gal-3 plays a role in B cell development, as well. Gal-3$^{-/-}$ mice have greater numbers of pro-B cells, which displayed elevated surface levels of IL-7R; this suggests higher IL-7R signaling due to Gal-3 loss leads to developmental arrest at the pro-B cell stage.[184]

Glycosylation and Galectins in B Cell Activation and Peripheral Functions

Most of what is known about glycosylation in B cells is in the context of sialylation and Siglecs (i.e., CD22) as mentioned above. While CD22 is primarily recognized as an inhibitory receptor with its four ITIMs and recruitment of SHP-1, a few studies suggest it regulates B cell survival and proliferation via Grb2 recruitment to a specific non-ITIM

motif and activation of a MAPK pathway depending on the context of stimulation.[185-188] The CD22 ligand (α2,6-linked sialic acid) is expressed on itself, surface IgM/IgD, CD45, and CD19, so CD22 can interact with other CD22s or other *cis* glycoproteins.[166] Protein-glycan crosslinking of steady-state B cells revealed CD22-CD22 associations form homomultimeric complexes, limiting its interaction with other receptors.[189] Advanced super-resolution microscopy and single particle tracking showed CD22 was in highly mobile nanoclusters that rapidly diffused across the cell membrane.[190] CD22 nanocluster formation was dependent on surface CD45 abundance – less CD45 led to bigger and slower CD22 nanoclusters due to reduced CD45 sialylated glycans enhancing CD22-CD22 associations. CD22 nanoclusters moved 4-5 times faster than surface IgD and CD19, and two times faster than surface IgM. In this manner, CD22 can quickly and globally survey BCR complexes to regulate tonic signaling and balance activation/inhibitory signaling upon B cell stimulation in the periphery.

CD22 also regulates TLR responses. Upon TLR3, 4, 7, and 9 stimulation $CD22^{-/-}$ B cells display a hyperactivated phenotype marked by increased proliferation and expression of MHCII and CD86.[168,191] Reports have also shown a role for CD22 in regulating T-dependent antibody responses[168,192,193], germinal center (GC) B cell formation[194], and memory B cell generation[195-197]. Similarly, $ST6Gal1^{-/-}$ mice have weakened antibody responses to influenza.[198]

CD22 has been heavily implicated in auto-antibody mediated diseases. A significant number of $CD22^{-/-}$ mice aged >8 months had high titers of high affinity anti-DNA IgG and shorter life spans.[199] In mice, three CD22 alleles (i.e., $CD22^{a}$, $CD22^{b}$, $CD22^{c}$) have been identified, with $CD22^{a}$ and $CD22^{c}$ found to be expressed in lupus-

prone strains.[200-202] *CD22*^{a} and *CD22*^{c} result in less CD22 expression and CD22 protein less efficiently binding α2,6-linked sialic acid compared to the non-autoimmune *CD22*^{b} allele. In humans, *CD22* polymorphisms are linked to SLE, RA, and cutaneous systemic sclerosis (SSc).[203,204] Apoptotic cells have been reported to lose surface α2,3- and α2,6-linked sialic acid.[205] Here, *trans* CD22 binding is lost, consequently reducing B cell self-tolerance; this could be a mechanism of SLE pathogenesis, in which inadequate apoptotic/necrotic cell clearance invokes aberrant B cell responses.[206]

Recently, Giovannone et al. examined human naïve, GC, and memory B cells by whole glycome mass spectrometry.[207] They found tri- and tetra-antennary complex N-glycans containing poly-LacNAc on all three B cell subsets. Uniquely, only GC B cells harbored poly-LacNAcs with internal β1,6-linked LacNAc, termed “I-branches”, which are catalyzed by β1,6-N-acetylglucosaminyltransferase-2 (β6GnT2, or “big I” IGnT).[207] They further demonstrated the consequence of N-glycan I-branches inhibits Gal-3 and Gal-9 binding on the surface of GC B cells. Gal-3 and Gal-9 suppress B cell activation[208], and preventing association on GC B cells is mechanistically conducive to their proliferating state.

Gal-1, -3, -9, and -8 play a role in B cell activation and function. Certain studies showed Gal-1 stimulates B cells in a manner comparable to BCR stimulation as seen by activation of similar downstream signaling pathways, induced Ca^{2+} flux, proliferation, and upregulation of CD69 and CD86.[209,210] However, other studies showed Gal-1 suppressed B cell activation[211,212], so the effects of Gal-1 on B cell function may be complex and context dependent, and possibly confounded by extrinsic Gal-1 secreted by other cells. Gal-3 is suggested to suppress B cell activation as Gal-3$^{-/-}$ mice have

spontaneous GC formation and higher titers of IgG2c and IgG3.[213] This was shown to be B cell intrinsic since adoptive transfer of Gal-3$^{-/-}$ B cells into B cell knockout mice had similar *in vivo* outcomes. Gal-9 has recently been shown to suppress B cell activity.[207,214,215] Gal-9$^{-/-}$ mice immunized with influenza were reported to have elevated circulating viral-specific antibodies compared to controls; CD8^{+} T cells also responded more robustly in this model, demonstrating Gal-9 extrinsic effect.[216] Gal-8 is reported to induce B cell arrest, limits mobility in order to enhance proteolytic extraction of antigens, and bolsters antigen presentation of B cells to CD4^{+} T cells.[217]

A growing body of evidence is establishing the importance of glycosylation in B cell development, tolerance, and peripheral functions. However, while we have shown how N-glycan branching regulates T cells, almost nothing is known in regards to B cells. For the first time, we have demonstrated how degree of N-glycan branching regulates B cell positive selection and maturation, as well as coupling of innate (TLR) and adaptive (CD19/BCR) signaling in the periphery with implications in autoimmunity.

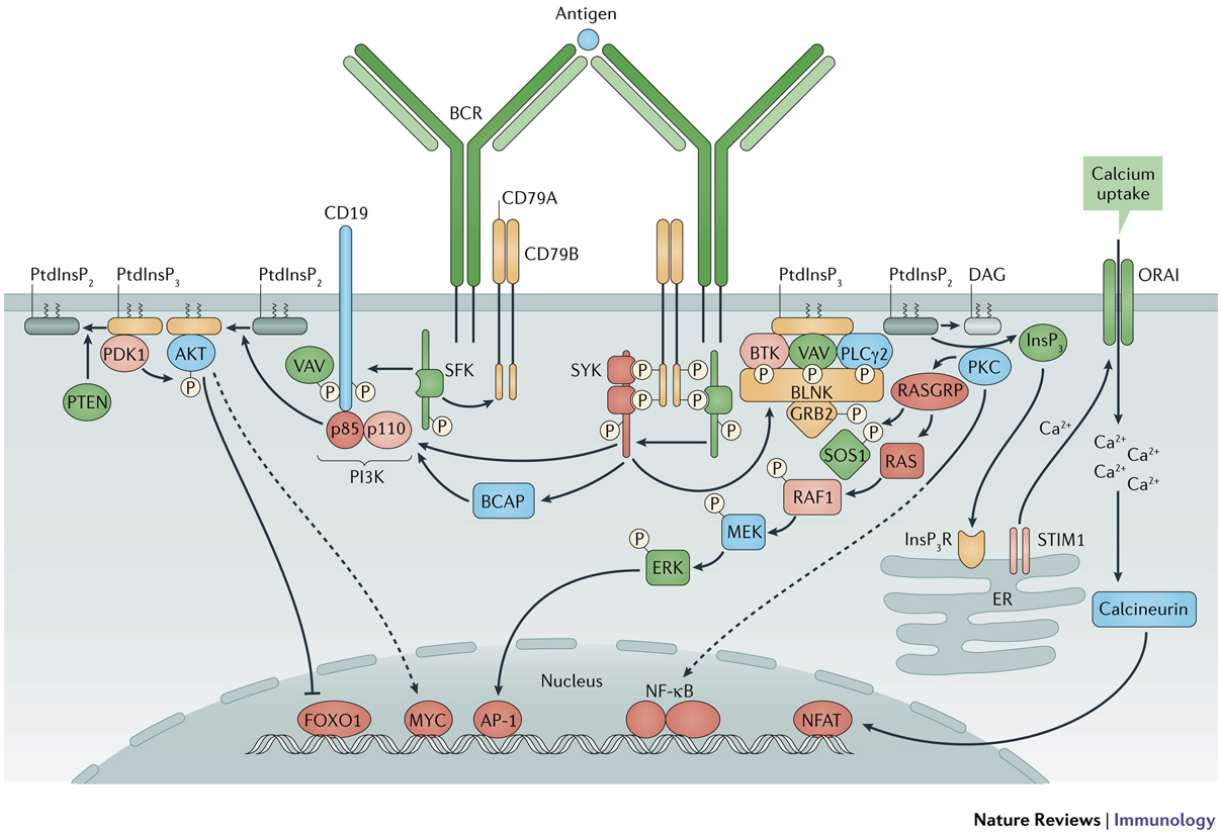

Figure 1.1. CD19/BCR signaling. At rest, a B cell's BCR Igα/Igβ units are weakly bound by SFKs. Antigen engagement induces conformational changes that allows Igα/Igβ ITAM phosphorylation by SFKs and the concurrent (1) recruitment and activation of SYK, and (2) tyrosine phosphorylation of the cytoplasmic tail of CD19.[218] Phosphorylated CD19 and/or BCAP recruits and activates PI3K which phosphorylates PIP_2 to generate PIP_3.[37,219] PIP_3 recruits AKT, PDK1, BTK and other enzymes to propagate downstream signaling. In another pathway, BLNK is phosphorylated by SFK/SYK and recruits PLCγ2.[220] Activated PLCγ2 induces Ca^{2+} mobilization by hydrolyzing PIP_2 to DAG and $InsP_3$ which engage IP_3R to open the Ca^{2+} channel ORAI. This activates calcineurin and the nuclear translocation of NFAT. In parallel, DAG recruits and activates PKC, which ultimately results in NF-κB and AP-1 nuclear translocation. Abbreviations: SFK, Src family kinases; SYK, spleen tyrosine kinase; BCAP, protein B cell adaptor for PI3K; PIP_2, phosphatidylinositol-4,5-bisphosphate; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; AKT, protein kinase B; PDK1, phosphoinositide-dependent protein kinase 1; BTK, Bruton's tyrosine kinase; BLNK, B cell linker protein; PLCγ2, phospholipase Cγ2; VAV, guanine nucleotide exchange factor; GRB2, growth factor receptor-bound protein 2; DAG, diacylglycerol; IP_3, inositol-1,4,5-trisphosphate; IP_3R, $InsP_3$ receptor; NFAT, nuclear factor of activated T cells; PKC, protein kinase C; RASGRP, RAS guanyl-releasing protein; NF-κB, nuclear factor-κB; AP-1, activator protein 1; SOS1, guanine nucleotide exchange factor son of sevenless 1; RAS, a small GTPase; RAF1, a serine kinase; MEK, MAPK/ERK kinase; ERK, extracellular signal-regulated kinases; PTEN, phosphatase and tensin homologue; ER, endoplasmic reticulum; STIM1, stromal interaction molecule 1.
Image © David Nemazee. *Nature Reviews Immunology* **Volume 17**, pages 281–294 (2017)

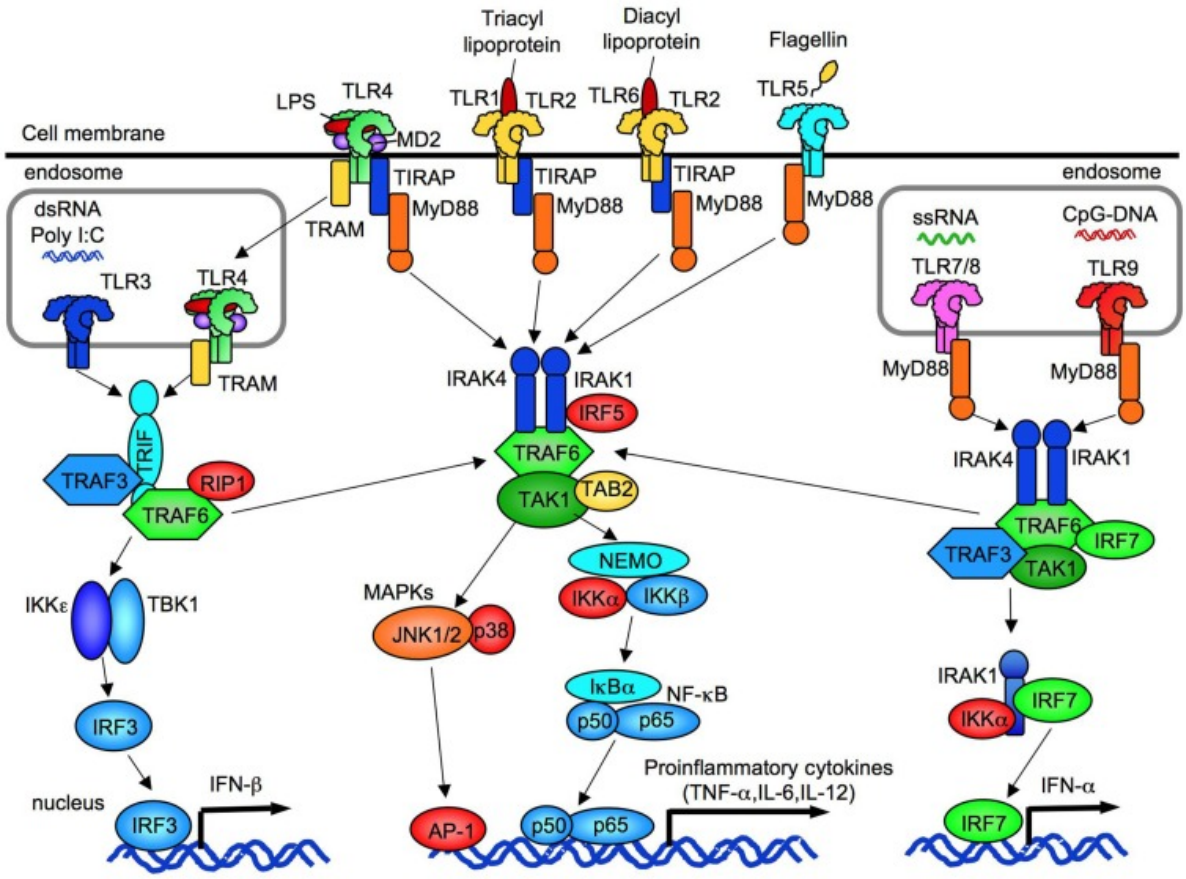

Figure 1.2. TLR signaling. TLRs are comprised of extracellular domains of LRRs and intracellular TIR domains.[221] TLRs 1, 2, 4, 5, and 6 are expressed on the cell surface and TLRs 3, 7, 8, and 9 exist in endosomes. Upon ligand binding, TLR4 can be endocytosed (mediated by CD14 on myeloid cells[222]) and signal from endosomes; MD2 is required for optimal TLR4 signaling[223]. TLRs either homo- or hetero-dimerize upon ligand binding; this induces conformational changes that allow the intracellular recruitment of adaptor proteins (i.e., TIRAP, MyD88, TRIF, TRAM) for signal transduction. TLR TIR and adaptor protein TIR domain association is required for signaling. Surface TLR signaling proceeds through a MyD88-dependant pathway to activating MAPKs (e.g., p38), AP-1, and canonical NF-κB for pro-inflammatory cytokine and co-stimulatory molecule expression.[224] Endosomal TLR signaling may proceed towards MyD88/AP-1/NF-κB activation, or towards TRAF3 mediated activation of IRF3 or IRF7 for type-1 interferon (IFNα/β) expression for viral clearance.[225] Endosomal TLR4 signaling is also suggested to result in anti-inflammatory IL-10 production.[226] Abbreviations: TLR, toll-like receptor; LRR, leucine-rich repeat; CD14, cluster of differentiation 14; MD2, lymphocyte antigen 96; TIR, toll/interleukin-1 receptor; TIRAP, TIR domain-containing adaptor protein; MyD88, myeloid differentiation factor 88; TRIF, TIR domain-containing adaptor-inducing IFN-β; TRAM, TRIF-related adaptor molecule; MAPK, mitogen-activated protein kinase; AP-1, activator protein 1; NF-κB, nuclear factor-κB; IRF, interferon-regulatory factor; IL-10, interleukin-10.

Image © Yang, L. and Seki, E. *Frontiers in Physiology* **Volume 3**, Issue 138 (2012)

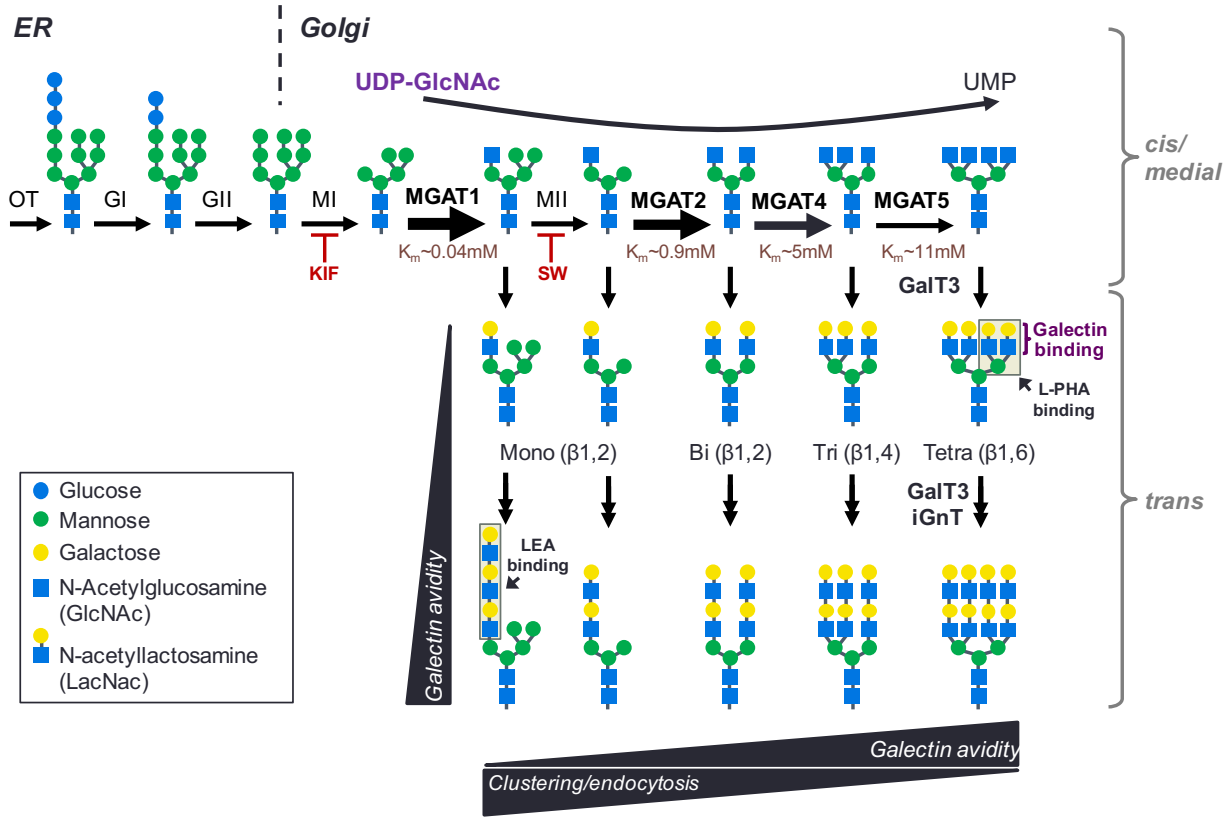

Figure 1.3. The N-glycosylation branching pathway. Oligosaccharyltransferase (OT) transfers a pre-assembled glycan, Glc3Man9GlcNAc2, onto Asp-X-Ser-Thr (X≠Pro) motifs on glycoproteins in the endoplasmic reticulum (ER). This glycan is extensively modified as glycoproteins transit through the Golgi by mannosidases (MI/MII) and the N-acetylglucosaminyltransferase (Mgat) enzymes, which act sequentially to generate N-acetylglucosamone (GlcNAc) branched N-glycans with increasing avidities for galectins. Mgat1, 2, 4, and 5 catalyze N-glycan branching using the same sugar-nucleotide donor (i.e. UDP-GlcNAc) but with declining efficiency (i.e., reducing K_m). GalT and iGnT extend branches to create N-acetyllactosamine (LacNAc) which can also be bound by galectins. Small molecule inhibitors (KIF/SW) can be used to eliminate/reduce branching. Plant lectins L-PHA and LEA binding sites are indicated. Abbreviations: OT, oligosaccharyltransferase; GI, glucosidase I; GII, glucosidase II; MI, mannosidase I; MII, mannosidase II; Mgat, N-acetylglucosaminyltransferase; GalT3, Galactosyltransferase 3; iGnT, little "i" β-1,3-N-acetylglucosaminyltransferase; KIF, kifunensine; SW, swainsonine; UDP, uridine diphosphate; UMP, uridine monophosphate

REFERENCES

1 Cooper, M. D. & Alder, M. N. The evolution of adaptive immune systems. *Cell* **124**, 815-822, doi:10.1016/j.cell.2006.02.001 (2006).

2 Tiselius, A. & Kabat, E. A. Electrophoresis of Immune Serum. *Science* **87**, 416-417, doi:10.1126/science.87.2262.416-a (1938).

3 Fagraeus, A. The plasma cellular reaction and its relation to the formation of antibodies in vitro. *J Immunol* **58**, 1-13 (1948).

4 Cooper, M. D., Peterson, R. D. & Good, R. A. Delineation of the Thymic and Bursal Lymphoid Systems in the Chicken. *Nature* **205**, 143-146, doi:10.1038/205143a0 (1965).

5 Cooper, M. D., Raymond, D. A., Peterson, R. D., South, M. A. & Good, R. A. The functions of the thymus system and the bursa system in the chicken. *J Exp Med* **123**, 75-102, doi:10.1084/jem.123.1.75 (1966).

6 Miller, J. F. & Mitchell, G. F. Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. *J Exp Med* **128**, 801-820, doi:10.1084/jem.128.4.801 (1968).

7 Mitchell, G. F. & Miller, J. F. Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. *J Exp Med* **128**, 821-837, doi:10.1084/jem.128.4.821 (1968).

8 Froland, S., Natvig, J. B. & Berdal, P. Surface-bound immunoglobulin as a marker of B lymphocytes in man. *Nat New Biol* **234**, 251-252, doi:10.1038/newbio234251a0 (1971).

9 Preud'homme, J. L. & Seligmann, M. Surface bound immunoglobulins as a cell marker in human lymphoproliferative diseases. *Blood* **40**, 777-794 (1972).

10 Coombs, R. R., Feinstein, A. & Wilson, A. B. Immunoglobulin determinants on the surface of human lymphocytes. *Lancet* **2**, 1157-1160, doi:10.1016/s0140-6736(69)92484-2 (1969).

11 Hardy, R. R., Kincade, P. W. & Dorshkind, K. The protean nature of cells in the B lymphocyte lineage. *Immunity* **26**, 703-714, doi:10.1016/j.immuni.2007.05.013 (2007).

12 LeBien, T. W. Fates of human B-cell precursors. *Blood* **96**, 9-23 (2000).

13 Schatz, D. G., Oettinger, M. A. & Baltimore, D. The V(D)J recombination activating gene, RAG-1. *Cell* **59**, 1035-1048, doi:10.1016/0092-8674(89)90760-5 (1989).

14 Oettinger, M. A., Schatz, D. G., Gorka, C. & Baltimore, D. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science* **248**, 1517-1523, doi:10.1126/science.2360047 (1990).

15 Ikuta, K., Uchida, N., Friedman, J. & Weissman, I. L. Lymphocyte development from stem cells. *Annu Rev Immunol* **10**, 759-783, doi:10.1146/annurev.iy.10.040192.003551 (1992).

16 Zuniga-Pflucker, J. C. & Lenardo, M. J. Regulation of thymocyte development from immature progenitors. *Curr Opin Immunol* **8**, 215-224, doi:10.1016/s0952-7915(96)80060-4 (1996).

17 LeBien, T. W. & Tedder, T. F. B lymphocytes: how they develop and function. *Blood* **112**, 1570-1580, doi:10.1182/blood-2008-02-078071 (2008).

18 Lin, Y. C. *et al.* A global network of transcription factors, involving E2A, EBF1 and Foxo1, that orchestrates B cell fate. *Nat Immunol* **11**, 635-643, doi:10.1038/ni.1891 (2010).

19 Pieper, K., Grimbacher, B. & Eibel, H. B-cell biology and development. *J Allergy Clin Immunol* **131**, 959-971, doi:10.1016/j.jaci.2013.01.046 (2013).

20 Martensson, I. L., Almqvist, N., Grimsholm, O. & Bernardi, A. I. The pre-B cell receptor checkpoint. *FEBS Lett* **584**, 2572-2579, doi:10.1016/j.febslet.2010.04.057 (2010).

21 Ochiai, K. *et al.* A self-reinforcing regulatory network triggered by limiting IL-7 activates pre-BCR signaling and differentiation. *Nat Immunol* **13**, 300-307, doi:10.1038/ni.2210 (2012).

22 von Freeden-Jeffry, U. *et al.* Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J Exp Med* **181**, 1519-1526, doi:10.1084/jem.181.4.1519 (1995).

23 Rolink, A. G., Winkler, T., Melchers, F. & Andersson, J. Precursor B cell receptor-dependent B cell proliferation and differentiation does not require the bone marrow or fetal liver environment. *J Exp Med* **191**, 23-32, doi:10.1084/jem.191.1.23 (2000).

24 Reth, M. Antigen receptors on B lymphocytes. *Annu Rev Immunol* **10**, 97-121, doi:10.1146/annurev.iy.10.040192.000525 (1992).

25 Monroe, J. G. ITAM-mediated tonic signalling through pre-BCR and BCR complexes. *Nat Rev Immunol* **6**, 283-294, doi:10.1038/nri1808 (2006).

26 Niiro, H. & Clark, E. A. Regulation of B-cell fate by antigen-receptor signals. *Nat Rev Immunol* **2**, 945-956, doi:10.1038/nri955 (2002).

27 Pelanda, R. & Torres, R. M. Central B-cell tolerance: where selection begins. *Cold Spring Harb Perspect Biol* **4**, a007146, doi:10.1101/cshperspect.a007146 (2012).

28 King, L. B., Norvell, A. & Monroe, J. G. Antigen receptor-induced signal transduction imbalances associated with the negative selection of immature B cells. *J Immunol* **162**, 2655-2662 (1999).

29 Nemazee, D. Mechanisms of central tolerance for B cells. *Nat Rev Immunol* **17**, 281-294, doi:10.1038/nri.2017.19 (2017).

30 Pillai, S. & Cariappa, A. The follicular versus marginal zone B lymphocyte cell fate decision. *Nat Rev Immunol* **9**, 767-777, doi:10.1038/nri2656 (2009).

31 Cariappa, A., Chase, C., Liu, H., Russell, P. & Pillai, S. Naive recirculating B cells mature simultaneously in the spleen and bone marrow. *Blood* **109**, 2339-2345, doi:10.1182/blood-2006-05-021089 (2007).

32 Duong, B. H. *et al.* Decoration of T-independent antigen with ligands for CD22 and Siglec-G can suppress immunity and induce B cell tolerance in vivo. *J Exp Med* **207**, 173-187, doi:10.1084/jem.20091873 (2010).

33 Macauley, M. S. *et al.* Antigenic liposomes displaying CD22 ligands induce antigen-specific B cell apoptosis. *J Clin Invest* **123**, 3074-3083, doi:10.1172/JCI69187 (2013).

34 Sabouri, Z. *et al.* Redemption of autoantibodies on anergic B cells by variable-region glycosylation and mutation away from self-reactivity. *Proceedings of the National Academy of Sciences of the United States of America* **111**, E2567-2575, doi:10.1073/pnas.1406974111 (2014).

35 Goodnow, C. C. *et al.* Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature* **334**, 676-682, doi:10.1038/334676a0 (1988).

36 Myers, D. R., Zikherman, J. & Roose, J. P. Tonic Signals: Why Do Lymphocytes Bother? *Trends Immunol* **38**, 844-857, doi:10.1016/j.it.2017.06.010 (2017).

37 Fujimoto, M. *et al.* CD19 regulates Src family protein tyrosine kinase activation in B lymphocytes through processive amplification. *Immunity* **13**, 47-57, doi:10.1016/s1074-7613(00)00007-8 (2000).

38 Mukherjee, S. *et al.* Monovalent and multivalent ligation of the B cell receptor exhibit differential dependence upon Syk and Src family kinases. *Sci Signal* **6**, ra1, doi:10.1126/scisignal.2003220 (2013).

39 Stamenkovic, I. & Seed, B. CD19, the earliest differentiation antigen of the B cell lineage, bears three extracellular immunoglobulin-like domains and an Epstein-Barr virus-related cytoplasmic tail. *J Exp Med* **168**, 1205-1210, doi:10.1084/jem.168.3.1205 (1988).

40 Engel, P. *et al.* Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. *Immunity* **3**, 39-50, doi:10.1016/1074-7613(95)90157-4 (1995).

41 Rickert, R. C., Rajewsky, K. & Roes, J. Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. *Nature* **376**, 352-355, doi:10.1038/376352a0 (1995).

42 Diamant, E., Keren, Z. & Melamed, D. CD19 regulates positive selection and maturation in B lymphopoiesis: lack of CD19 imposes developmental arrest of immature B cells and consequential stimulation of receptor editing. *Blood* **105**, 3247-3254, doi:10.1182/blood-2004-08-3165 (2005).

43 Anzelon, A. N., Wu, H. & Rickert, R. C. Pten inactivation alters peripheral B lymphocyte fate and reconstitutes CD19 function. *Nat Immunol* **4**, 287-294, doi:10.1038/ni892 (2003).

44 Benhamou, D. *et al.* A c-Myc/miR17-92/Pten Axis Controls PI3K-Mediated Positive and Negative Selection in B Cell Development and Reconstitutes CD19 Deficiency. *Cell Rep* **16**, 419-431, doi:10.1016/j.celrep.2016.05.084 (2016).

45 Chalupny, N. J. *et al.* Tyrosine phosphorylation of CD19 in pre-B and mature B cells. *Embo J* **12**, 2691-2696 (1993).

46 Carter, R. H. & Fearon, D. T. CD19: lowering the threshold for antigen receptor stimulation of B lymphocytes. *Science* **256**, 105-107, doi:10.1126/science.1373518 (1992).

47 Uckun, F. M. *et al.* Signal transduction through the CD19 receptor during discrete developmental stages of human B-cell ontogeny. *J Biol Chem* **268**, 21172-21184 (1993).

48 Otero, D. C., Anzelon, A. N. & Rickert, R. C. CD19 function in early and late B cell development: I. Maintenance of follicular and marginal zone B cells requires CD19-dependent survival signals. *J Immunol* **170**, 73-83, doi:10.4049/jimmunol.170.1.73 (2003).

49 Keppler, S. J. *et al.* Wiskott-Aldrich Syndrome Interacting Protein Deficiency Uncovers the Role of the Co-receptor CD19 as a Generic Hub for PI3 Kinase Signaling in B Cells. *Immunity* **43**, 660-673, doi:10.1016/j.immuni.2015.09.004 (2015).

50 Morbach, H. *et al.* CD19 controls Toll-like receptor 9 responses in human B cells. *J Allergy Clin Immunol* **137**, 889-898 e886, doi:10.1016/j.jaci.2015.08.040 (2016).

51 Baumgarth, N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol* **11**, 34-46, doi:10.1038/nri2901 (2011).

52 Cariappa, A. *et al.* Perisinusoidal B cells in the bone marrow participate in T-independent responses to blood-borne microbes. *Immunity* **23**, 397-407, doi:10.1016/j.immuni.2005.09.004 (2005).

53 Pillai, S., Cariappa, A. & Moran, S. T. Marginal zone B cells. *Annu Rev Immunol* **23**, 161-196, doi:10.1146/annurev.immunol.23.021704.115728 (2005).

54 Mesin, L., Ersching, J. & Victora, G. D. Germinal Center B Cell Dynamics. *Immunity* **45**, 471-482, doi:10.1016/j.immuni.2016.09.001 (2016).

55 Batista, F. D. & Harwood, N. E. The who, how and where of antigen presentation to B cells. *Nat Rev Immunol* **9**, 15-27, doi:10.1038/nri2454 (2009).

56 Vinuesa, C. G., Linterman, M. A., Yu, D. & MacLennan, I. C. Follicular Helper T Cells. *Annu Rev Immunol* **34**, 335-368, doi:10.1146/annurev-immunol-041015-055605 (2016).

57 Nutt, S. L., Hodgkin, P. D., Tarlinton, D. M. & Corcoran, L. M. The generation of antibody-secreting plasma cells. *Nat Rev Immunol* **15**, 160-171, doi:10.1038/nri3795 (2015).

58 Forthal, D. N. Functions of Antibodies. *Microbiol Spectr* **2**, AID-0019-2014, doi:10.1128/microbiolspec.AID-0019-2014 (2014).

59 Hoffman, W., Lakkis, F. G. & Chalasani, G. B Cells, Antibodies, and More. *Clin J Am Soc Nephrol* **11**, 137-154, doi:10.2215/CJN.09430915 (2016).

60 Browne, E. P. Regulation of B-cell responses by Toll-like receptors. *Immunology* **136**, 370-379, doi:10.1111/j.1365-2567.2012.03587.x (2012).

61 Gururajan, M., Jacob, J. & Pulendran, B. Toll-like receptor expression and responsiveness of distinct murine splenic and mucosal B-cell subsets. *PLoS One* **2**, e863, doi:10.1371/journal.pone.0000863 (2007).

62 Bell, E. TLR4 signalling. *Nature Reviews Immunology* **8**, 241-241, doi:10.1038/nri2301 (2008).

63 Hornung, V. *et al.* Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol* **168**, 4531-4537, doi:10.4049/jimmunol.168.9.4531 (2002).

64 Agrawal, S. & Gupta, S. TLR1/2, TLR7, and TLR9 signals directly activate human peripheral blood naive and memory B cell subsets to produce cytokines, chemokines, and hematopoietic growth factors. *J Clin Immunol* **31**, 89-98, doi:10.1007/s10875-010-9456-8 (2011).

65 Bernasconi, N. L., Onai, N. & Lanzavecchia, A. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. *Blood* **101**, 4500-4504, doi:10.1182/blood-2002-11-3569 (2003).

66 Magna, M. & Pisetsky, D. S. The role of HMGB1 in the pathogenesis of inflammatory and autoimmune diseases. *Mol Med* **20**, 138-146, doi:10.2119/molmed.2013.00164 (2014).

67 Roh, J. S. & Sohn, D. H. Damage-Associated Molecular Patterns in Inflammatory Diseases. *Immune Netw* **18**, e27, doi:10.4110/in.2018.18.e27 (2018).

68 Land, W. G. The Role of Damage-Associated Molecular Patterns (DAMPs) in Human Diseases: Part II: DAMPs as diagnostics, prognostics and therapeutics in clinical medicine. *Sultan Qaboos Univ Med J* **15**, e157-170 (2015).

69 Andersson, A. *et al.* Pivotal advance: HMGB1 expression in active lesions of human and experimental multiple sclerosis. *Journal of leukocyte biology* **84**, 1248-1255, doi:10.1189/jlb.1207844 (2008).

70 Gitlin, A. D., Shulman, Z. & Nussenzweig, M. C. Clonal selection in the germinal centre by regulated proliferation and hypermutation. *Nature* **509**, 637-640, doi:10.1038/nature13300 (2014).

71 Shulman, Z. *et al.* Dynamic signaling by T follicular helper cells during germinal center B cell selection. *Science* **345**, 1058-1062, doi:10.1126/science.1257861 (2014).

72 Misumi, I. & Whitmire, J. K. B cell depletion curtails CD4+ T cell memory and reduces protection against disseminating virus infection. *J Immunol* **192**, 1597-1608, doi:10.4049/jimmunol.1302661 (2014).

73 Adler, L. N. *et al.* The Other Function: Class II-Restricted Antigen Presentation by B Cells. *Front Immunol* **8**, 319, doi:10.3389/fimmu.2017.00319 (2017).

74 Yuseff, M. I., Pierobon, P., Reversat, A. & Lennon-Dumenil, A. M. How B cells capture, process and present antigens: a crucial role for cell polarity. *Nat Rev Immunol* **13**, 475-486, doi:10.1038/nri3469 (2013).

75 Natkanski, E. *et al.* B cells use mechanical energy to discriminate antigen affinities. *Science* **340**, 1587-1590, doi:10.1126/science.1237572 (2013).

76 Yuseff, M. I. *et al.* Polarized secretion of lysosomes at the B cell synapse couples antigen extraction to processing and presentation. *Immunity* **35**, 361-374, doi:10.1016/j.immuni.2011.07.008 (2011).

77 Roche, P. A. & Furuta, K. The ins and outs of MHC class II-mediated antigen processing and presentation. *Nat Rev Immunol* **15**, 203-216, doi:10.1038/nri3818 (2015).

78 Collins, M., Ling, V. & Carreno, B. M. The B7 family of immune-regulatory ligands. *Genome Biol* **6**, 223, doi:10.1186/gb-2005-6-6-223 (2005).

79 Boussiotis, V. A. *et al.* Activated human B lymphocytes express three CTLA-4 counterreceptors that costimulate T-cell activation. *Proceedings of the National*

Academy of Sciences of the United States of America **90**, 11059-11063, doi:10.1073/pnas.90.23.11059 (1993).

80 Hathcock, K. S., Laszlo, G., Pucillo, C., Linsley, P. & Hodes, R. J. Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression and function. *J Exp Med* **180**, 631-640, doi:10.1084/jem.180.2.631 (1994).

81 Hamze, M., Desmetz, C. & Guglielmi, P. B cell-derived cytokines in disease. *Eur Cytokine Netw* **24**, 20-26, doi:10.1684/ecn.2013.0327 (2013).

82 Wong, S. C. *et al.* Macrophage polarization to a unique phenotype driven by B cells. *Eur J Immunol* **40**, 2296-2307, doi:10.1002/eji.200940288 (2010).

83 Noseworthy, J. H., Lucchinetti, C., Rodriguez, M. & Weinshenker, B. G. Multiple sclerosis. *N Engl J Med* **343**, 938-952, doi:10.1056/NEJM200009283431307 (2000).

84 Hohlfeld, R. & Steinman, L. T Cell-Transfer Experimental Autoimmune Encephalomyelitis: Pillar of Multiple Sclerosis and Autoimmunity. *J Immunol* **198**, 3381-3383, doi:10.4049/jimmunol.1700346 (2017).

85 Ben-Nun, A. *et al.* From classic to spontaneous and humanized models of multiple sclerosis: impact on understanding pathogenesis and drug development. *J Autoimmun* **54**, 33-50, doi:10.1016/j.jaut.2014.06.004 (2014).

86 Steinman, L. Immunology of relapse and remission in multiple sclerosis. *Annu Rev Immunol* **32**, 257-281, doi:10.1146/annurev-immunol-032713-120227 (2014).

87 Kaskow, B. J. & Baecher-Allan, C. Effector T Cells in Multiple Sclerosis. *Cold Spring Harbor perspectives in medicine* **8**, doi:10.1101/cshperspect.a029025 (2018).

88 Hafler, D. A. *et al.* Risk alleles for multiple sclerosis identified by a genomewide study. *The New England journal of medicine* **357**, 851-862, doi:10.1056/NEJMoa073493 (2007).

89 Gregory, S. G. *et al.* Interleukin 7 receptor alpha chain (IL7R) shows allelic and functional association with multiple sclerosis. *Nat Genet* **39**, 1083-1091, doi:10.1038/ng2103 (2007).

90 Lundmark, F. *et al.* Variation in interleukin 7 receptor alpha chain (IL7R) influences risk of multiple sclerosis. *Nat Genet* **39**, 1108-1113, doi:10.1038/ng2106 (2007).

91 Sawcer, S. *et al.* Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* **476**, 214-219, doi:10.1038/nature10251 (2011).

92 Hussman, J. P. *et al.* GWAS analysis implicates NF-kappaB-mediated induction of inflammatory T cells in multiple sclerosis. *Genes and immunity* **17**, 305-312, doi:10.1038/gene.2016.23 (2016).

93 Canto, E. & Oksenberg, J. R. Multiple sclerosis genetics. *Multiple sclerosis* **24**, 75-79, doi:10.1177/1352458517737371 (2018).

94 Sabatino, J. J., Jr., Zamvil, S. S. & Hauser, S. L. B-Cell Therapies in Multiple Sclerosis. *Cold Spring Harbor perspectives in medicine*, doi:10.1101/cshperspect.a032037 (2018).

95 Hauser, S. L. *et al.* B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N Engl J Med* **358**, 676-688, doi:10.1056/NEJMoa0706383 (2008).

96 Hauser, S. L. *et al.* Ocrelizumab versus Interferon Beta-1a in Relapsing Multiple Sclerosis. *N Engl J Med* **376**, 221-234, doi:10.1056/NEJMoa1601277 (2017).

97 Hauser, S. L., Belachew, S. & Kappos, L. Ocrelizumab in Primary Progressive and Relapsing Multiple Sclerosis. *N Engl J Med* **376**, 1694, doi:10.1056/NEJMc1702076 (2017).

98 Magliozzi, R. *et al.* Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. *Brain* **130**, 1089-1104, doi:10.1093/brain/awm038 (2007).

99 Mitsdoerffer, M. & Peters, A. Tertiary Lymphoid Organs in Central Nervous System Autoimmunity. *Front Immunol* **7**, 451, doi:10.3389/fimmu.2016.00451 (2016).

100 Link, H. & Huang, Y. M. Oligoclonal bands in multiple sclerosis cerebrospinal fluid: an update on methodology and clinical usefulness. *J Neuroimmunol* **180**, 17-28, doi:10.1016/j.jneuroim.2006.07.006 (2006).

101 Kanter, J. L. *et al.* Lipid microarrays identify key mediators of autoimmune brain inflammation. *Nat Med* **12**, 138-143, doi:10.1038/nm1344 (2006).

102 Genain, C. P., Cannella, B., Hauser, S. L. & Raine, C. S. Identification of autoantibodies associated with myelin damage in multiple sclerosis. *Nat Med* **5**, 170-175, doi:10.1038/5532 (1999).

103 Kennedy, B. P. *et al.* Contributions of the sympathetic nervous system, glutathione, body mass and gender to blood pressure increase with normal

aging: influence of heredity. *J Hum Hypertens* **19**, 951-969, doi:10.1038/sj.jhh.1001912 (2005).

104 Cross, A. H., Stark, J. L., Lauber, J., Ramsbottom, M. J. & Lyons, J. A. Rituximab reduces B cells and T cells in cerebrospinal fluid of multiple sclerosis patients. *J Neuroimmunol* **180**, 63-70, doi:10.1016/j.jneuroim.2006.06.029 (2006).

105 Hauser, S. L. The Charcot Lecture | beating MS: a story of B cells, with twists and turns. *Multiple sclerosis* **21**, 8-21, doi:10.1177/1352458514561911 (2015).

106 Monson, N. L. *et al.* Rituximab therapy reduces organ-specific T cell responses and ameliorates experimental autoimmune encephalomyelitis. *PLoS One* **6**, e17103, doi:10.1371/journal.pone.0017103 (2011).

107 Molnarfi, N. *et al.* MHC class II-dependent B cell APC function is required for induction of CNS autoimmunity independent of myelin-specific antibodies. *J Exp Med* **210**, 2921-2937, doi:10.1084/jem.20130699 (2013).

108 Weber, M. S. *et al.* B-cell activation influences T-cell polarization and outcome of anti-CD20 B-cell depletion in central nervous system autoimmunity. *Annals of neurology* **68**, 369-383, doi:10.1002/ana.22081 (2010).

109 Pierson, E. R., Stromnes, I. M. & Goverman, J. M. B cells promote induction of experimental autoimmune encephalomyelitis by facilitating reactivation of T cells in the central nervous system. *J Immunol* **192**, 929-939, doi:10.4049/jimmunol.1302171 (2014).

110 Barr, T. A. *et al.* B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells. *J Exp Med* **209**, 1001-1010, doi:10.1084/jem.20111675 (2012).

111 Bar-Or, A. *et al.* Abnormal B-cell cytokine responses a trigger of T-cell-mediated disease in MS? *Annals of neurology* **67**, 452-461, doi:10.1002/ana.21939 (2010).

112 Iwata, Y. *et al.* Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. *Blood* **117**, 530-541, doi:10.1182/blood-2010-07-294249 (2011).

113 Okada, Y. *et al.* Signaling via toll-like receptor 4 and CD40 in B cells plays a regulatory role in the pathogenesis of multiple sclerosis through interleukin-10 production. *J Autoimmun* **88**, 103-113, doi:10.1016/j.jaut.2017.10.011 (2018).

114 Correale, J. & Farez, M. Helminth antigens modulate immune responses in cells from multiple sclerosis patients through TLR2-dependent mechanisms. *J Immunol* **183**, 5999-6012, doi:10.4049/jimmunol.0900897 (2009).

115 Fillatreau, S., Sweenie, C. H., McGeachy, M. J., Gray, D. & Anderton, S. M. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol* **3**, 944-950, doi:10.1038/ni833 (2002).

116 Kelleher, D. J. & Gilmore, R. An evolving view of the eukaryotic oligosaccharyltransferase. *Glycobiology* **16**, 47r-62r, doi:DOI 10.1093/glycob/cwj066 (2006).

117 Dennis, J. W., Nabi, I. R. & Demetriou, M. Metabolism, cell surface organization, and disease. *Cell* **139**, 1229-1241, doi:10.1016/j.cell.2009.12.008 (2009).

118 Schachter, H. The 'yellow brick road' to branched complex N-glycans. *Glycobiology* **1**, 453-461 (1991).

119 Kornfeld, R. & Kornfeld, S. Assembly of asparagine-linked oligosaccharides. *Annu Rev Biochem* **54**, 631-664, doi:10.1146/annurev.bi.54.070185.003215 (1985).

120 Helenius, A. & Aebi, M. Roles of N-linked glycans in the endoplasmic reticulum. *Annual review of biochemistry* **73**, 1019-1049, doi:10.1146/annurev.biochem.73.011303.073752 (2004).

121 Kowarik, M. *et al.* N-linked glycosylation of folded proteins by the bacterial oligosaccharyltransferase. *Science* **314**, 1148-1150 (2006).

122 Banerjee, S. *et al.* The evolution of N-glycan-dependent endoplasmic reticulum quality control factors for glycoprotein folding and degradation. *Proc Natl Acad Sci U S A* **104**, 11676-11681 (2007).

123 Ioffe, E. & Stanley, P. Mice lacking N-acetylglucosaminyltransferase I activity die at mid-gestation, revealing an essential role for complex or hybrid N-linked carbohydrates. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 728-732 (1994).

124 Jaeken, J. *et al.* Carbohydrate deficient glycoprotein syndrome type II: a deficiency in Golgi localised N-acetyl-glucosaminyltransferase II. *Archives of disease in childhood* **71**, 123-127 (1994).

125 Brewer, C. F., Miceli, M. C. & Baum, L. G. Clusters, bundles, arrays and lattices: novel mechanisms for lectin-saccharide-mediated cellular interactions. *Current opinion in structural biology* **12**, 616-623 (2002).

126 Hirabayashi, J. *et al.* Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. *Biochimica et biophysica acta* **1572**, 232-254, doi:10.1016/s0304-4165(02)00311-2 (2002).

127 Ahmad, N. *et al.* Galectin-3 precipitates as a pentamer with synthetic multivalent carbohydrates and forms heterogeneous cross-linked complexes. *J Biol Chem* **279**, 10841-10847, doi:10.1074/jbc.M312834200 (2004).

128 Demetriou, M., Granovsky, M., Quaggin, S. & Dennis, J. W. Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature* **409**, 733-739, doi:10.1038/35055582 (2001).

129 Lau, K. S. *et al.* Complex N-glycan number and degree of branching cooperate to regulate cell proliferation and differentiation. *Cell* **129**, 123-134, doi:10.1016/j.cell.2007.01.049 (2007).

130 Partridge, E. A. *et al.* Regulation of cytokine receptors by Golgi N-glycan processing and endocytosis. *Science* **306**, 120-124, doi:10.1126/science.1102109 (2004).

131 Chen, I. J., Chen, H. L. & Demetriou, M. Lateral compartmentalization of T cell receptor versus CD45 by galectin-N-glycan binding and microfilaments coordinate basal and activation signaling. *The Journal of biological chemistry* **282**, 35361-35372, doi:10.1074/jbc.M706923200 (2007).

132 Demotte, N. *et al.* Restoring the association of the T cell receptor with CD8 reverses anergy in human tumor-infiltrating lymphocytes. *Immunity* **28**, 414-424, doi:10.1016/j.immuni.2008.01.011 (2008).

133 Pace, K. E., Lee, C., Stewart, P. L. & Baum, L. G. Restricted receptor segregation into membrane microdomains occurs on human T cells during apoptosis induced by galectin-1. *J Immunol* **163**, 3801-3811 (1999).

134 Lajoie, P. *et al.* Plasma membrane domain organization regulates EGFR signaling in tumor cells. *J Cell Biol* **179**, 341-356, doi:10.1083/jcb.200611106 (2007).

135 Ohtsubo, K. *et al.* Dietary and genetic control of glucose transporter 2 glycosylation promotes insulin secretion in suppressing diabetes. *Cell* **123**, 1307-1321, doi:10.1016/j.cell.2005.09.041 (2005).

136 Cha, S. K. *et al.* Removal of sialic acid involving Klotho causes cell-surface retention of TRPV5 channel via binding to galectin-1. *Proc Natl Acad Sci U S A* **105**, 9805-9810, doi:10.1073/pnas.0803223105 (2008).

137 Kuball, J. *et al.* Increasing functional avidity of TCR-redirected T cells by removing defined N-glycosylation sites in the TCR constant domain. *J Exp Med* **206**, 463-475, doi:10.1084/jem.20082487 (2009).

138 Grigorian, A., Torossian, S. & Demetriou, M. T-cell growth, cell surface organization, and the galectin-glycoprotein lattice. *Immunol Rev* **230**, 232-246, doi:10.1111/j.1600-065X.2009.00796.x (2009).

139 Rabinovich, G. A. & Croci, D. O. Regulatory circuits mediated by lectin-glycan interactions in autoimmunity and cancer. *Immunity* **36**, 322-335, doi:10.1016/j.immuni.2012.03.004 (2012).

140 Liu, F. T. & Rabinovich, G. A. Galectins: regulators of acute and chronic inflammation. *Ann N Y Acad Sci* **1183**, 158-182, doi:10.1111/j.1749-6632.2009.05131.x (2010).

141 Rabinovich, G. A. & Conejo-Garcia, J. R. Shaping the Immune Landscape in Cancer by Galectin-Driven Regulatory Pathways. *J Mol Biol* **428**, 3266-3281, doi:10.1016/j.jmb.2016.03.021 (2016).

142 Toscano, M. A., Martinez Allo, V. C., Cutine, A. M., Rabinovich, G. A. & Marino, K. V. Untangling Galectin-Driven Regulatory Circuits in Autoimmune Inflammation. *Trends Mol Med* **24**, 348-363, doi:10.1016/j.molmed.2018.02.008 (2018).

143 Mkhikian, H. *et al.* Golgi self-correction generates bioequivalent glycans to preserve cellular homeostasis. *Elife* **5**, doi:10.7554/eLife.14814 (2016).

144 Pace, K. E., Lee, C., Stewart, P. L. & Baum, L. G. Restricted receptor segregation into membrane microdomains occurs on human T cells during apoptosis induced by galectin-1. *J Immunol* **163**, 3801-3811 (1999).

145 Cha, S. K. *et al.* Removal of sialic acid involving Klotho causes cell-surface retention of TRPV5 channel via binding to galectin-1. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 9805-9810, doi:10.1073/pnas.0803223105 (2008).

146 Lee, S. U. *et al.* N-glycan processing deficiency promotes spontaneous inflammatory demyelination and neurodegeneration. *The Journal of biological chemistry* **282**, 33725-33734, doi:10.1074/jbc.M704839200 (2007).

147 Grigorian, A. *et al.* Control of T Cell-mediated autoimmunity by metabolite flux to N-glycan biosynthesis. *J Biol Chem* **282**, 20027-20035, doi:10.1074/jbc.M701890200 (2007).

148 Mkhikian, H. *et al.* Genetics and the environment converge to dysregulate N-glycosylation in multiple sclerosis. *Nat Commun* **2**, 334, doi:10.1038/ncomms1333 (2011).

149 Smith-Garvin, J. E., Koretzky, G. A. & Jordan, M. S. T cell activation. *Annu Rev Immunol* **27**, 591-619, doi:10.1146/annurev.immunol.021908.132706 (2009).

150 Grigorian, A., Torossian, S. & Demetriou, M. T-cell growth, cell surface organization, and the galectin-glycoprotein lattice. *Immunological reviews* **230**, 232-246, doi:10.1111/j.1600-065X.2009.00796.x (2009).

151 Zhou, R. W. *et al.* N-glycosylation bidirectionally extends the boundaries of thymocyte positive selection by decoupling Lck from Ca(2)(+) signaling. *Nat Immunol* **15**, 1038-1045, doi:10.1038/ni.3007 (2014).

152 Togayachi, A. *et al.* Polylactosamine on glycoproteins influences basal levels of lymphocyte and macrophage activation. *Proc Natl Acad Sci U S A* **104**, 15829-15834, doi:10.1073/pnas.0707426104 (2007).

153 Kuball, J. *et al.* Increasing functional avidity of TCR-redirected T cells by removing defined N-glycosylation sites in the TCR constant domain. *J Exp Med* **206**, 463-475, doi:10.1084/jem.20082487 (2009).

154 Alegre, M. L., Frauwirth, K. A. & Thompson, C. B. T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol* **1**, 220-228, doi:10.1038/35105024 (2001).

155 Tao, X., Constant, S., Jorritsma, P. & Bottomly, K. Strength of TCR signal determines the costimulatory requirements for Th1 and Th2 CD4+ T cell differentiation. *J Immunol* **159**, 5956-5963 (1997).

156 Tao, X., Grant, C., Constant, S. & Bottomly, K. Induction of IL-4-producing CD4+ T cells by antigenic peptides altered for TCR binding. *J Immunol* **158**, 4237-4244 (1997).

157 Morgan, R. *et al.* N-acetylglucosaminyltransferase V (Mgat5)-mediated N-glycosylation negatively regulates Th1 cytokine production by T cells. *J Immunol* **173**, 7200-7208 (2004).

158 Araujo, L., Khim, P., Mkhikian, H., Mortales, C. L. & Demetriou, M. Glycolysis and glutaminolysis cooperatively control T cell function by limiting metabolite supply to N-glycosylation. *Elife* **6**, doi:10.7554/eLife.21330 (2017).

159 Grigorian, A. *et al.* N-acetylglucosamine inhibits T-helper 1 (Th1) / T-helper 17 (Th17) responses and treats experimental autoimmune encephalomyelitis. *J Biol Chem*, doi:10.1074/jbc.M111.277814 (2011).

160 Li, C. F. *et al.* Hypomorphic MGAT5 polymorphisms promote multiple sclerosis cooperatively with MGAT1 and interleukin-2 and 7 receptor variants. *Journal of neuroimmunology*, doi:10.1016/j.jneuroim.2012.12.008 (2013).

161 Mkhikian, H. *et al.* Genetics and the environment converge to dysregulate N-glycosylation in multiple sclerosis. *Nature communications* **2**, 334, doi:10.1038/ncomms1333 (2011).

162 Yu, Z. *et al.* Family studies of type 1 diabetes reveal additive and epistatic effects between MGAT1 and three other polymorphisms. *Genes Immun*, doi:10.1038/gene.2014.7 (2014).

163 Koch, U. & Radtke, F. Mechanisms of T cell development and transformation. *Annu Rev Cell Dev Biol* **27**, 539-562, doi:10.1146/annurev-cellbio-092910-154008 (2011).

164 Starr, T. K., Jameson, S. C. & Hogquist, K. A. Positive and negative selection of T cells. *Annu Rev Immunol* **21**, 139-176, doi:10.1146/annurev.immunol.21.120601.141107 (2003).

165 Hennet, T., Chui, D., Paulson, J. C. & Marth, J. D. Immune regulation by the ST6Gal sialyltransferase. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 4504-4509, doi:10.1073/pnas.95.8.4504 (1998).

166 Clark, E. A. & Giltiay, N. V. CD22: A Regulator of Innate and Adaptive B Cell Responses and Autoimmunity. *Front Immunol* **9**, 2235, doi:10.3389/fimmu.2018.02235 (2018).

167 Collins, B. E., Smith, B. A., Bengtson, P. & Paulson, J. C. Ablation of CD22 in ligand-deficient mice restores B cell receptor signaling. *Nat Immunol* **7**, 199-206, doi:10.1038/ni1283 (2006).

168 Nitschke, L., Carsetti, R., Ocker, B., Kohler, G. & Lamers, M. C. CD22 is a negative regulator of B-cell receptor signalling. *Curr Biol* **7**, 133-143, doi:10.1016/s0960-9822(06)00057-1 (1997).

169 Otipoby, K. L. *et al.* CD22 regulates thymus-independent responses and the lifespan of B cells. *Nature* **384**, 634-637, doi:10.1038/384634a0 (1996).

170 Santos, L. *et al.* Dendritic cell-dependent inhibition of B cell proliferation requires CD22. *J Immunol* **180**, 4561-4569, doi:10.4049/jimmunol.180.7.4561 (2008).

171 Danzer, C. P., Collins, B. E., Blixt, O., Paulson, J. C. & Nitschke, L. Transitional and marginal zone B cells have a high proportion of unmasked CD22: implications for BCR signaling. *Int Immunol* **15**, 1137-1147, doi:10.1093/intimm/dxg114 (2003).

172 Samardzic, T. *et al.* Reduction of marginal zone B cells in CD22-deficient mice. *Eur J Immunol* **32**, 561-567, doi:10.1002/1521-4141(200202)32:2<561::AID-IMMU561>3.0.CO;2-H (2002).

173 Poe, J. C. & Tedder, T. F. CD22 and Siglec-G in B cell function and tolerance. *Trends Immunol* **33**, 413-420, doi:10.1016/j.it.2012.04.010 (2012).

174 Jellusova, J., Wellmann, U., Amann, K., Winkler, T. H. & Nitschke, L. CD22 x Siglec-G double-deficient mice have massively increased B1 cell numbers and develop systemic autoimmunity. *J Immunol* **184**, 3618-3627, doi:10.4049/jimmunol.0902711 (2010).

175 Manhardt, C. T., Punch, P. R., Dougher, C. W. L. & Lau, J. T. Y. Extrinsic sialylation is dynamically regulated by systemic triggers in vivo. *J Biol Chem* **292**, 13514-13520, doi:10.1074/jbc.C117.795138 (2017).

176 Irons, E. E. & Lau, J. T. Y. Systemic ST6Gal-1 Is a Pro-survival Factor for Murine Transitional B Cells. *Front Immunol* **9**, 2150, doi:10.3389/fimmu.2018.02150 (2018).

177 Ubelhart, R. *et al.* N-linked glycosylation selectively regulates autonomous precursor BCR function. *Nat Immunol* **11**, 759-765, doi:10.1038/ni.1903 (2010).

178 Li, W. *et al.* Core fucosylation of mu heavy chains regulates assembly and intracellular signaling of precursor B cell receptors. *J Biol Chem* **287**, 2500-2508, doi:10.1074/jbc.M111.303123 (2012).

179 Bonzi, J. *et al.* Pre-B cell receptor binding to galectin-1 modifies galectin-1/carbohydrate affinity to modulate specific galectin-1/glycan lattice interactions. *Nature communications* **6**, 6194, doi:10.1038/ncomms7194 (2015).

180 Gauthier, L., Rossi, B., Roux, F., Termine, E. & Schiff, C. Galectin-1 is a stromal cell ligand of the pre-B cell receptor (BCR) implicated in synapse formation between pre-B and stromal cells and in pre-BCR triggering. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 13014-13019, doi:10.1073/pnas.202323999 (2002).

181 Rossi, B., Espeli, M., Schiff, C. & Gauthier, L. Clustering of pre-B cell integrins induces galectin-1-dependent pre-B cell receptor relocalization and activation. *J Immunol* **177**, 796-803, doi:10.4049/jimmunol.177.2.796 (2006).

182 Mourcin, F. *et al.* Galectin-1-expressing stromal cells constitute a specific niche for pre-BII cell development in mouse bone marrow. *Blood* **117**, 6552-6561, doi:10.1182/blood-2010-12-323113 (2011).

183 Espeli, M., Mancini, S. J., Breton, C., Poirier, F. & Schiff, C. Impaired B-cell development at the pre-BII-cell stage in galectin-1-deficient mice due to inefficient pre-BII/stromal cell interactions. *Blood* **113**, 5878-5886, doi:10.1182/blood-2009-01-198465 (2009).

184 de Oliveira, F. L. *et al.* Lack of galectin-3 modifies differentially Notch ligands in bone marrow and spleen stromal cells interfering with B cell differentiation. *Sci Rep* **8**, 3495, doi:10.1038/s41598-018-21409-7 (2018).

185 Otipoby, K. L., Draves, K. E. & Clark, E. A. CD22 regulates B cell receptor-mediated signals via two domains that independently recruit Grb2 and SHP-1. *J Biol Chem* **276**, 44315-44322, doi:10.1074/jbc.M105446200 (2001).

186 Poe, J. C., Fujimoto, M., Jansen, P. J., Miller, A. S. & Tedder, T. F. CD22 forms a quaternary complex with SHIP, Grb2, and Shc. A pathway for regulation of B lymphocyte antigen receptor-induced calcium flux. *J Biol Chem* **275**, 17420-17427, doi:10.1074/jbc.M001892200 (2000).

187 Yohannan, J., Wienands, J., Coggeshall, K. M. & Justement, L. B. Analysis of tyrosine phosphorylation-dependent interactions between stimulatory effector proteins and the B cell co-receptor CD22. *J Biol Chem* **274**, 18769-18776, doi:10.1074/jbc.274.26.18769 (1999).

188 Fujimoto, M. *et al.* B cell antigen receptor and CD40 differentially regulate CD22 tyrosine phosphorylation. *J Immunol* **176**, 873-879, doi:10.4049/jimmunol.176.2.873 (2006).

189 Han, S., Collins, B. E., Bengtson, P. & Paulson, J. C. Homomultimeric complexes of CD22 in B cells revealed by protein-glycan cross-linking. *Nature chemical biology* **1**, 93-97, doi:10.1038/nchembio713 (2005).

190 Gasparrini, F. *et al.* Nanoscale organization and dynamics of the siglec CD22 cooperate with the cytoskeleton in restraining BCR signalling. *Embo J* **35**, 258-280, doi:10.15252/embj.201593027 (2016).

191 Kawasaki, N., Rademacher, C. & Paulson, J. C. CD22 regulates adaptive and innate immune responses of B cells. *J Innate Immun* **3**, 411-419, doi:10.1159/000322375 (2011).

192 Sato, S. *et al.* CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: altered signaling in CD22-deficient mice. *Immunity* **5**, 551-562, doi:10.1016/s1074-7613(00)80270-8 (1996).

193 Tuscano, J., Engel, P., Tedder, T. F. & Kehrl, J. H. Engagement of the adhesion receptor CD22 triggers a potent stimulatory signal for B cells and blocking CD22/CD22L interactions impairs T-cell proliferation. *Blood* **87**, 4723-4730 (1996).

194 Onodera, T., Poe, J. C., Tedder, T. F. & Tsubata, T. CD22 regulates time course of both B cell division and antibody response. *J Immunol* **180**, 907-913, doi:10.4049/jimmunol.180.2.907 (2008).

195 Horikawa, K. *et al.* Enhancement and suppression of signaling by the conserved tail of IgG memory-type B cell antigen receptors. *J Exp Med* **204**, 759-769, doi:10.1084/jem.20061923 (2007).

196 Waisman, A. *et al.* IgG1 B cell receptor signaling is inhibited by CD22 and promotes the development of B cells whose survival is less dependent on Ig alpha/beta. *J Exp Med* **204**, 747-758, doi:10.1084/jem.20062024 (2007).

197 Chappell, C. P., Draves, K. E. & Clark, E. A. CD22 is required for formation of memory B cell precursors within germinal centers. *PLoS One* **12**, e0174661, doi:10.1371/journal.pone.0174661 (2017).

198 Zeng, J. *et al.* The generation of influenza-specific humoral responses is impaired in ST6Gal I-deficient mice. *J Immunol* **182**, 4721-4727, doi:10.4049/jimmunol.0802833 (2009).

199 O'Keefe, T. L., Williams, G. T., Batista, F. D. & Neuberger, M. S. Deficiency in CD22, a B cell-specific inhibitory receptor, is sufficient to predispose to development of high affinity autoantibodies. *J Exp Med* **189**, 1307-1313, doi:10.1084/jem.189.8.1307 (1999).

200 Mary, C. *et al.* Dysregulated expression of the Cd22 gene as a result of a short interspersed nucleotide element insertion in Cd22a lupus-prone mice. *J Immunol* **165**, 2987-2996, doi:10.4049/jimmunol.165.6.2987 (2000).

201 Law, C. L. *et al.* Organization of the murine Cd22 locus. Mapping to chromosome 7 and characterization of two alleles. *J Immunol* **151**, 175-187 (1993).

202 Nitschke, L. *et al.* Expression of aberrant forms of CD22 on B lymphocytes in Cd22a lupus-prone mice affects ligand binding. *Int Immunol* **18**, 59-68, doi:10.1093/intimm/dxh349 (2006).

203 Hatta, Y. *et al.* Identification of the gene variations in human CD22. *Immunogenetics* **49**, 280-286, doi:10.1007/s002510050494 (1999).

204 Hitomi, Y. *et al.* Association of CD22 gene polymorphism with susceptibility to limited cutaneous systemic sclerosis. *Tissue Antigens* **69**, 242-249, doi:10.1111/j.1399-0039.2007.00801.x (2007).

205 Meesmann, H. M. *et al.* Decrease of sialic acid residues as an eat-me signal on the surface of apoptotic lymphocytes. *J Cell Sci* **123**, 3347-3356, doi:10.1242/jcs.066696 (2010).

206 Colonna, L., Lood, C. & Elkon, K. B. Beyond apoptosis in lupus. *Curr Opin Rheumatol* **26**, 459-466, doi:10.1097/BOR.0000000000000083 (2014).

207 Giovannone, N. *et al.* Galectin-9 suppresses B cell receptor signaling and is regulated by I-branching of N-glycans. *Nature communications* **9**, 3287, doi:10.1038/s41467-018-05770-9 (2018).

208 Giovannone, N., Smith, L. K., Treanor, B. & Dimitroff, C. J. Galectin-Glycan Interactions as Regulators of B Cell Immunity. *Front Immunol* **9**, 2839, doi:10.3389/fimmu.2018.02839 (2018).

209 Tsai, C. M. *et al.* Phosphoproteomic analyses reveal that galectin-1 augments the dynamics of B-cell receptor signaling. *J Proteomics* **103**, 241-253, doi:10.1016/j.jprot.2014.03.031 (2014).

210 Tsai, C. M. *et al.* Galectin-1 and galectin-8 have redundant roles in promoting plasma cell formation. *J Immunol* **187**, 1643-1652, doi:10.4049/jimmunol.1100297 (2011).

211 Yu, X., Siegel, R. & Roeder, R. G. Interaction of the B cell-specific transcriptional coactivator OCA-B and galectin-1 and a possible role in regulating BCR-mediated B cell proliferation. *J Biol Chem* **281**, 15505-15516, doi:10.1074/jbc.M509041200 (2006).

212 Tabrizi, S. J. *et al.* T cell leukemia/lymphoma 1 and galectin-1 regulate survival/cell death pathways in human naive and IgM+ memory B cells through altering balances in Bcl-2 family proteins. *J Immunol* **182**, 1490-1499, doi:10.4049/jimmunol.182.3.1490 (2009).

213 Beccaria, C. G. *et al.* Galectin-3 deficiency drives lupus-like disease by promoting spontaneous germinal centers formation via IFN-gamma. *Nature communications* **9**, 1628, doi:10.1038/s41467-018-04063-5 (2018).

214 Ungerer, C. *et al.* Galectin-9 is a suppressor of T and B cells and predicts the immune modulatory potential of mesenchymal stromal cell preparations. *Stem Cells Dev* **23**, 755-766, doi:10.1089/scd.2013.0335 (2014).

215 Cao, A. *et al.* Galectin-9 binds IgM-BCR to regulate B cell signaling. *Nature communications* **9**, 3288, doi:10.1038/s41467-018-05771-8 (2018).

216 Sharma, S. *et al.* T cell immunoglobulin and mucin protein-3 (Tim-3)/Galectin-9 interaction regulates influenza A virus-specific humoral and CD8 T-cell responses. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 19001-19006, doi:10.1073/pnas.1107087108 (2011).

217 Obino, D. *et al.* Galectin-8 Favors the Presentation of Surface-Tethered Antigens by Stabilizing the B Cell Immune Synapse. *Cell Rep* **25**, 3110-3122 e3116, doi:10.1016/j.celrep.2018.11.052 (2018).

218 Rolli, V. *et al.* Amplification of B cell antigen receptor signaling by a Syk/ITAM positive feedback loop. *Mol Cell* **10**, 1057-1069, doi:10.1016/s1097-2765(02)00739-6 (2002).

219 Okkenhaug, K. Signaling by the phosphoinositide 3-kinase family in immune cells. *Annu Rev Immunol* **31**, 675-704, doi:10.1146/annurev-immunol-032712-095946 (2013).

220 Rickert, R. C. New insights into pre-BCR and BCR signalling with relevance to B cell malignancies. *Nat Rev Immunol* **13**, 578-591, doi:10.1038/nri3487 (2013).

221 Botos, I., Segal, D. M. & Davies, D. R. The structural biology of Toll-like receptors. *Structure* **19**, 447-459, doi:10.1016/j.str.2011.02.004 (2011).

222 Zanoni, I. *et al.* CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. *Cell* **147**, 868-880, doi:10.1016/j.cell.2011.09.051 (2011).

223 Akashi-Takamura, S. & Miyake, K. TLR accessory molecules. *Curr Opin Immunol* **20**, 420-425, doi:10.1016/j.coi.2008.07.001 (2008).

224 Kawasaki, T. & Kawai, T. Toll-like receptor signaling pathways. *Front Immunol* **5**, 461, doi:10.3389/fimmu.2014.00461 (2014).

225 Blasius, A. L. & Beutler, B. Intracellular toll-like receptors. *Immunity* **32**, 305-315, doi:10.1016/j.immuni.2010.03.012 (2010).

226 Siegemund, S. & Sauer, K. Balancing pro- and anti-inflammatory TLR4 signaling. *Nat Immunol* **13**, 1031-1033, doi:10.1038/ni.2452 (2012).

Chapter 2

N-glycan Branching is Required for B Cell Positive Selection

SUMMARY

Cell function and differentiation is dynamically controlled by the branching, extension and number of complex Asn (N) linked-glycans per protein molecule. Branched N-glycans regulate binding to the galectin family of lectins, forming a cell-surface galectin-glycoprotein lattice that controls the distribution, clustering, endocytosis and signaling of surface glycoproteins in a coordinated manner. During T cell development, N-glycan branching is absolutely required for positive selection of thymocytes, inhibiting both death by neglect and negative selection via enhanced surface retention of the CD4/CD8 co-receptors and limiting TCR clustering/signaling, respectively. In B cells, galectins have been implicated in inhibiting B cell receptor (BCR) signaling in mature B cells but promoting pre-B cell receptor signaling during early development. Here we report that N-glycan branching is absolutely required for positive selection of B cells. Elimination of branched N-glycans in developing B cells via targeted deletion of N-acetylglucosaminyl transferase I (Mgat1) markedly reduced cellularity in the bone marrow and spleen, and blocked B cell maturation. Branching deficiency induced spontaneous death of bone marrow derived $B220^{+}IgM^{+}$ cells *in vitro*, a phenotype rescued by low dose BCR stimulation and exacerbated by high dose BCR stimulation. Over-expression of anti-apoptotic Bcl_{xL} and deletion of pro-apoptotic Bim failed to rescue death. Branching deficiency markedly lowered surface expression of the BCR co-receptor CD19 and enhanced Nur77 induction. Thus, N-glycan branching promotes positive selection of B cells by augmenting BCR signaling via CD19 surface retention and concurrently limiting negative selection from excessive BCR engagement, paralleling activity in T cells.

INTRODUCTION

T and B cells (or lymphocytes) undergo similar developmental events and checkpoints that involve the dynamic expression of transcription factors, response to extracellular environmental cues such as cytokines, and the somatic rearrangement, expression, and screening of their antigen receptors in order to generate a functionally diverse, self-tolerant lymphocyte repertoire[1-4]. Abnormalities in lymphocyte development and selection give rise to congenital immunodeficiencies, leukemias/lymphomas, and autoimmune diseases.[5] In the bone marrow, developing B cells rearrange immunoglobulin (Ig) μ heavy (H)-chain and light (L)-chain genes to express surface bound IgM that complexes with immunoreceptor tyrosine-based activation motif (ITAM) containing Igα and Igβ (CD79A and CD79B) signaling proteins to form the B cell receptor (BCR) on immature B cells[6-11]. BCR binding to antigen directs immature B cell fate – when the BCR is engaged by self-antigen, immature B cells die by clonal deletion (negative selection by apoptosis) or undergo receptor editing (continuous L-chain rearrangement) to reduce/eliminate autoreactivity[12]. Immature B cells expressing a self-tolerant BCR continue through transitional developmental stages (T1 and T2) in the bone marrow and the spleen, but predominantly continue maturation into follicular (FO) or marginal zone (MZ) B cells in the spleen and co-express surface bound IgM and IgD.[13] About 25% of developing B cells can become mature B cells in the bone marrow.[14] Unengaged BCRs induce tonic signaling to drive developmental progression and survival of immature B cells in a CD19 dependent manner[15]. CD19 is a transmembrane glycoprotein first expressed in pro-B cells in the bone marrow and augments BCR signaling at multiple points during B cell development, maturation, and

differentiation[16-20]. Mice with targeted deletion of CD19 have greatly reduced B cell numbers, impaired B cell function, and defective immune responses[21,22].

The branching of Asparagine (N)-linked glycans with N-acetylglucosamine by the N-acetylglucosaminyl transferases Mgat1, Mgat2, Mgat4a/b and Mgat5 sequentially increase production of ligands for the galectin family of sugar-binding proteins[23,24]. At the cell surface, interactions of galectins with branched N-glycans attached to glycoproteins generates a macromolecular lattice, thereby controlling receptor localization, mobility, clustering, and surface retention to impact cell function/differentiation and disease states[25-33]. Our group demonstrated that N-glycan branching regulates thymic positive selection by defining the upper and lower bounds of TCR affinity for peptide-MHC[33]. Using a T cell specific (Lck driven cre) knockout of the N-glycan branching enzyme Mgat1, we observed markedly reduced thymic and splenic T cell numbers. Mgat1 deletion enhanced thymocyte death by neglect via decreased CD4/CD8 co-receptor surface retention (i.e., enhanced CD4/CD8 endocytosis) and associated reduced Lck induced Erk signaling, which are important for augmenting low affinity TCR engagement. N-glycan deficient thymocytes simultaneously exhibited increased death by negative selection due to excessive Ca^{2+} flux driven by enhanced TCR clustering. Thus, during T cell development N-glycan branching provides a mechanism for decoupling CD4/CD8 co-receptor and TCR signaling to maintain the appropriate range of TCR signal intensity necessary for thymocyte positive selection and generation of functional circulating $CD4^+$ and $CD8^+$ T cells.

Various studies have demonstrated glycosylation to be important in B cell development, selection, and maturation. This includes the sialic acid-binding

immunoglobulin-type lectin (Siglec) and B cell inhibitory co-receptor CD22[34-37], the sialyltransferace ST6Gal1[38], fucosylation[39,40], and galectins[41-46]. The role of N-glycan branching, however, has not been investigated. Here, we demonstrate N-glycan branching promotes positive selection of B cells by enhancing low affinity BCR engagement via promoting CD19 surface levels while also reducing high affinity BCR engagement to prevent negative selection.

MATERIALS AND METHODS

Mice

Mgat1$^{f/f}$ (006891), *Mgat2*$^{f/f}$ (006892), *CD19-cre* (006785), and *Bim*$^{-/-}$ (004525) mice were obtained from Jackson Laboratory. *Eμ-Bcl*$_{xL}$ mice were transferred to us by Dr. Brian Iritani from the Department of Comparative Medicine at the University of Washington, Seattle. Inter-breeding generated all other mice. Mice used were 5-7 weeks old but otherwise selected randomly for experiments and approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Flow cytometry

Flow cytometric analysis was performed as previously described (Demetriou et al., 2001; Grigorian et al., 2007; Zhou et al., 2014). The fluorophore conjugated mouse specific antibodies from Thermo Fisher/eBioscience were CD43 (R2/60), IgM (II/41), B220 (RA3-6B2), CD19 (eBio1D3), CD23 (B3B4), and CD21 (8D9). For flow cytometric analysis of glycan expression, cells were stained with 2 μg/mL L-PHA-FITC or biotinylated L-PHA followed by streptavidin conjugated fluorophore (Vector Labs and Thermo Fisher Scientific, respectively). Fluorophore conjugated Annexin-V was from BD Pharmingen, and fluorophore conjugated Nur77 from Cell Signaling Technologies. Samples were acquired on the Attune NxT (Thermo Fisher Scientific) flow cytometer. Data analysis was performed using FlowJo software.

Cell culture and stimulation

For *in vitro* experiments, isolated bone marrow cells were cultured in RPMI-1640 media (Thermo Fisher Scientific) supplemented with 10% heat inactivated fetal bovine serum (VWR), 2 μM L-glutamine and 100 U/mL penicillin/streptomycin (Gibco), and 50 μM β-mercaptoethanol (Gibco). Cells were cultured at 2.5 $x10^5$ cells per well in a 96-well plate in triplicates without stimulation, or in the presence of functional grade goat anti-mouse IgM $F(ab')_2$ (polyclonal, Thermo Fisher Scientific) at various concentrations.

RESULTS

N-glycan branching is required for B cell generation.

To investigate a role for N-glycan branching in B cell development, we generated B cell specific *Mgat1* and *Mgat2* deficient mice using *CD19-cre*. *Mgat1* deletion completely eliminates while *Mgat2* deletion reduces N-glycan branching (Fig. 2.1a). CD19 expression is initiated at the pro-B cell stage[16,17], however, membrane turnover of glycans is delayed. Flow cytometry with the plant lectin L-PHA, which binds to branched N-glycans eliminated by deletion of the *Mgat1* or *2* genes, was used to assess deletion of *Mgat1* and *Mgat2* in the bone marrow and spleen of $B220^+$ B cells from $Mgat1^{f/f}/CD19\text{-}cre^{+/-}$ and $Mgat2^{f/f}/CD19\text{-}cre^{+/-}$ mice, respectively (Fig. 2.2a). In $Mgat2^{f/f}/CD19\text{-}cre^{+/-}$ mice, pro-B cells displayed a small population with reduced L-PHA binding ($L\text{-}PHA^{int}$) that became a larger population of $L\text{-}PHA^{lo/-}$ while transitioning to the mature B cell stage and entering the periphery (Fig. 2.2a). In contrast, only a small proportion of $L\text{-}PHA^{int}$ pre-B and immature B cells are observed in $Mgat1^{f/f}/CD19\text{-}cre^{+/-}$ mice, with the population largely disappearing at the mature B cell stage and in peripheral splenic B cells (Fig. 2.2a). This suggests deletion of *Mgat1* triggers death while the immature B cells are losing branched N-glycans from the cell surface, with all cells dying prior to the complete loss of branching. The population of $L\text{-}PHA^+$ B cells likely result from inefficient CD19-cre mediated excision of $Mgat1^{f/f}$ and continued expansion of this $L\text{-}PHA^+$ cell population (Fig. 2.2a). Consistent with loss of N-glycan branching leading to death of developing B cells, bone marrow and spleen cellularity (Fig. 2.2b) as well as number of $B220^+$ B cells (Fig. 2.2c) were all markedly reduced in $Mgat1^{f/f}/CD19\text{-}cre^{+/-}$ but not in $Mgat2^{f/f}/CD19\text{-}cre^{+/-}$ mice. These results demonstrate

partial N-glycan branching levels is permissible for B cell generation, while complete elimination of N-glycan branching fails to generate mature B cells.

N-glycan branching drives late bone marrow development and splenic maturation of B cells.

Next, we delineated at what stage loss of N-glycan branching impacts B cell development and maturation in the bone marrow. Cell surface markers and gating strategy used to identify specific B cell populations were as previously described (Hardy et al., 2007; Carsetti, 2004) and is depicted in Figure 2.1b,c. We focused on *Mgat1*$^{f/f}$*/CD19-cre*$^{+/-}$ mice as they displayed significant loss of B cells in the bone marrow and spleen. In the bone marrow of *Mgat1*$^{f/f}$*/CD19-cre*$^{+/-}$ mice, cellularity at each developmental stage revealed little difference at the pro-, pre-, and immature stages, but a drastic loss in the number of mature B cells in (Fig. 2.3a). In the spleen, transitional T1, transitional T2, follicular (FO) and marginal zone (MZ) B cells are similarly reduced by *Mgat1* deficiency (Fig. 2.3b). These data suggest that branching is essential for late B cell development in bone marrow, particularly during the immature to mature B cell transition.

Inhibiting programmed cell death fails to rescue B cell death from the loss of N-glycan branching.

In T cells, we previously observed that the death of developing thymocytes induced by *Mgat1* deletion was rescued by over-expression of anti-apoptotic Bcl_{xL} as well as targeted deletion of pro-apoptotic Bim[33]. Therefore we investigated whether

manipulating these programmed cell death pathways (i.e., the mitochondrial apoptotic pathway[47]) could similarly rescue death of N-glycan branching deficient B cells. We generated *Mgat1*$^{f/f}$*/CD19-cre*$^{+/-}$*/Eμ-Bcl*$_{xL}$ to induce B cell specific anti-apoptotic Bcl_{xL} overexpression, as well as *Mgat1*$^{f/f}$*/CD19-cre*$^{+/-}$*/Bim*$^{-/-}$ for deletion of pro-apoptotic Bim. As *Mgat1* deficiency primarily altered the transition from immature to mature B cells in the bone marrow, we focused on changes in mature B cells. Neither *Mgat1*$^{f/f}$*/CD19-cre*$^{+/-}$*/Eμ-Bcl*$_{xL}$ nor *Mgat1*$^{f/f}$*/CD19-cre*$^{+/-}$*/Bim*$^{-/-}$ displayed an increase in the proportion of mature B cells (Fig. 2.4). Thus, Bcl_{xL} overexpression and Bim deletion both fail to overcome the developmental block of N-glycan branching deficient immature B cells into mature B cells.

N-glycan branching promotes CD19-BCR tonic signaling to inhibit immature B cell death by neglect.

Immature B cells expressing a BCR are subject to selection. Only intermediate (i.e., tonic) BCR signaling allows continued development into mature B cells. As *Mgat1*$^{f/f}$*/CD19-cre*$^{+/-}$ mice displayed a major defect in the immature to mature B cell transition in bone marrow, we assessed whether this was due to dysregulated selection. CD19 surface expression was drastically reduced in L-PHA^{int}B220^{+} B cells from the bone marrow of *Mgat1*$^{f/f}$*/CD19-cre*$^{+/-}$ mice (Fig. 2.5a,b). To assess whether N-glycan branching deficiency drives cell death, we cultured bone marrow cells for one day in media without stimulation and assessed cell death by Annexin V labeling. L-PHA^{int}B220^{+}IgM^{+} cells from *Mgat1*$^{f/f}$*/CD19-cre*$^{+/-}$ mice displayed a marked increase in spontaneous death relative to control cells (Fig. 2.5c), consistent with increased

sensitivity to death by loss of CD19 dependent BCR tonic signaling. Bcl_{xL} overexpression in L-PHA^{int}B220^{+}IgM^{+} cells led to a small reduction in cell death, but was still markedly greater than control cells (Fig. 2.5c). Next, we assessed whether providing minimal BCR signaling would reduce the death induced by N-glycan branching deficiency and loss of CD19. Indeed, low doses of anti-IgM F(ab')$_2$ partially rescued L-PHA^{int}B220^{+}IgM^{+} cells from death (Fig. 2.5d). These data suggest that N-glycan branching promotes CD19 surface expression and associated BCR tonic signaling to prevent death by neglect of IgM^{+} B cells.

N-glycan branching inhibits negative selection of immature B cells.

Nur77 is a transcription factor whose expression corresponds with BCR tonic signaling levels and has been found to be expressed at higher levels in B cells subject to excessive antigen encounter and eventual deletion by negative selection[48-50]. *Ex vivo* L-PHA^{int}B220^{+}IgM^{+} cells displayed greater Nur77 levels compared to control (Fig 2.6a). This suggests N-glycan branching deficient IgM^{+} B cells from *Mgat1$^{f/f}$/CD19-cre$^{+/-}$* mice have enhanced BCR engagement and negative selection *in vivo*. To further support this conclusion, we examined whether providing high BCR signaling further enhances the death of B cells deficient in N-glycan branching. Indeed, BCR stimulation with high doses of anti-IgM F(ab')$_2$ enhanced cell death of L-PHA^{int}B220^{+}IgM^{+} cells, as evidenced by Annexin V labeling (Fig. 2.6b). Together, these data suggest that N-glycan branching also inhibits negative selection of immature B cells.

DISCUSSION

The effects of N-glycan branching during B cell positive selection is similar to what we have observed in T cells[33]. As with T cells, N-glycan branching appears to be required for maintaining the boundaries of BCR signaling thresholds to permit immature B cell positive selection, acting by inhibiting both death by neglect and negative selection. N-glycan branching promotes CD19 surface expression to enhance low affinity BCR engagement and maintain tonic signaling to inhibit death by neglect. The mechanism by which N-glycan branching inhibits death by negative selection is less clear, but may be similar to that in T cells, namely limiting antigen induced BCR clustering to prevent excessive signaling and death by negative selection.

Peripheral *Mgat5*$^{-/-}$ B cells were reported to respond similarly to wildtype B cells when stimulated with various agonists[25]. However, here we observed that loss of branching has a marked effect on B cell development. Interestingly, *Mgat2*$^{f/f}$*/CD19-cre*$^{+/-}$ mice were grossly comparable to controls, yet we expected them to have an intermediate phenotype between *Mgat5* and *Mgat1* deficiency due to having a single N-glycan branch, which should allow formation of a partially intact galectin-glycoprotein lattice. However, we recently showed loss of branching in *Mgat2* deficient T cells is partially compensated for by poly-N-acetyllactosamine (poly-LacNAc) extension of the remaining single GlcNAc branch (Fig. 2.1a), resulting in similar levels of cell surface galectin-3 binding and function as *Mgat5*$^{-/-}$ T cells.[31] Thus, partial N-glycan branching loss in B cells from *Mgat2*$^{f/f}$*/CD19-cre*$^{+/-}$ mice may be compensated for by poly-LacNAc extension to maintain lattice integrity and appropriate BCR signaling thresholds for B cell positive selection. Indeed, unpublished data indicates that *Mgat2* deficient B cells

display the same increase in poly-LacNAc extension that we observed in *Mgat2*$^{-/-}$ T cells.

As CD19 is coupled to BCR signaling, CD19 and BCR deletion studies yield similar phenotypes. For immature B cells, lack of CD19 results in loss of basal phosphoinositide 3-kinase (PI3K) signaling, continued recombination-activating genes (RAG) expression, and L-chain receptor editing, thus inhibiting positive selection.[15] Phosphatase and tensin homologue (PTEN) opposes PI3K activity; the absence of PTEN activity reverses the effects of CD19 loss to promote immature B cell positive selection.[51,52] Similarly, BCR deletion results in loss of mature B cells. Constitutive PI3K signaling was necessary and sufficient to rescue the survival of BCR deficient B cells, whereas other interventions such as constitutive nuclear factor-kappa B (NF-κB) signaling, mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinases (ERK) kinase (MEK) signaling, or Bcl2 overexpression were not.[53,54] While Bcl2 and Bcl_{xL} are both anti-apoptotic factors in programmed cell death, Bcl_{xL} has been shown to be superior in promoting B cell survival[55]; thus we chose to overexpress Bcl_{xL}. In contrast to N-glycan branching deficient thymocytes, neither Bcl_{xL} overexpression nor Bim deletion rescued branching deficient mature B cells *in vivo*. Bcl_{xL} overexpression modestly enhanced survival of bone marrow B cells *in vitro*, so Bcl_{xL} may have a limited effect on survival of N-glycan branching deficient B cells. Notably, branching deficiency induced a much greater loss of cell surface CD19 in developing B cells than CD4 and CD8 co-receptor loss induced in thymocytes (~90% versus ~25-50%, respectively).[33] Therefore, B cell N-glycan branching deficiency largely phenocopies CD19/BCR deficiency that could not be rescued with Bcl2 over-expression, whereas a partial loss of

CD/CD8 in thymocytes is a more mild phenotype that could be rescued by altering apoptotic pathways. Future research examining mechanisms by which N-glycan branching deficiency affects PI3K and apoptosis in immature B cells would provide insight in how branching regulates CD19-BCR downstream signaling pathways and subsequent functional outcomes such as death.

Our recent work on N-glycan branching in thymocytes[33] and now B cell positive selection gives great emphasis to the role branching plays in maintaining central tolerance. Future research in delineating the role of N-glycan branching in B cell peripheral tolerance may provide new insight on the role of B cells in diseases such as systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis.

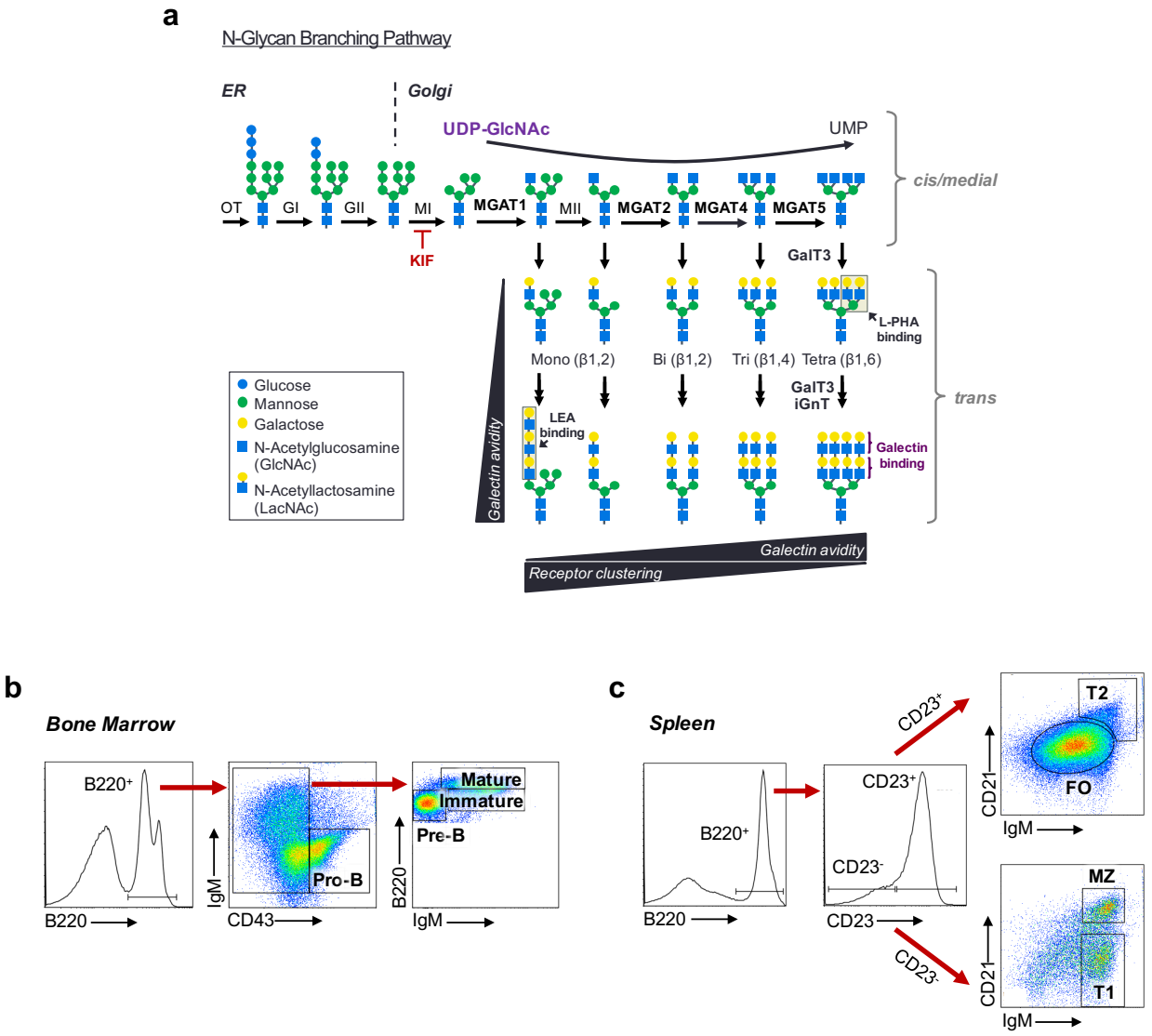

Figure 2.1. N-glycan branching pathway and gating strategies for B cell subset analysis. (**a**) Oligosaccharyltransferase (OT) transfers a pre-assembled glycan, Glc3Man9GlcNAc2, onto Asp-X-Ser-Thr (X≠Pro) motifs on glycoproteins in the endoplasmic reticulum (ER). This glycan is extensively modified as glycoproteins transit through the Golgi by mannosidases and the N-acetylglucosaminyltransferase (Mgat) enzymes, which act sequentially to generate branched N-glycans with increasing affinities for galectins. GalT and iGnT extend branches to create N-acetyllactosamine (LacNac) which can also be bound by galectins. Plant lectin L-PHA and LEA binding sites are indicated. OT, oligosaccharyltransferase; GI, glucosidase I; GII, glucosidase II; MI, mannosidase I; MII, mannosidase II; Mgat, N-acetylglucosaminyltransferase; GalT3, Galactosyltransferase 3; iGnT, "little i" β-1,3-N-acetylglucosaminyltransferase; KIF, kifunensine. (**b**-**c**) Flow cytometric analysis of B cell subsets in the bone marrow (b) and spleen (c).

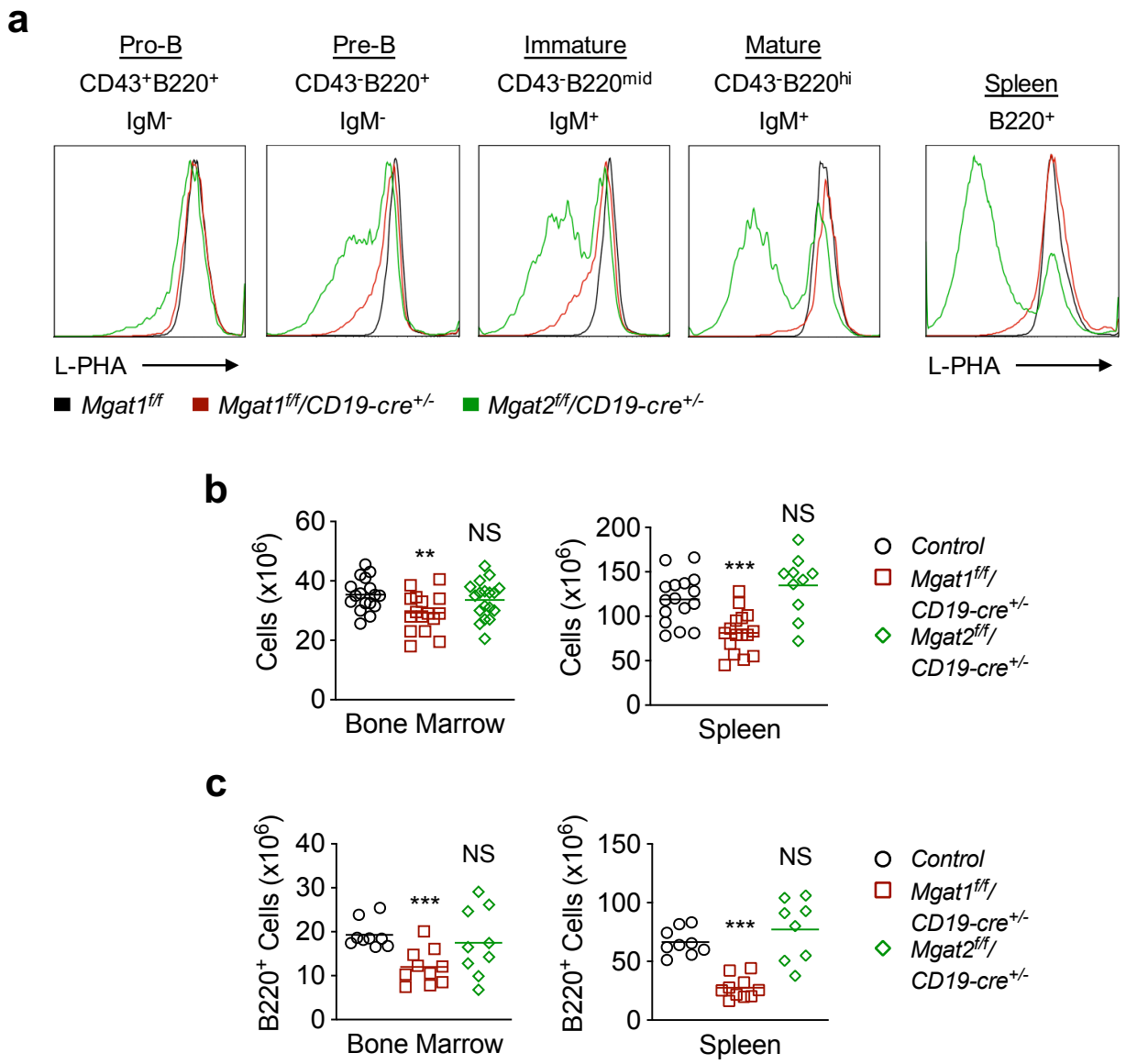

Figure 2.2. Late B cell development requires N-glycan branching. (**a**) Flow cytometric analysis of L-PHA binding on bone marrow B cell subsets and splenic B cells. (**b**) Total cellularity of bone marrow and spleens. (**c**) Total B220$^+$ cell count calculated by multiplying percentage of B220$^+$ cells acquired by flow cytometry by total cellularity of bone marrow and spleens. Each symbol represents one mouse, unpaired two-tailed *t*-tests with Bonferroni's correction (b-c). Bars indicate mean. NS, not significant; ***$P < 0.0005$; **$P < 0.005$.

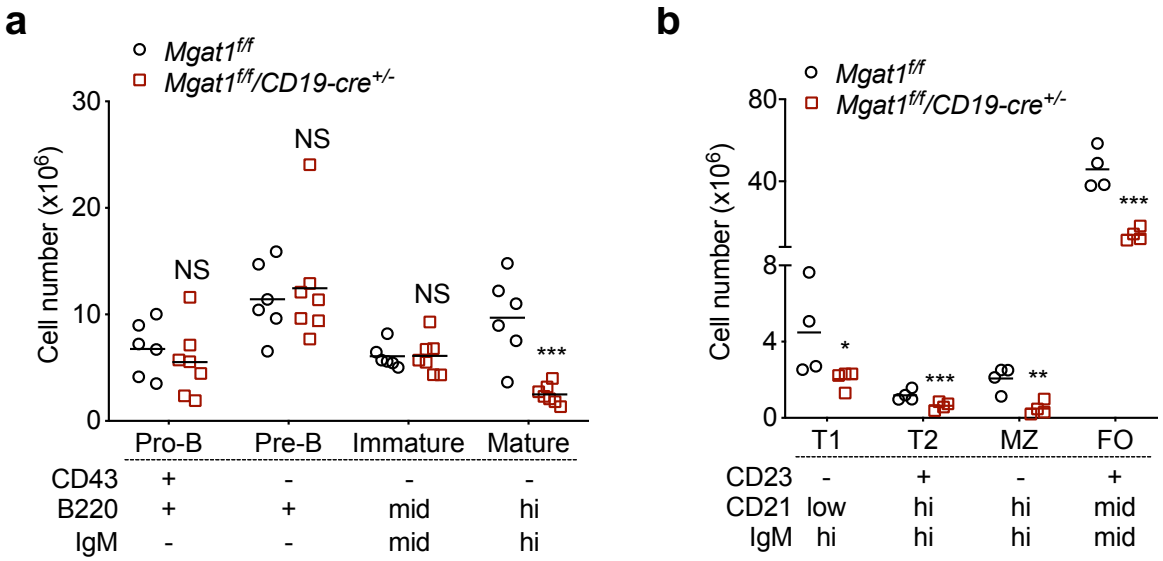

Figure 2.3. N-glycan branching promotes transition of immature to mature B cells in bone marrow and B cell maturation in the spleen. (**a-b**) Flow cytometric analysis of total number of B cell subtypes in the bone marrow (a) and spleen (b) using indicated markers. Each symbol represents one mouse, unpaired two-tailed *t*-tests with Welch's correction. Bars indicate mean. NS, not significant; ***$P < 0.0005$; **$P < 0.005$; *$P < 0.05$.

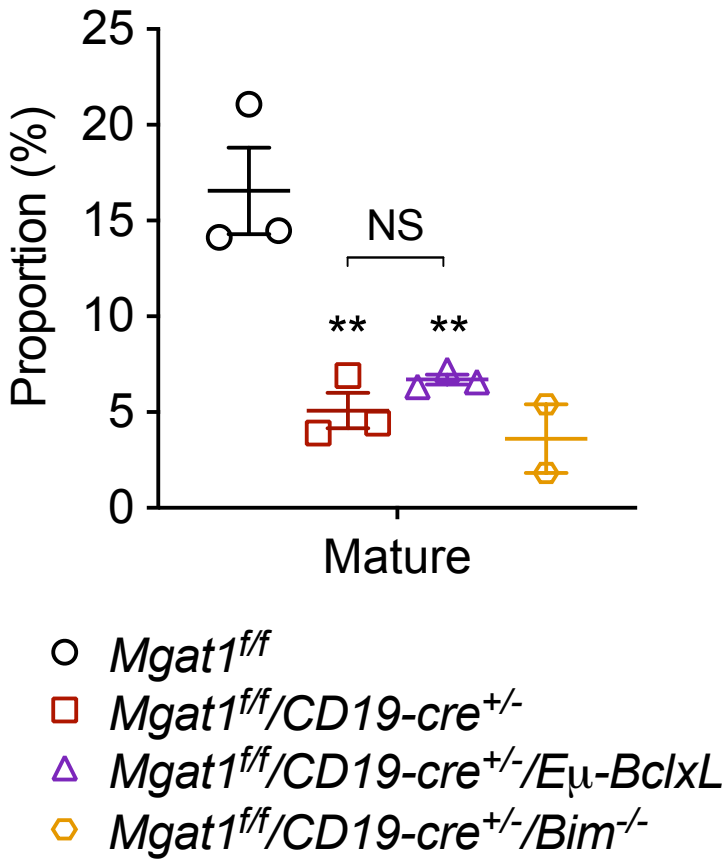

Figure 2.4. Bcl_{xL} overexpression and Bim deletion fail to rescue N-glycan deficient mature B cells. Flow cytometric analysis of mature bone marrow $B220^+$ B cells. Each symbol represents one mouse, unpaired two-tailed *t*-tests with Bonferroni's correction. Error bars indicate mean ± s.e.m. NS, not significant; $^{**}P < 0.005$.

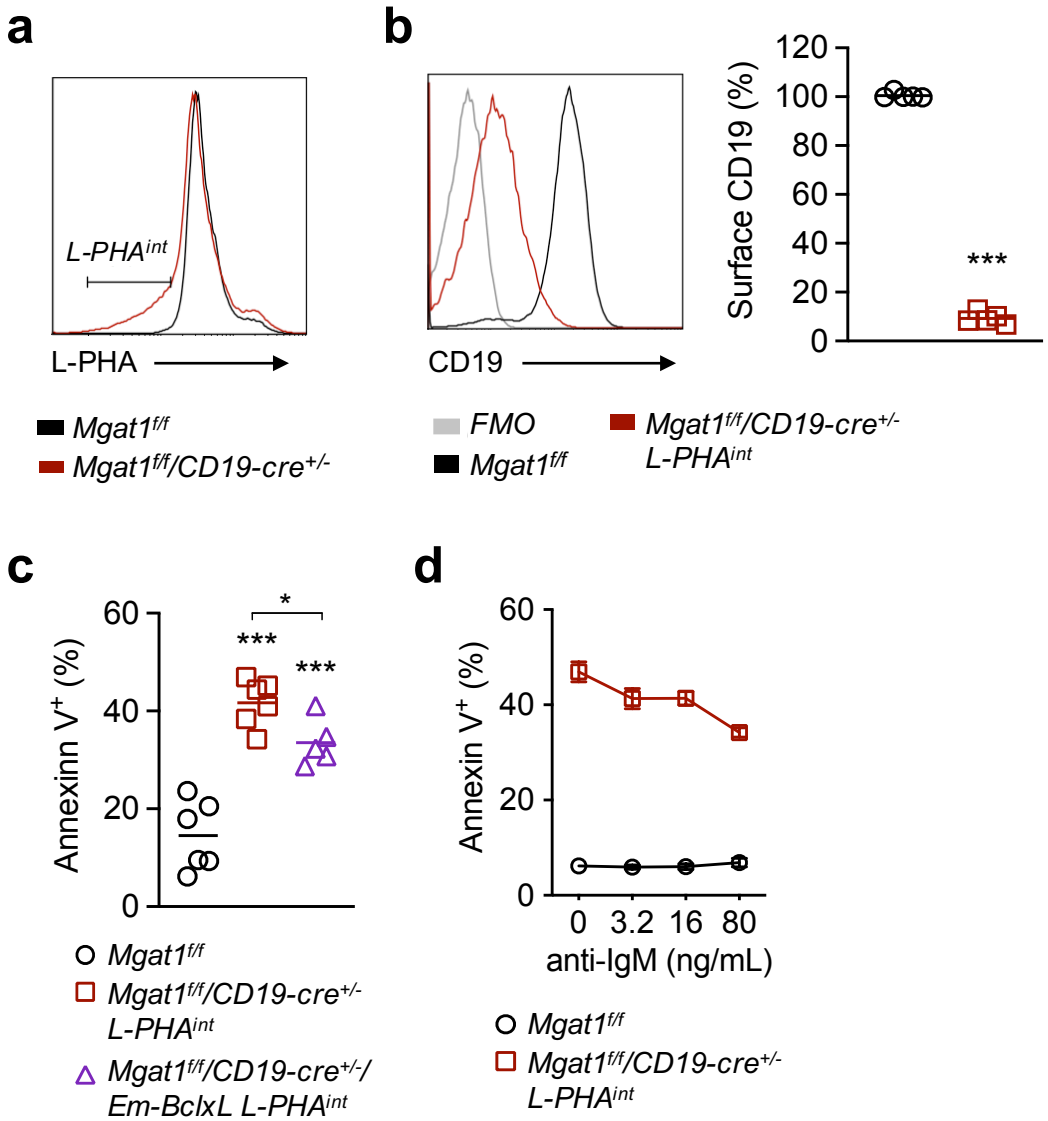

Figure 2.5. N-glycan branching promotes immature B cell positive selection via CD19 surface expression. (**a-d**) Flow cytometric analysis of *ex vivo* $B220^+$ bone marrow B cells for L-PHA^{int} gating (a) and CD19 surface levels (b), Annexin V binding to $B220^+IgM^+$ bone marrow B cells cultured for 1 day without BCR stimulation (c) and with low doses of anti-IgM $F(ab')_2$ (d). $n \geq 3$. Each symbol represents one mouse, unpaired two-tailed *t*-tests (b,c) with Bonferroni's correction (c). Error bars indicate mean ± s.e.m. ***$P < 0.0005$; *$P < 0.05$.

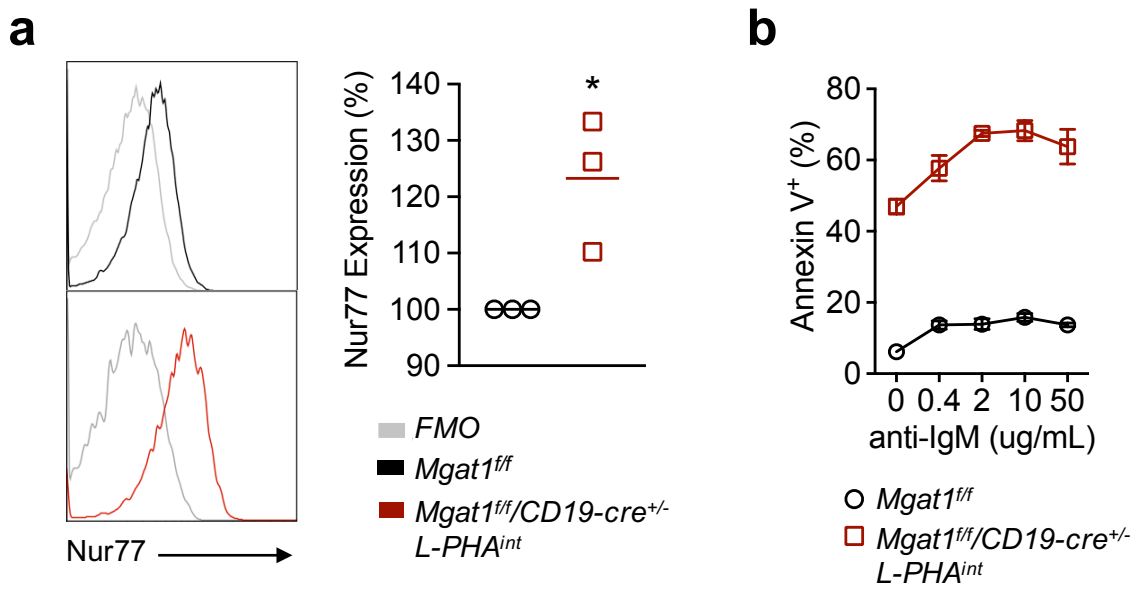

Figure 2.6. N-glycan branching limits immature B cell negative selection by limiting BCR engagement. (**a-b**) Flow cytometric analysis of *ex vivo* $B220^{+}IgM^{+}$ bone marrow B cells for intracellular staining of Nur77 (a), and Annexin V binding after 1 day culture with high doses of anti-IgM $F(ab')_2$ (b). n = 3. Each symbol represents one mouse, unpaired two-tailed *t*-test (a). Error bars indicate mean ± s.e.m. **P* < 0.05.

REFERENCES

1 Ikuta, K., Uchida, N., Friedman, J. & Weissman, I. L. Lymphocyte development from stem cells. *Annu Rev Immunol* **10**, 759-783, doi:10.1146/annurev.iy.10.040192.003551 (1992).

2 Zuniga-Pflucker, J. C. & Lenardo, M. J. Regulation of thymocyte development from immature progenitors. *Curr Opin Immunol* **8**, 215-224, doi:10.1016/s0952-7915(96)80060-4 (1996).

3 LeBien, T. W. & Tedder, T. F. B lymphocytes: how they develop and function. *Blood* **112**, 1570-1580, doi:10.1182/blood-2008-02-078071 (2008).

4 Lin, Y. C. *et al.* A global network of transcription factors, involving E2A, EBF1 and Foxo1, that orchestrates B cell fate. *Nat Immunol* **11**, 635-643, doi:10.1038/ni.1891 (2010).

5 Cooper, M. D. & Alder, M. N. The evolution of adaptive immune systems. *Cell* **124**, 815-822, doi:10.1016/j.cell.2006.02.001 (2006).

6 Pieper, K., Grimbacher, B. & Eibel, H. B-cell biology and development. *J Allergy Clin Immunol* **131**, 959-971, doi:10.1016/j.jaci.2013.01.046 (2013).

7 Niiro, H. & Clark, E. A. Regulation of B-cell fate by antigen-receptor signals. *Nat Rev Immunol* **2**, 945-956, doi:10.1038/nri955 (2002).

8 Pelanda, R. & Torres, R. M. Central B-cell tolerance: where selection begins. *Cold Spring Harb Perspect Biol* **4**, a007146, doi:10.1101/cshperspect.a007146 (2012).

9 King, L. B., Norvell, A. & Monroe, J. G. Antigen receptor-induced signal transduction imbalances associated with the negative selection of immature B cells. *J Immunol* **162**, 2655-2662 (1999).

10 Reth, M. Antigen receptors on B lymphocytes. *Annu Rev Immunol* **10**, 97-121, doi:10.1146/annurev.iy.10.040192.000525 (1992).

11 Monroe, J. G. ITAM-mediated tonic signalling through pre-BCR and BCR complexes. *Nat Rev Immunol* **6**, 283-294, doi:10.1038/nri1808 (2006).

12 Nemazee, D. Mechanisms of central tolerance for B cells. *Nat Rev Immunol* **17**, 281-294, doi:10.1038/nri.2017.19 (2017).

13 Pillai, S. & Cariappa, A. The follicular versus marginal zone B lymphocyte cell fate decision. *Nat Rev Immunol* **9**, 767-777, doi:10.1038/nri2656 (2009).

14 Cariappa, A., Chase, C., Liu, H., Russell, P. & Pillai, S. Naive recirculating B cells mature simultaneously in the spleen and bone marrow. *Blood* **109**, 2339-2345, doi:10.1182/blood-2006-05-021089 (2007).

15 Diamant, E., Keren, Z. & Melamed, D. CD19 regulates positive selection and maturation in B lymphopoiesis: lack of CD19 imposes developmental arrest of immature B cells and consequential stimulation of receptor editing. *Blood* **105**, 3247-3254, doi:10.1182/blood-2004-08-3165 (2005).

16 Hardy, R. R., Kincade, P. W. & Dorshkind, K. The protean nature of cells in the B lymphocyte lineage. *Immunity* **26**, 703-714, doi:10.1016/j.immuni.2007.05.013 (2007).

17 Stamenkovic, I. & Seed, B. CD19, the earliest differentiation antigen of the B cell lineage, bears three extracellular immunoglobulin-like domains and an Epstein-

Barr virus-related cytoplasmic tail. *J Exp Med* **168**, 1205-1210, doi:10.1084/jem.168.3.1205 (1988).

18 Carter, R. H. & Fearon, D. T. CD19: lowering the threshold for antigen receptor stimulation of B lymphocytes. *Science* **256**, 105-107, doi:10.1126/science.1373518 (1992).

19 Chalupny, N. J. *et al.* Tyrosine phosphorylation of CD19 in pre-B and mature B cells. *Embo J* **12**, 2691-2696 (1993).

20 Uckun, F. M. *et al.* Signal transduction through the CD19 receptor during discrete developmental stages of human B-cell ontogeny. *J Biol Chem* **268**, 21172-21184 (1993).

21 Engel, P. *et al.* Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. *Immunity* **3**, 39-50, doi:10.1016/1074-7613(95)90157-4 (1995).

22 Rickert, R. C., Rajewsky, K. & Roes, J. Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. *Nature* **376**, 352-355, doi:10.1038/376352a0 (1995).

23 Dennis, J. W., Nabi, I. R. & Demetriou, M. Metabolism, cell surface organization, and disease. *Cell* **139**, 1229-1241, doi:10.1016/j.cell.2009.12.008 (2009).

24 Grigorian, A., Torossian, S. & Demetriou, M. T-cell growth, cell surface organization, and the galectin-glycoprotein lattice. *Immunological reviews* **230**, 232-246, doi:10.1111/j.1600-065X.2009.00796.x (2009).

25 Demetriou, M., Granovsky, M., Quaggin, S. & Dennis, J. W. Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature* **409**, 733-739, doi:10.1038/35055582 (2001).

26 Brewer, C. F., Miceli, M. C. & Baum, L. G. Clusters, bundles, arrays and lattices: novel mechanisms for lectin-saccharide-mediated cellular interactions. *Current opinion in structural biology* **12**, 616-623 (2002).

27 Lau, K. S. *et al.* Complex N-glycan number and degree of branching cooperate to regulate cell proliferation and differentiation. *Cell* **129**, 123-134, doi:10.1016/j.cell.2007.01.049 (2007).

28 Chen, I. J., Chen, H. L. & Demetriou, M. Lateral compartmentalization of T cell receptor versus CD45 by galectin-N-glycan binding and microfilaments coordinate basal and activation signaling. *J Biol Chem* **282**, 35361-35372, doi:10.1074/jbc.M706923200 (2007).

29 Lee, S. U. *et al.* N-glycan processing deficiency promotes spontaneous inflammatory demyelination and neurodegeneration. *J Biol Chem* **282**, 33725-33734, doi:10.1074/jbc.M704839200 (2007).

30 Mkhikian, H. *et al.* Genetics and the environment converge to dysregulate N-glycosylation in multiple sclerosis. *Nature communications* **2**, 334, doi:10.1038/ncomms1333 (2011).

31 Mkhikian, H. *et al.* Golgi self-correction generates bioequivalent glycans to preserve cellular homeostasis. *Elife* **5**, doi:10.7554/eLife.14814 (2016).

32 Araujo, L., Khim, P., Mkhikian, H., Mortales, C. L. & Demetriou, M. Glycolysis and glutaminolysis cooperatively control T cell function by limiting metabolite supply to N-glycosylation. *Elife* **6**, doi:10.7554/eLife.21330 (2017).

33 Zhou, R. W. *et al.* N-glycosylation bidirectionally extends the boundaries of thymocyte positive selection by decoupling Lck from Ca(2)(+) signaling. *Nat Immunol* **15**, 1038-1045, doi:10.1038/ni.3007 (2014).

34 Nitschke, L., Carsetti, R., Ocker, B., Kohler, G. & Lamers, M. C. CD22 is a negative regulator of B-cell receptor signalling. *Curr Biol* **7**, 133-143, doi:10.1016/s0960-9822(06)00057-1 (1997).

35 Otipoby, K. L. *et al.* CD22 regulates thymus-independent responses and the lifespan of B cells. *Nature* **384**, 634-637, doi:10.1038/384634a0 (1996).

36 Danzer, C. P., Collins, B. E., Blixt, O., Paulson, J. C. & Nitschke, L. Transitional and marginal zone B cells have a high proportion of unmasked CD22: implications for BCR signaling. *Int Immunol* **15**, 1137-1147, doi:10.1093/intimm/dxg114 (2003).

37 Samardzic, T. *et al.* Reduction of marginal zone B cells in CD22-deficient mice. *Eur J Immunol* **32**, 561-567, doi:10.1002/1521-4141(200202)32:2<561::AID-IMMU561>3.0.CO;2-H (2002).

38 Irons, E. E. & Lau, J. T. Y. Systemic ST6Gal-1 Is a Pro-survival Factor for Murine Transitional B Cells. *Front Immunol* **9**, 2150, doi:10.3389/fimmu.2018.02150 (2018).

39 Li, W. *et al.* Core fucosylation of mu heavy chains regulates assembly and intracellular signaling of precursor B cell receptors. *J Biol Chem* **287**, 2500-2508, doi:10.1074/jbc.M111.303123 (2012).

40 Yao, D. *et al.* Protein O-fucosyltransferase 1 (Pofut1) regulates lymphoid and myeloid homeostasis through modulation of Notch receptor ligand interactions. *Blood* **117**, 5652-5662, doi:10.1182/blood-2010-12-326074 (2011).

41 Bonzi, J. *et al.* Pre-B cell receptor binding to galectin-1 modifies galectin-1/carbohydrate affinity to modulate specific galectin-1/glycan lattice interactions. *Nature communications* **6**, 6194, doi:10.1038/ncomms7194 (2015).

42 Gauthier, L., Rossi, B., Roux, F., Termine, E. & Schiff, C. Galectin-1 is a stromal cell ligand of the pre-B cell receptor (BCR) implicated in synapse formation between pre-B and stromal cells and in pre-BCR triggering. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 13014-13019, doi:10.1073/pnas.202323999 (2002).

43 Rossi, B., Espeli, M., Schiff, C. & Gauthier, L. Clustering of pre-B cell integrins induces galectin-1-dependent pre-B cell receptor relocalization and activation. *J Immunol* **177**, 796-803, doi:10.4049/jimmunol.177.2.796 (2006).

44 Mourcin, F. *et al.* Galectin-1-expressing stromal cells constitute a specific niche for pre-BII cell development in mouse bone marrow. *Blood* **117**, 6552-6561, doi:10.1182/blood-2010-12-323113 (2011).

45 Espeli, M., Mancini, S. J., Breton, C., Poirier, F. & Schiff, C. Impaired B-cell development at the pre-BII-cell stage in galectin-1-deficient mice due to

inefficient pre-BII/stromal cell interactions. *Blood* **113**, 5878-5886, doi:10.1182/blood-2009-01-198465 (2009).

46 de Oliveira, F. L. *et al.* Lack of galectin-3 modifies differentially Notch ligands in bone marrow and spleen stromal cells interfering with B cell differentiation. *Sci Rep* **8**, 3495, doi:10.1038/s41598-018-21409-7 (2018).

47 Kalkavan, H. & Green, D. R. MOMP, cell suicide as a BCL-2 family business. *Cell Death Differ* **25**, 46-55, doi:10.1038/cdd.2017.179 (2018).

48 Zikherman, J., Parameswaran, R. & Weiss, A. Endogenous antigen tunes the responsiveness of naive B cells but not T cells. *Nature* **489**, 160-164, doi:10.1038/nature11311 (2012).

49 Myers, D. R., Zikherman, J. & Roose, J. P. Tonic Signals: Why Do Lymphocytes Bother? *Trends Immunol* **38**, 844-857, doi:10.1016/j.it.2017.06.010 (2017).

50 Tan, C. *et al.* Nur77 Links Chronic Antigen Stimulation to B Cell Tolerance by Restricting the Survival of Self-Reactive B Cells in the Periphery. *J Immunol* **202**, 2907-2923, doi:10.4049/jimmunol.1801565 (2019).

51 Anzelon, A. N., Wu, H. & Rickert, R. C. Pten inactivation alters peripheral B lymphocyte fate and reconstitutes CD19 function. *Nat Immunol* **4**, 287-294, doi:10.1038/ni892 (2003).

52 Benhamou, D. *et al.* A c-Myc/miR17-92/Pten Axis Controls PI3K-Mediated Positive and Negative Selection in B Cell Development and Reconstitutes CD19 Deficiency. *Cell Rep* **16**, 419-431, doi:10.1016/j.celrep.2016.05.084 (2016).

53 Lam, K. P., Kuhn, R. & Rajewsky, K. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell* **90**, 1073-1083, doi:10.1016/s0092-8674(00)80373-6 (1997).

54 Srinivasan, L. *et al.* PI3 kinase signals BCR-dependent mature B cell survival. *Cell* **139**, 573-586, doi:10.1016/j.cell.2009.08.041 (2009).

55 Fang, W. *et al.* Self-reactive B lymphocytes overexpressing Bcl-xL escape negative selection and are tolerized by clonal anergy and receptor editing. *Immunity* **9**, 35-45, doi:10.1016/s1074-7613(00)80586-5 (1998).

Chapter 3

N-glycan Branching Decouples B Cell Innate and Adaptive Immunity to Control Inflammatory Demyelination

SUMMARY

B cells are unique within the immune system, harboring both innate and adaptive immune activity. B cell depletion potently reduces episodes of inflammatory demyelination in multiple sclerosis (MS), predominantly through loss of innate immunity. However, molecular mechanisms controlling innate versus adaptive B cell function are poorly understood. Branched N-glycans regulate endocytosis and signaling of cell surface receptors to control cell function. Here we report that N-glycan branching in mouse B cells reduces pro-inflammatory innate responses by decreasing Toll Like Receptor-4 (TLR4) and TLR2 surface expression via endocytosis. In contrast, adaptive immunity via the B cell receptor (BCR) is promoted by N-glycan branching mediated cell surface retention of the BCR co-receptor CD19. Branched N-glycans in B cells reduce pro-inflammatory T-helper cell-17 (T_H17)/T_H1 differentiation and inflammatory demyelination in mice. Thus, N-glycan branching is a critical negative regulator of B cell innate versus adaptive immunity and a potential therapeutic target for MS.

INTRODUCTION

Multiple sclerosis (MS) is the most common autoimmune disease of the central nervous system, causing inflammatory demyelination and neurodegeneration[1]. Long-standing data from animal models of MS, particularly experimental autoimmune encephalomyelitis (EAE), implicate $CD4^+$ T cells and their differentiation into pro-inflammatory T_H1 and T_H17 cells as critical to inflammatory demyelination[2,3]. Prominent T_H1 and T_H17 responses are also observed in MS patients[4,5]. Genetic analysis in MS has largely been consistent with this data, highlighting the relevance of the immune system, particularly T cells, in promoting development of MS. This is best exemplified by the strongest genetic risk factor for MS being variants of the molecules that present antigen to T cells, namely the human leukocyte antigen (HLA) complex[6,7]. Moreover, gene enrichment analysis of the >200 genetic regions associated with MS has implicated multiple immune pathways, including factors that alter T cell growth (IL-2, IL-7 pathways) and their differentiation into pro-inflammatory T_H1 and T_H17 cells versus anti-inflammatory T regulatory cells[6,8-11].

The dominant role of T cells in MS has recently been challenged by the potent therapeutic activity of B cell depleting therapies in MS[12], with the anti-CD20 monoclonal antibody ocrelizumab shown to be superior to high-dose interferon-β-1a (Rebif) at reducing relapse rates as well as displaying positive activity in progressive MS[13,14]. B cells are unique within the immune system, having both innate and adaptive immune activity, with the former exemplified by activation via pattern recognition receptors like Toll-like receptors (TLRs) and the ability to directly present antigen to $CD4^+$ T cells. The therapeutic benefit of B cell depletion in MS appears to largely arise from altered innate

activity and reduced antigen presentation to $CD4^+$ T cells, rather than reduced B cell adaptive immune responses and auto-antibody production[15]. For example, anti-CD20 therapy reduces pro-inflammatory T_H1 and T_H17 responses as well as B and T cell numbers but not IgG concentration, IgG index, IgG synthesis rate or oligoclonal band number in the cerebral spinal fluid of treated MS patients[16,17]. Data in EAE also supports this conclusion, with B cells promoting T_H1 and/or T_H17 responses as well as inflammatory demyelination via their antigen-presenting cell (APC) function rather than through antibody production[18-21]. B cells may also play an important role in MS via TLR4 and/or TLR2 signaling mediated alterations in anti-inflammatory IL-10 production[22-24], a cytokine utilized by B regulatory cells to inhibit autoimmunity[25].

Although pro-inflammatory innate immune activity of B cells appears to be a central player in the immuno-pathogenesis of MS, the regulatory mechanisms that drive B cell innate activity to trigger inflammatory demyelination in MS are poorly understood. We have previously shown that branched N-glycans produced by the N-acetylglucosaminyl transferase enzymes Mgat1, 2, 4 and 5 play a critical role in limiting pro-inflammatory T cell responses and autoimmunity[26-34]. Galectin's bind the T cell receptor (TCR) and other glycoproteins at the cell surface in proportion to the branching, extension and number of complex N-glycans, forming a molecular lattice that controls clustering, signaling and endocytosis of surface receptors and transporters in a coordinated fashion[26-34]. N-glycan branching negatively regulates TCR clustering/signaling, promotes surface retention of anti-autoimmune Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), directly inhibits T_H1 and T_H17 while promoting anti-autoimmune induced T regulatory cell (iT_{REG}) differentiation, suppresses inflammatory

demyelinating disease in mice and is associated with risk of MS[26-43]. However, the function of N-glycan branching in B cells is unknown. Here we demonstrate that N-glycan branching has a unique dual activity in B cells, promoting adaptive immunity while simultaneously suppressing innate activity in B cells; the latter reducing B cell dependent pro-inflammatory T_H1/T_H17 differentiation of T cells and inflammatory demyelination in mice.

MATERIALS AND METHODS

Mice

Mgat1$^{f/f}$ (006891), *Mgat2*$^{f/f}$ (006892), *CD19-cre* (006785), *tetO-cre* (006234), *ROSA26-rtTA* (006965), 2D2 TCR^{MOG} transgenic (006912), and PL/J (000680) mice were obtained from Jackson Laboratory. Inter-breeding generated all other mice maintained on the C57BL/6 background. Mice were selected randomly for experiments and approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. For inducible deletion of Mgat1 in peripheral B cells, doxycycline was provided in 1% sucrose drinking water at 2 mg/mL to *Mgat1*$^{f/f}$*/tetO-cre/ROSA26-rtTA* mice for four weeks. For *in vivo* Mgat1 activity inhibition, intraperitoneal injections of kifunensine (KIF, GlycoSyn) at 250 μg/mL were done for 4 consecutive days.

B Cell Purification, Culture, and Stimulation

For all *in vitro* experiments, splenic B cells were immuno-magnetically purified using the EasySep^{TM} Mouse B Cell Isolation Kit (STEMCELL Technologies) according to manufacturer's instructions with resulting purity >95%; 20 μg/mL biotinylated L-PHA (Vector Labs) was supplemented to deplete L-PHA^{+} (non- *Mgat1* or *Mgat2* deleted) B cells. Cells were cultured in "complete" media: RPMI-1640 (Thermo Fisher Scientific) supplemented with 10% heat inactivated fetal bovine serum (VWR), 2 μM L-glutamine and 100 U/mL penicillin/streptomycin (Gibco), and 50 μM β-mercaptoethanol (Gibco). Stimulation conditions with TLR agonists (Invivogen) were 5 μg/mL LPS for TLR4, 500 ng/mL Pam2CSK4 for TLR2:6, and 500 ng/mL Pam3CSK4 for TLR2:1 unless indicated otherwise. B cells activated through BCR were stimulated with 10 μg/mL functional

grade goat anti-mouse IgM $F(ab')_2$ (eBioscience/Thermo Fisher Scientific) unless indicated otherwise. B cells from KIF injected mice were cultured in the presence of 5 μM KIF added once at day 0. Cytokine secretion in cell culture supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA) with ELISA MAX™ Deluxe Sets (BioLegend) according to the manufacturer's instructions.

Human PBMC Culture and Stimulation

Procedures with human subjects were approved by the Institutional Review Board of the University of California, Irvine. Human PBMCs were cultured in complete media as described above, with or without 10 μM KIF for 4 days prior to stimulation. PBMCs were then stimulated with 10 μg/mL anti-Ig (AffiniPure F(ab')2 Fragment Goat Anti-Human IgA + IgG + IgM, Jackson ImmunoResearch), 1 μg/mL CD40L (recombinant human CD40 ligand, Enzo Life Sciences) and/or 0.1 μM CpG ODN (class B CpG oligonucleotide 2006 (ODN 2006), Invivogen), with or without 10 μM KIF.

Flow Cytometry and Proliferation

Fluorophore conjugated mouse specific antibodies from eBioscience/Thermo Fisher Scientific were B220 (RA3-6B2), CD19 (eBio1D3), CD69 (H1.2F3), CD80 (16-10A1), CD86 (GL1), MHCII (M5/114.15.2, TLR2 (6C2), TLR4 (UT41), IgM (II/41), CD4 (RM4-5), IFNγ(XMG1.2), IL-17A (eBio17B7), and FOXP3 (FJK-16s). Fluorophore conjugated antibodies from BioLegend were mouse specific CD22 (OX-97), and human specific CD19 (HIB19), and TLR4 (HTA125). For flow cytometric analysis of glycan expression, cells were stained with 2 μg/mL L-PHA-FITC or LEA-FITC (Vector Labs). To assess

proliferation, cells were stained with 5 μM 5,6-carboxyfluorescein diacetate succinimidyl ester (CellTrace™ CFSE dye; Invitrogen/Thermo Fisher Scientific) and stimulated for 2 days. Samples were stained in FACS buffer (PBS with 1% BSA and 0.1% Na-azide) and acquired on the Attune NxT flow cytometer (Invitrogen/Thermo Fisher Scientific). Data analysis was performed using FlowJo software.

Endocytosis assay

The endocytosis assay was done as previously described[34]. Briefly, purified B cells were stained with fluorophore conjugated anti- TLR4, TLR2, or CD19, re-suspended in complete RPMI 1640 medium and incubated at 37°C for 1.5 hours. Cells were washed in FACS buffer or acidic buffer (150 mM NaCl and 20 mM HCl, pH 1.7) for 3 minutes at room temperature and then fixed in 1% PFA before analyzing by flow cytometry. The acidic buffer removes surface-bound antibody, and the MFI of acid-washed cells is divided by the MFI of FACS buffer washed cells to determine internalized antibody.

Calcium Flux

Purified B cells were concurrently stained with 9.2 μM Fura Red AM and 4.4 μM fluo-3 AM dyes (Life Technologies/Thermo Fisher Scientific). After establishing baseline Ca^{2+} levels, anti-mouse IgM $F(ab')_2$ was added to induce Ca^{2+} flux. Samples were acquired on a BD LSR II flow cytometer, and Ca^{2+} mobilization was determined by the ratio of fluo-3 to Fura Red fluorescence intensity using the kinetics tool in FlowJo software.

Western Blot

Purified B cells were lysed in RIPA buffer with 100x Halt™ protease and phosphatase

inhibitor cocktail (Thermo Fisher Scientific). For total CD19 protein analysis, lysates were treated with Rapid PNGase F (New England BioLabs) to remove all N-glycans. Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. Western blot antibodies to CD19 (#3574), TLR4 (D8L5W), TRAF3 (#4729), phospho-NF-κB p65 (Ser536) (93H1), phospho-Akt (Ser473) (D9E), phospho-ERK1/2 (Thr202/Tyr204) (197G2), phospho-TBK1 (Ser172) (D52C2), phospho-CD19 (Tyr531) (#3571), phospho-Syk (Tyr525/526) (C87C1), phospho-PLCγ2 (Tyr759) (#3874), β-actin (13E5), and HRP-conjugated anti- rabbit or mouse IgG were from Cell Signaling Technology. Abundance was measured by chemiluminescence and quantified by normalization to β-actin and relative to control using ImageJ software.

B and $CD4^+$ T cell Co-cultures and Intracellular Cytokine Staining

Purified B cells were stimulated with 20 μg/mL LPS for 2 hours and co-cultured at a 10:1 ratio with purified splenic $CD4^+$ T cells (EasySep™ Mouse $CD4^+$ T Cell Isolation Kit, STEMCELL Technologies) for 3-4 days in two *in vitro* co-culture systems: (1) a mixed lymphocyte reaction with allogeneic $CD4^+$ T cells from PL/J mice, and (2) with $CD4^+$ T cells from congenic 2D2 TCR^{MOG} transgenic C57BL/6 mice in the presence of human MOG_{35-55} ($hMOG_{35-55}$) or $OVA_{323-339}$ peptide (AnaSpec). Cytokine combinations for $CD4^+$ T cell differentiation conditions were as follows: 10 μg/mL of anti-IL-4 (eBioscience/Thermo Fisher Scientific) and 25ng/mL of mouse IL-12 (BioLegend) for T_H1; 10 μg/mL of anti-IL-4, 10 μg/mL of anti-IFNγ (eBioscience/Thermo Fisher Scientific), 20 ng/mL of mouse IL-23 (BioLegend), 20 ng/mL of mouse IL-6 (BioLegend), and 5 ng/mL of human TGFβ1 (eBioscience/Thermo Fisher Scientific) for T_H17; and 10

μg/mL of anti-IL-4, 10 μg/mL of anti-IFNγ 5 ng/mL of human TGFβ1 for T_{REG}. For intracellular cytokine flow cytometric analysis, cells were re-stimulated at 37°C for 4 hours with 50 ng/mL PMA (phorbol 12-myristate 13-acetate, Sigma-Aldrich) and 750 ng/mL ionomycin (Sigma-Aldrich) in the presence of GolgiPlug (1000x, BD Biosciences) and stained using the FOXP3 Transcription Factor Fixation/Permeabilization Kit (eBioscience/Thermo Fisher Scientific) according to manufacturer's instructions.

Experimental Autoimmune Encephalomyelitis

EAE was induced in 8-12 week old male and female mice by subcutaneous immunization at day 0 with 100 μg of recombinant human MOG (rhMOG) protein (AnaSpec) emulsified in Complete Freund's Adjuvant containing 4 mg/mL heat-inactivated Mycobacterium tuberculosis (H37RA; Difco). On days 0 and 2, mice received 200 ng of pertussis toxin (List Biological Laboratories) by intraperitoneal injection. Mice were examined daily for clinical signs of EAE over the next 35 days with observer blinded to mice genotypes. Mice were scored as previously described[44]: 0, no disease; 1, loss of tail tone; 2, hindlimb weakness; 3, partial hindlimb paralysis; 4, forelimb weakness or paralysis and hindlimb paralysis; 5, moribund or dead. At the end of the EAE experiment (day 35), spinal cords were harvested by hydraulic extrusion as previously described[45] and processed into single cell suspension for flow cytometric analysis of $CD4^+$ T cell and $B220^+$ B cell infiltration. Additionally, *in vivo* cytokine production of $CD4^+$ T cells was assessed by culturing splenocytes for 4 hours in the presence of PMA+ionomycin and GolgiPlug prior to intracellular cytokine staining and flow cytometric analysis.

RESULTS

N-glycan branching inhibits pro-inflammatory TLR4 and TLR2 signaling in B cells.

The Toll-like receptor (TLR) family plays critical roles in initiating innate inflammatory responses[46,47]. Cell surface TLR4 signaling is pro-inflammatory via MyD88/IRAK/TRAF6/AP-1/NF-κB, while signaling from endosomes is anti-inflammatory via TRIF/IRF3[48]. Thus, regulating TLR4 endocytosis is critical to pro- versus anti-inflammatory responses to lipopolysaccharide (LPS). CD14 controls LPS induced TLR4 endocytosis and associated anti-inflammatory signaling in myeloid cells[49]; however, B cells do not express CD14. TLR2, which hetero-dimerizes with TLR1 or TLR6, is the other major class of pro-inflammatory cell-surface TLR receptors expressed in mouse B cells and, like TLR4, has been implicated in MS[22-24]. As N-glycan branching regulates the endocytosis of multiple glycoproteins, we hypothesized that branching may regulate TLR4 and TLR2 endocytosis to control pro- versus anti-inflammatory innate responses in B cells.

To directly assess the role of N-glycan branching on cell surface TLR function in B cells, we generated two genetic models. First, we utilized the tet-on system to generate mice with doxycycline inducible deletion of the Mgat1 branching enzyme (i.e. *Mgat1*$^{f/f}$*/tetO-cre/ROSA-rtTA*). Second, we generated mice with B cell specific deletion of the Mgat2 branching enzyme (i.e. *Mgat2*$^{f/f}$*CD19cre*$^{+/-}$). *Mgat1* deficiency is expected to produce a more severe phenotype than *Mgat2* deficiency, as the former eliminates while the latter only reduces branching (Fig. 3.2a)[34,50]. *Mgat1*$^{-/-}$ and *Mgat2*$^{-/-}$ splenic B220^{+} B cells were purified by negative selection with L-PHA^{+} cell depletion from doxycycline treated *Mgat1*$^{f/f}$*/tetO-cre/ROSA-rtTA* or *Mgat2*$^{f/f}$*CD19-cre*$^{+/-}$ mice,

respectively (Fig. 3.2b). L-PHA binds β1,6-branched N-glycans, structures that are eliminated by *Mgat1* or *Mgat2* deficiency[26]. A third approach to reduce branching was to treat wildtype B cells with the small molecule Golgi inhibitor kifunensine (KIF), which blocks branching upstream of Mgat1 (Fig. 3.2a).

Mgat1$^{-/-}$ and *Mgat2*$^{-/-}$ B cells hypo-proliferated in response to both LPS (TLR4 agonist) and the bacterial lipopeptides Pam_2CSK_4 (TLR2:6 heterodimer agonist) and Pam_3CSK_4 (TLR2:1 heterodimer agonist), as measured by CFSE dilution (Fig. 3.1a). The magnitude of hypo-proliferation was less in *Mgat2*$^{-/-}$ than *Mgat1*$^{-/-}$ B cells, consistent with a less severe loss of branching in the former. Remarkably, despite hypo-proliferation, up-regulation of the activation marker CD69 induced by TLR4 and TLR2 agonists was enhanced by *Mgat1* and *Mgat2* deficiency (Fig. 3.1b). Consistent with this, pro-inflammatory NF-κB activation by cell surface TLR4 and TLR2 signaling was enhanced by *Mgat1* and *Mgat2* deficiency (Fig. 3.1c). TLR4 also signals through SYK to activate ERK and AKT[51] and branching deficiency led to enhanced LPS induced SYK, ERK, and AKT phosphorylation (Fig. 3.2c,d). In contrast, anti-inflammatory endosomal TLR4 signaling via TBK1 and TRAF3 was unchanged by *Mgat1* deficiency (Fig. 3.1d). NF-κB activation drives secretion of pro-inflammatory TNFα over anti-inflammatory IL-10[25]. *Mgat1*$^{-/-}$ B cells stimulated with TLR4 or TLR2 agonists displayed increased TNFα and reduced IL-10 secretion compared to control B cells (Fig. 3.1e). B cells express the T cell co-stimulatory ligands CD80 and CD86, which promote pro-inflammatory T_H1 and humoral/immunomodulatory T_H2 responses, respectively, to influence inflammatory demyelination in EAE[52,53]. LPS stimulated *Mgat1*$^{-/-}$ and *Mgat2*$^{-/-}$ B cells displayed increased induction of CD80 and less CD86 on their cell surface compared to controls

(Fig. 3.1f). Induction of MHCII was also elevated in *Mgat1*$^{-/-}$ and *Mgat2*$^{-/-}$ B cells (Fig. 3.1g). In resting B cells, CD80, CD86, and MHCII surface levels on *Mgat1*$^{-/-}$ and *Mgat2*$^{-/-}$ B cells were similar to control (Fig. 3.2e,f). Together, these data demonstrate that N-glycan branching inhibits TLR4 and TLR2 pro-inflammatory cell surface signaling.

N-glycan branching promotes TLR4 and TLR2 endocytosis to reduce cell surface expression in B cells.

Next, we investigated the mechanism for enhanced TLR4 and TLR2 cell surface signaling. In resting B cells, cell surface expression of TLR4 is minimal (Fig. 3.4a). Resting *Mgat1*$^{-/-}$ B cells displayed no significant difference in TLR4 or TLR2 surface expression, endocytosis rate or mRNA levels (Fig. 3.4a-c). Binding of LPS to cell surface TLR4 was also unaltered by *Mgat1* deficiency, indicating that loss of branching did not impact the interaction of LPS to TLR4 (Fig. 3.4d). However, 2-3 days after TLR4 or TLR2 stimulation, N-glycan branching deficient B cells had greater TLR4 and TLR2 surface expression than control cells, with *Mgat1* > *Mgat2* deficiency (Fig. 3.3a). This coincided with a physiological increase in N-glycan branching in activated wildtype B cells stimulated with TLR4 and TLR2 ligands, a phenotype blocked by *Mgat1* and *Mgat2* deficiency (Fig. 3.3b). Comparing cell surface versus total TLR4 and TLR2 by flow cytometry indicated that loss of branching did not alter total TLR protein levels and specifically enhanced cell surface TLR4 and TLR2 expression (Fig. 3.3c). Western blotting confirmed that total TLR4 protein was not altered (Fig. 3.4e). Consistent with increased surface levels, the rate of TLR4 and TLR2 endocytosis in activated B cells was significantly reduced by *Mgat1* > *Mgat2* deficiency (Fig. 3.3d) and KIF treatment (Fig. 3.4f).

In contrast to mouse B cells, human B cells were originally reported to not express TLR4 at the cell surface. However, more recent data indicates that surface TLR2 and TLR4 is induced in human B cells under inflammatory conditions and by certain signaling pathways such as BCR, CD40 and TLR9 (CpG)[54-57]. Consistent with this data and our mouse data, inhibiting branching in human B cells with KIF enhanced TLR4 surface expression induced by co-stimulation with CpG + CD40L and CpG + anti-IgM/G/A (Fig. 3.4g). Collectively, these data show that N-glycan branching negatively regulates pro-inflammatory TLR4 and TLR2 signaling in activated B cells by enhancing surface loss to endocytosis.

N-glycan branching regulates CD19 to promote activation signaling by the B cell receptor independently of CD22.

As N-glycan branching regulates both basal and ligand induced TCR signaling, we explored whether branching similarly regulates B cell receptor (BCR) signaling. *Mgat1*$^{-/-}$ B cells hypo-proliferated in response to anti-IgM F(ab')$_2$ compared to control B cells (Fig. 3.5a), a result opposite to that in T cells but similar to TLR4 and TLR2 stimulated B cell proliferation. A potential mechanism for altered BCR signaling in *Mgat1* deficient B cells is via CD22, a negative regulator of BCR signaling. CD22 is a Siglec (sialic-acid-binding immunoglobulin-like lectin) that binds α2,6 linked sialic acid, a terminal sugar attached to galactose in a wide variety of glycans including N-glycans, O-glycans and glycolipids. CD22 deficient B cells hyper-proliferate in response to anti-IgM F(ab')$_2$ by preventing delivery of the phosphatase SHP-1 to BCR[58,59]. Deficiency of ST6Gal-I, the enzyme that generates the CD22 ligand (α2,6 linked sialic acid), promotes association of CD22 with BCR to inhibit signaling and reduce proliferation[60,61]. As *Mgat1*

deficiency eliminates terminal sialic acids on N-glycans, but not other glycan types, it is possible that loss of branching may inhibit BCR signaling by promoting association of CD22 with BCR. To assess this hypothesis, we generated *CD22$^{-/-}$/Mgat1$^{f/f}$/tetO-cre/ROSA-rtTA* mice, where *Mgat1$^{-/-}$CD22$^{-/-}$* B cells are isolated following doxycycline treatment. As previously reported, *CD22$^{-/-}$* B cells were hyper-proliferative in response to anti-IgM F(ab')$_2$. However, eliminating CD22 on N-glycan branching deficient B cells failed to rescue proliferation (Fig. 3.5a). This is in contrast to ST6Gal-I deficiency, where CD22 loss reversed the negative effects of α2,6 linked sialic acid deficiency on BCR signaling to rescue proliferation[61].

Next, we explored whether N-glycan branching directly alters BCR signaling via reductions in surface expression of BCR and/or the CD19 co-receptor. CD19 facilitates the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on Igα/Igβ at the BCR complex by recruiting the protein kinases Lyn and Syk to initiate signaling cascades including PLCγ/Ca^{2+} dependent proliferation[62]. *Mgat1$^{-/-}$* B cells displayed reduced anti-IgM F(ab')$_2$ induced Ca^{2+} flux (Fig. 3.5b) and phosphorylation of CD19, Syk, and PLCγ2 (Fig. 3.5c). Cell surface levels of IgM were similarly reduced in *Mgat1$^{-/-}$, CD22$^{-/-}$*, and *Mgat1$^{-/-}$CD22$^{-/-}$* B cells compared to control (Fig. 3.5d), a phenotype comparable to ST6Gal-I and/or CD22 deficiency[61]. There was also a reduction in CD22 surface levels in *Mgat1$^{-/-}$* B cells (Fig. 3.6a). However, *Mgat1$^{-/-}$* and *Mgat1$^{-/-}$CD22$^{-/-}$* B cells had markedly reduced surface levels of CD19, while *CD22$^{-/-}$* B cells had no significant difference compared to control (Fig. 3.5e). Indeed, CD19 surface loss to endocytosis was enhanced by *Mgat1* deficiency (Fig. 3.5f) and KIF treatment (Fig. 3.6b). While there was no difference in CD19 mRNA levels (Fig. 3.6c), total CD19

protein was reduced in *Mgat1*$^{-/-}$ B cells (Fig. 3.6d), consistent with protein loss via degradation of endocytosed protein. These results demonstrate that N-glycan branching promotes BCR signaling and proliferation by enhancing CD19 surface expression independent of regulation by CD22.

In contrast to *Mgat1* deficiency and KIF treatment, which completely blocks all branching, limiting N-glycans to a single branch via *Mgat2* deficiency in B cells did not alter anti-IgM F(ab')$_2$ induced proliferation, Ca^{2+} mobilization, or IgM and CD19 surface levels (Fig. 3.6e-h). We recently reported that in *Mgat2* deficient T cells, loss of branching is partially compensated for by poly-N-acetyllactosamine (poly-LacNAc) extension of the remaining single GlcNAc branch, which leads to the same level of galetin-3 binding at the cell surface and function as *Mgat5*$^{-/-}$ T cells[50]. Indeed, *Mgat2*$^{-/-}$ B cells similarly displayed up-regulated poly-LacNAc expression, as measured by flow cytometry with *Lycopersicon Esculentum* lectin (LEA) (Fig. 3.6i)[50]. Together these results demonstrate that a minimal threshold level of LacNAc (galectin ligand) within branched N-glycans is required to maintain CD19 at the cell surface and drive robust BCR responses.

N-glycan branching deficiency in B cells promotes pro-inflammatory APC function.

As *Mgat2* deficiency impacted innate TLR2 and TLR4 but not adaptive BCR/CD19 responses, we utilized this model to explore the impact of branching on antigen presenting cell (APC) function of B cells. Enhanced LPS induced up-regulation of MHCII and CD80 in *Mgat2*$^{-/-}$ B cells should increase B cell triggered T cell activation.

To examine this, LPS pre-stimulated *Mgat2*$^{-/-}$ and control B cells from C57BL/6 mice were co-cultured with wildtype allogeneic splenic CD4^{+} T cells from PL/J mice under neutral, T_H1, T_H17, or T_{REG} inducing conditions. *Mgat2* branching deficiency in LPS pre-stimulated B cells markedly enhanced the differentiation of CD4^{+} T cells to T_H1 and T_H17 cells under their respective conditions (Fig. 3.7a,b) while also significantly inhibiting T_{REG} differentiation under T_{REG} inducing conditions (Fig. 3.7c). Under neutral conditions, addition of LPS also directly enhanced T_H1 differentiation by *Mgat2*$^{-/-}$ B cells (Fig. 3.8a). To confirm these results in an antigen specific *in vitro* model, we utilized congenic 2D2 TCR^{MOG} transgenic mice that harbor CD4^{+} T cells specific for the human MOG_{35-55} peptide ($hMOG_{35-55}$). Relative to control B cells, *Mgat2*$^{-/-}$ B cells significantly increased the differentiation of 2D2 CD4^{+} T cells to T_H1 and T_H17 cells in response to $hMOG_{35-55}$ (Fig. 3.7d,e), but had little effect on T_{REG} differentiation (Fig. 3.7f). 2D2 CD4^{+} T cell differentiation in response to $hMOG_{35-55}$ was dose dependent (Fig. 3.8b), whereas $OVA_{323-339}$ peptide did not induce robust CD4^{+} T cell responses under any conditions (Fig. 3.8c). Thus, enhanced pro-inflammatory TLR4 signaling induced by N-glycan branching deficiency directly enhances the ability of B cells to induce pro-autoimmune T_H1 and T_H17 responses via co-stimulatory APC function.

N-glycan branching deficiency in B cells promotes inflammatory demyelination.

Given the importance of pro-inflammatory innate immune activity of B cells in the immuno-pathogenesis of MS, we explored whether the enhanced innate activity induced by Mgat2 deficiency in B cells promotes the MS model EAE. As C57BL/6 mice lacking B cells are resistant to EAE induced with whole recombinant human MOG protein

(rhMOG) but remain sensitive to EAE induced by the MOG_{35-55} peptide and whole recombinant mouse MOG[19,25], we utilized rhMOG to induce B cell dependent EAE in *Mgat2*$^{f/f}$*CD19cre*$^{+/-}$ and control *CD19cre*$^{+/-}$ C57BL/6 mice. *Mgat2* deficiency in B cells significantly enhanced the incidence and severity of clinical EAE (Fig. 3.9a-c) as well as infiltration of the spinal cord by $CD4^+$ T cells and $B220^+$ B cells (Fig. 3.9d). Consistent with this and our *in vitro* co-culture data, *Mgat2* deficiency in B cells also enhanced rhMOG induction of T_H1 and T_H17 cells, but not T_{REG} cells, *in vivo* (Fig.3.9e).

DISCUSSION

B cell innate immune activity plays an important role in MS pathogenesis. Here we identify N-glycan branching as a novel regulatory mechanism in B cells that decouples innate surface TLR and APC function from adaptive activity via BCR to negatively regulate APC induced pro-inflammatory T_H1/T_H17 T cell responses and demyelinating disease (Fig. 3.10). N-glycan branching titrates reductions in the surface expression of TLR4 and TLR2 via endocytosis in activated B cells over a broad continuum of expression. In contrast, a minimal level of branching maintains CD19 at the cell surface to drive BCR mediated adaptive immune responses. The APC function of B cells is inhibited by N-glycan branching via suppression of TLR4 and TLR2 signaling induced up-regulation of MHCII and CD80 over CD86. CD80 exists as a dimer while CD86 is a monomer[63], allowing CD80 to bind CD28 with much greater affinity and thereby drive demyelinating disease[52,53]. Co-suppression of MHCII and CD80 by N-glycan branching reduces both primary TCR signaling as well as co-stimulatory signals via CD28, thereby inhibiting B cell triggered pro-inflammatory T_H1/T_H17 responses and inflammatory demyelination.

B cell aggregates are found in the meninges of secondary progressive MS patients[64,65]. B cell depletion with anti-CD20 antibodies inhibits relapses and progression in MS, a blunt tool that non-specifically reduces both innate and adaptive immunity and therefore is broadly immunosuppressive. In contrast, enhancing N-glycan branching in B cells may provide a safer therapeutic strategy by suppressing APC function and innate immunity, while promoting/maintaining adaptive immunity via BCR. In this regard, we have previously demonstrated that metabolically supplementing cells

or mice with the simple sugar N-acetylglucosamine (GlcNAc) increases N-glycan branching via enhanced substrate supply to the Golgi (Mgat) branching enzymes, suppresses T cell growth, enhances CTLA-4 retention, blocks T_H1/T_H17 responses, enhances iTreg differentiation, and inhibits EAE[29,36,37]. Based on this activity, we are currently conducting a dose-finding Phase 1 clinical trial of GlcNAc in MS. Our new data on B cells raise the possibility that oral GlcNAc may also function to suppress T cell responses and inflammatory demyelination by raising branching in B cells *in vivo*. Further investigations are warranted to determine the effects of GlcNAc on B cells in MS patients.

In myeloid cells, CD14 controls TLR4 endocytosis to regulate pro-inflammatory (cell surface) versus anti-inflammatory (endosomal) signaling[49]. As B cells lack CD14, how TLR4 cell surface vs endosomal signaling is regulated in B cells was poorly understood. Our data identifies N-glycan branching in activated B cells as a critical determinant of pro- vs anti-inflammatory TLR4 signaling. Conditions that drive low branching would be expected to trigger TLR4 surface expression and innate pro-inflammatory B cell activity.

TLR4 and TLR2 surface levels were elevated in *Mgat1*$^{-/-}$ > *Mgat2*$^{-/-}$ B cells, demonstrating a dose dependent effect of N-glycan branching on TLR surface retention and activation. In addition, the effects on TLR4 surface retention were greater than TLR2. This may be explained by differences in the number of attached N-glycosylation sites, which also determines interaction with galectins[29]. TLR4 has 14 and TLR2 has 3. The TLR2 binding partners TLR1 has 9, and TLR6 has 10. When respective agonists bind, these TLRs dimerize to form TLR4:4 with 28, TLR2:1 with 12, and TLR2:6 with 13

combined N-glycosylation sites, respectively. Thus, TLR4:4 homodimers have more than double the number of N-glycans, making the consequences for complete loss of N-glycan branching more severe than that of TLR2:1 or TLR2:6 heterodimers.

In contrast to promoting TLR4/2 endocytosis, N-glycan branching inhibits CD19 endocytosis to support BCR signaling and downstream adaptive immunity. As only Mgat1 but not Mgat2 deficiency resulted in loss of CD19 to endocytosis, at least a single GlcNAc branch is required to maintain CD19 at the cell surface. *Mgat2*$^{-/-}$ B cells have a marked increase in poly-LacNAc extension, which serves to enhance galectin avidity and thereby likely contributes to maintaining surface CD19 and signaling similar to control. The molecular mechanisms by which N-glycan branching simultaneously enhances TLR4/TLR2 but inhibits CD19 endocytosis may be secondary to galectin-mediated segregation of the proteins to different membrane microdomains. In T cells, the galectin lattice excludes the T cell receptor from GM3^{+} lipid rafts, while having the opposite effect on CD45; both by opposing the activity of actin microfilaments tethered to cytoplasmic domains of the respective proteins[28]. Similar activity in B cells may promote TLR4/2 but oppose CD19 partition to endocytic pits.

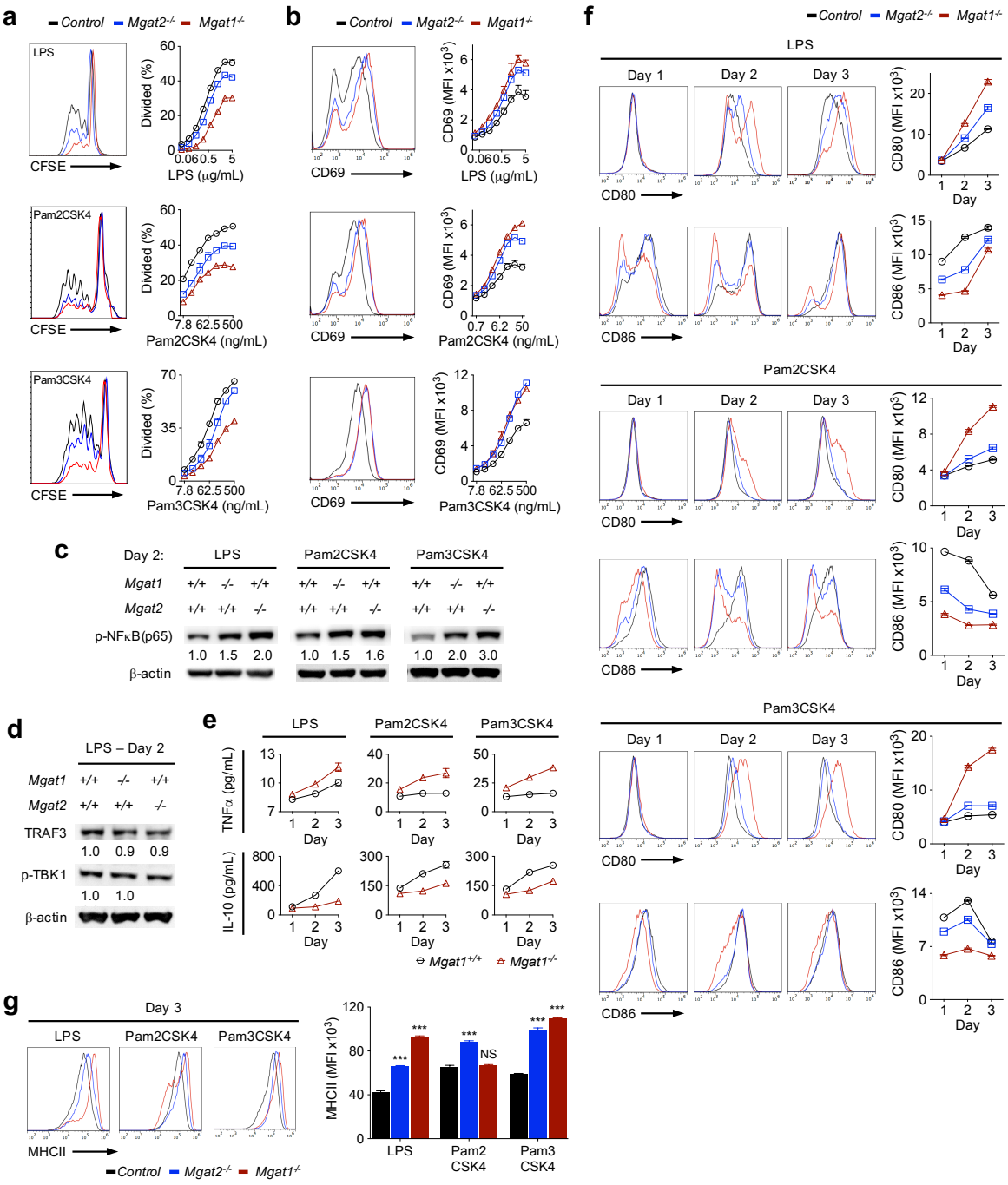

Figure 3.1. N-glycan branching inhibits pro-inflammatory TLR4 and TLR2 activation in B cells. (**a**,**b**,**g**,**h**) TLR4 and TLR2 stimulated B cells were assessed by flow cytometry for proliferation by CFSE dilution after 2 days (**a**), CD69 expression after 1 day (**b**), surface expression of CD80/CD86 after 1-3 days (**g**) and MHCII after 3 days (**h**). Histograms in (**b**,**c**) represent highest agonist dose. (**c,d**) Western blot analysis of phospho-NFκB (p65) (**c**), total TRAF3 protein and phospho-TBK1 (**d**) in B cells stimulated for 2 days. (**e**) TNFα and IL-10 secretion measure by ELISA from culture supernatants of stimulated B cells over 1-3 days. Data shown are mean ± s.e.m of triplicate samples (**a**,**b**,**e-g**) and representative of n ≥ 3 experiments. Unpaired two-tailed *t*-tests with Welch's correction (**g**). NS, not significant; ***p<0.001. MFI, mean fluorescence intensity.

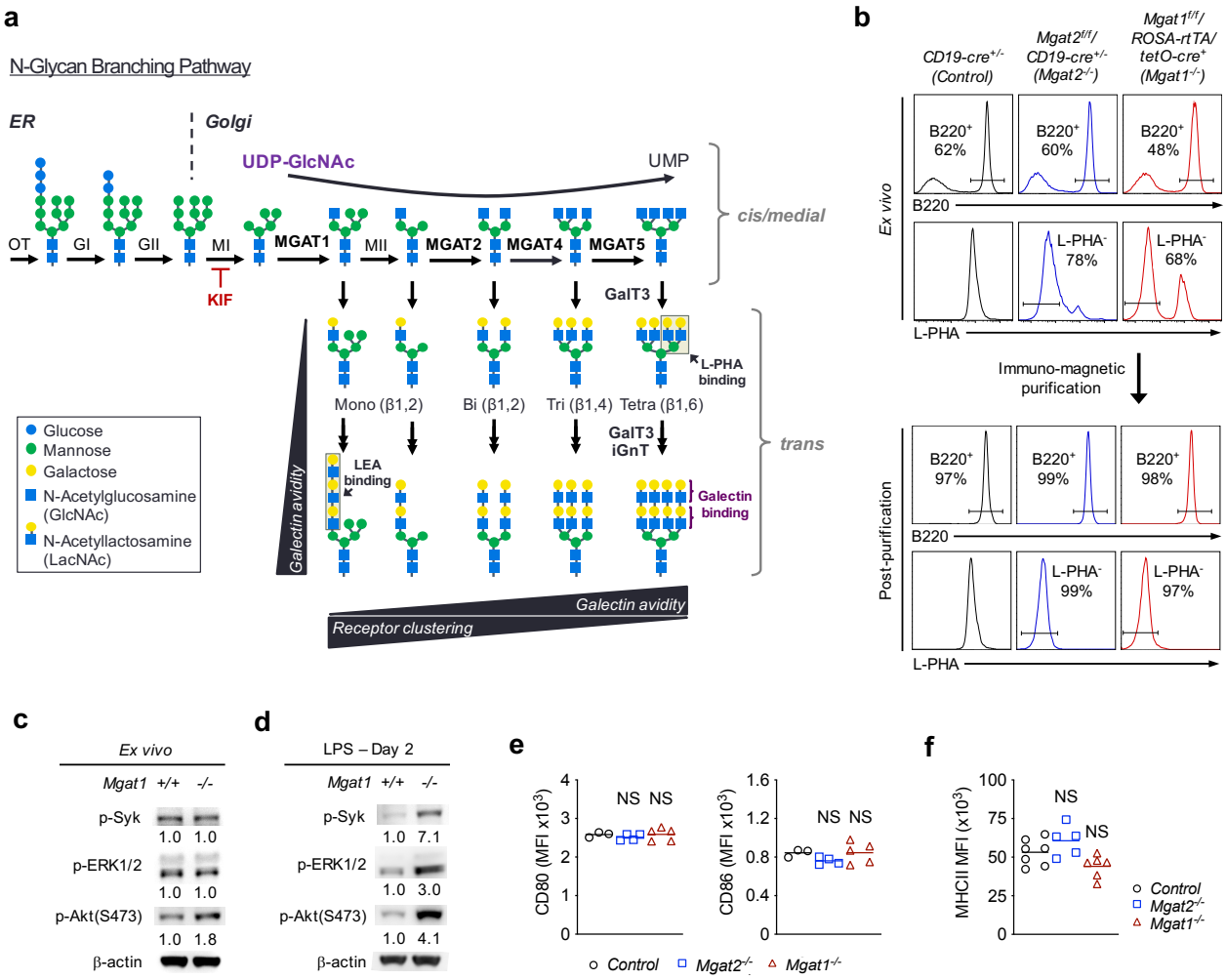

Figure 3.2. Supplement to Figure 3.1. (**a**) Oligosaccharyltransferase (OT) transfers a pre-assembled glycan, Glc3Man9GlcNAc2, onto Asp-X-Ser-Thr (X≠Pro) motifs on glycoproteins in the endoplasmic reticulum (ER). This glycan is extensively modified as glycoproteins transit through the Golgi by mannosidases and the N-acetylglucosaminyltransferase (Mgat) enzymes, which act sequentially to generate branched N-glycans with increasing affinities for galectins. GalT and iGnT extend branches to create N-acetyllactosamine (LacNac) which can also be bound by galectins. Plant lectin L-PHA and LEA binding sites are indicated. OT, oligosaccharyltransferase; GI, glucosidase I; GII, glucosidase II; MI, mannosidase I; MII, mannosidase II; Mgat, N-acetylglucosaminyltransferase; GalT3, Galactosyltransferase 3; iGnT, little "i" β-1,3-N-acetylglucosaminyltransferase; KIF, kifunensine. (**b**) Representative histograms of B220 and L-PHA staining on *ex vivo* splenocytes and immuno-magnetic purified B cells to demonstrate purity of (L-PHA$^-$) B220$^+$ B cells used for *in vitro* experiments. (**c**,**d**) Western blot analysis of phospho-Syk, phospho-ERK1/2 and phospho-Akt (Ser473) in B cells *ex vivo* (**c**) and stimulated for 2 days (**d**). (**e**,**f**) Flow cytometric analysis of *ex vivo* (L-PHA$^-$) B220$^+$ B cells for CD80 and CD86 (**e**) and MHCII (**f**) surface expression. Data shown are representative of n = 3 experiments (**c**,**d**). Each symbol represents one mouse and horizontal line represents the mean (**e**,**f**). NS, not significant. MFI, mean fluorescence intensity.

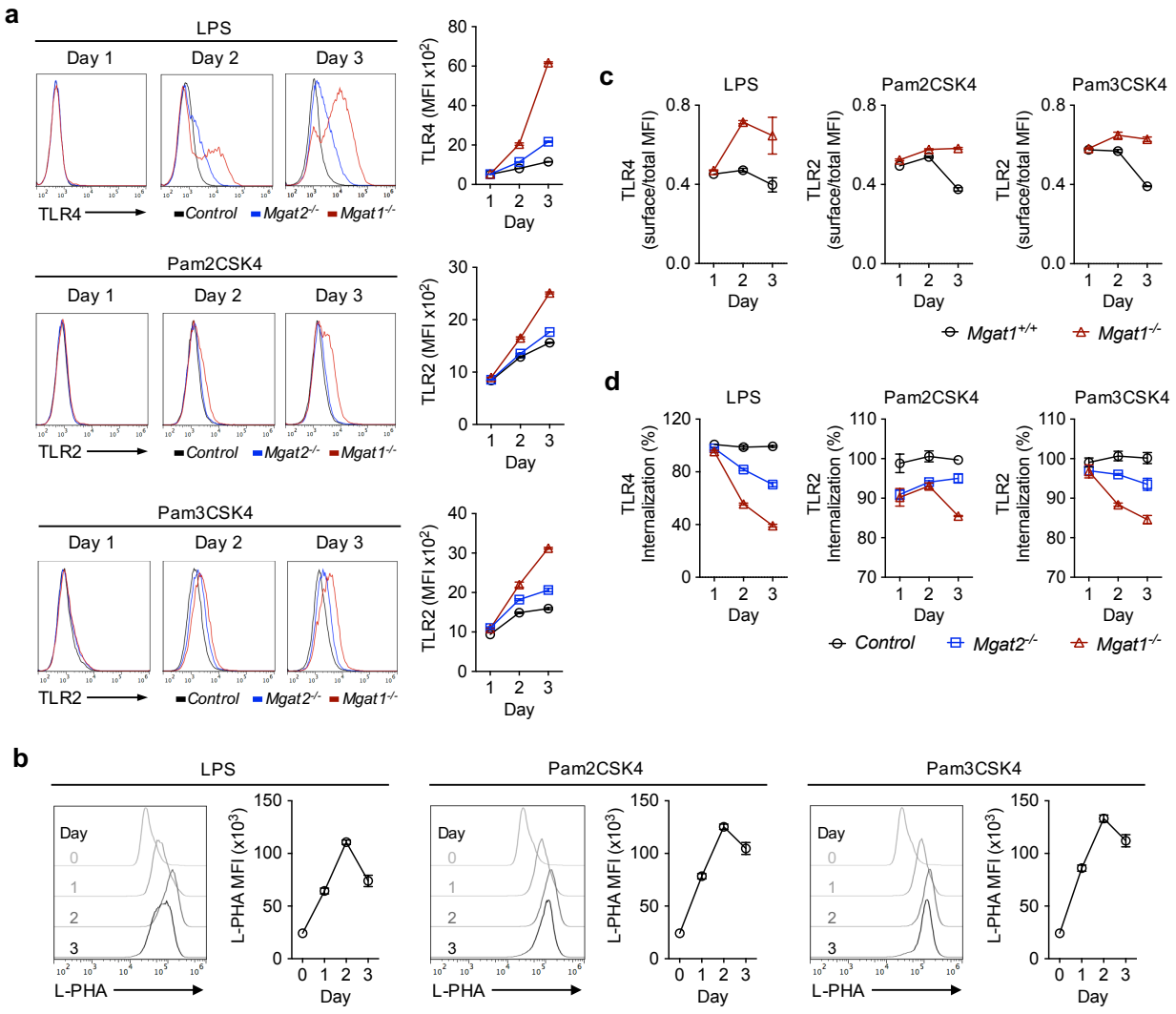

Figure 3.3. N-glycan branching reduces TLR4 and TLR2 surface levels by promoting endocytosis. (**a**-**d**) Flow cytometric analysis of B cells stimulated for 1-3 days and assessed for TLR4 and TLR2 surface expression (**a**), L-PHA binding (**b**), TLR4 and TLR2 surface-to-total ratio (**c**), and TLR4 and TLR2 endocytosis (**d**). Data shown are mean ± s.e.m of triplicate samples and representative of n ≥ 3 experiments. MFI, mean fluorescence intensity.

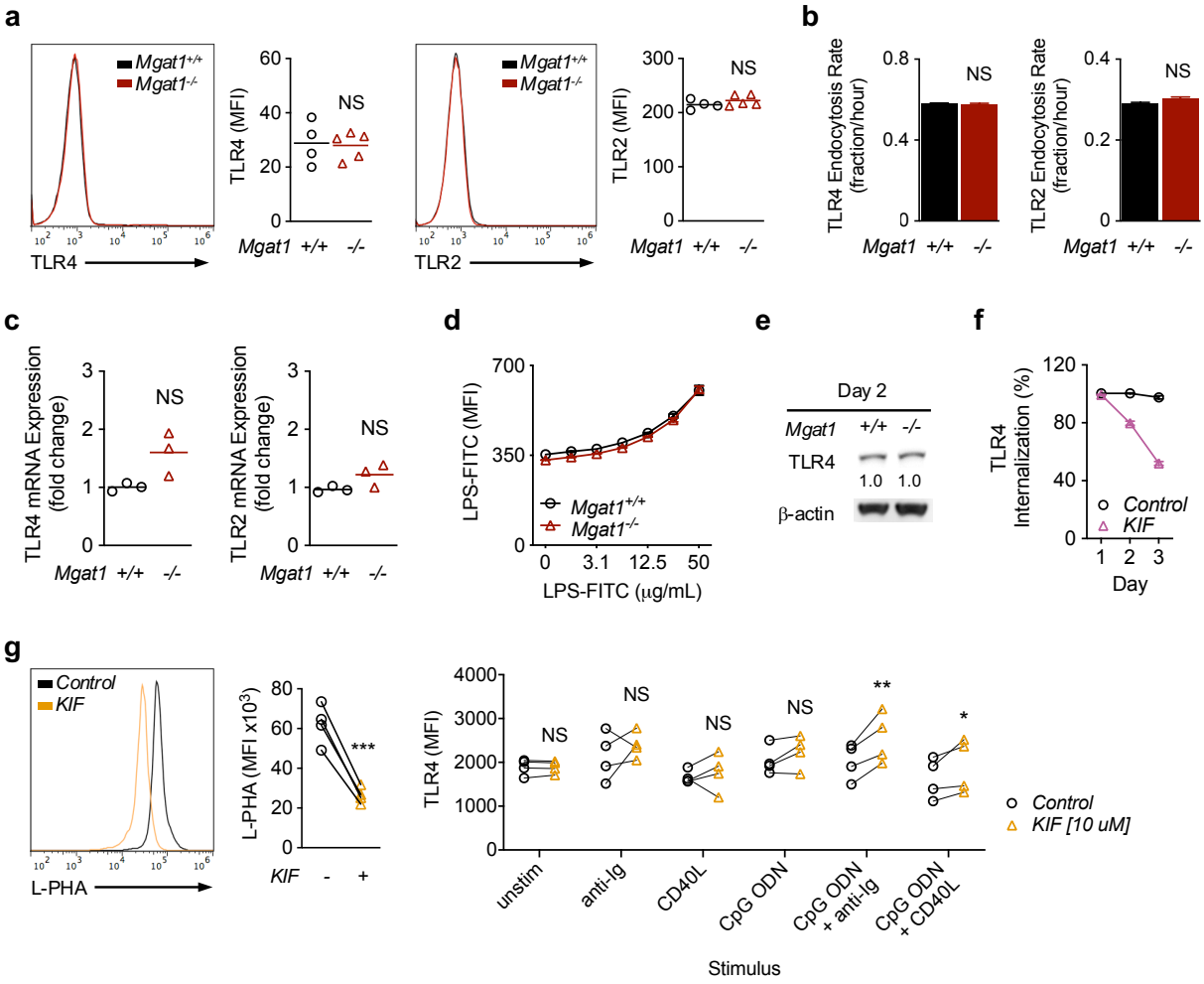

Figure 3.4. Supplement to Figure 3.3. (**a-d**) *Ex vivo* unstimulated B cells were assessed for TLR4 and TLR2 surface levels by flow cytometry (MFIs shown are with isotype MFIs subtracted) (**a**), TLR4 and TLR2 endocytosis rates by flow cytometry (**b**), TLR4 and TLR2 mRNA expression by real-time qPCR (**c**), and LPS-FITC binding by flow cytometry (**d**). (**e**) Western blot analysis of total TLR4 levels in B cells stimulated with LPS for 2 days. (**f**) B cells ± KIF stimulated with LPS for 1-3 days and assessed for TLR4 endocytosis rate by flow cytometry. (**g**) Gated $CD19^+$ B cells from human PBMCs cultured for 4 days ± KIF assessed for L-PHA binding, then stimulated for 3 days as indicated and assessed for TLR4 surface levels (MFIs shown are with isotype MFIs subtracted) by flow cytometry. Data shown are mean ± s.e.m of triplicate samples (**b**,**d**,**f**) and representative of n ≥ 3 experiments. Each symbol represents one mouse or blood donor, horizontal line represents the mean (**a**,**c**). Unpaired two-tailed *t*-tests with Welch's correction (**a-d**,**f**). Paired one-tailed *t*-test (**g**). NS, not significant; *p<0.05; **p<0.01; ***p<0.001. MFI, mean fluorescence intensity.

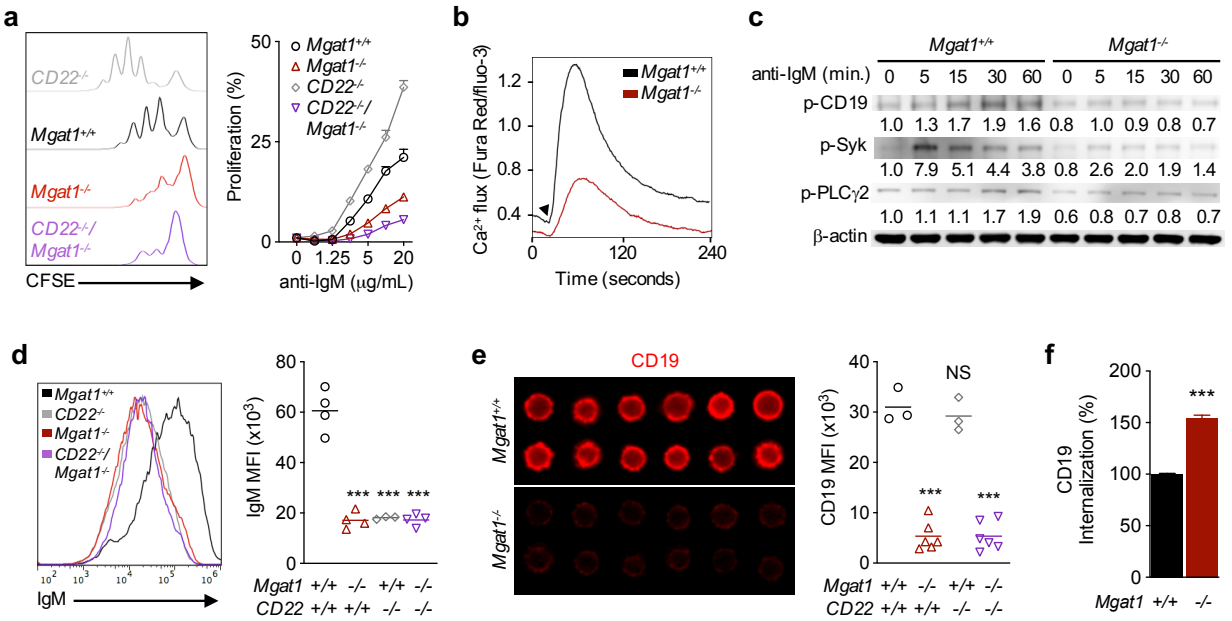

Figure 3.5. N-glycan branching promotes activation signaling via CD19 and the B cell receptor independent of CD22. (**a**,**b**,**d**-**f**) Flow cytometric analysis of anti-IgM F(ab')$_2$ induced proliferation by CFSE dilution after 2 days (**a**) and Ca^{2+} mobilization over 4 minutes (**b**), and *ex vivo* B cells for IgM surface expression (**d**), CD19 surface expression (**e**), and CD19 endocytosis (**f**). Histograms in (**a**) represent highest stimulation concentration, arrow in (**b**) indicates addition of 2.5 µg/mL anti-IgM F(ab')$_2$, and immunofluorescent images in (**e**) were acquired on an Amnis ImageStream Imaging Flow Cytometer. (**c**) Western blot analysis of phospho-CD19, phospho-Syk, and phospho-PLCγ2 in B cells stimulated with 10 µg/mL anti-IgM F(ab')$_2$. Data shown are mean ± s.e.m of triplicate samples (**a**,**f**) and representative of n ≥ 3 experiments. Each symbol represents one mouse and horizontal line represents the mean (**d**,**e**). Unpaired two-tailed *t*-tests with Bonferroni's (**d**,**e**) and Welch's corrections (**f**). NS, not significant; ***p<0.001. MFI, mean fluorescence intensity.

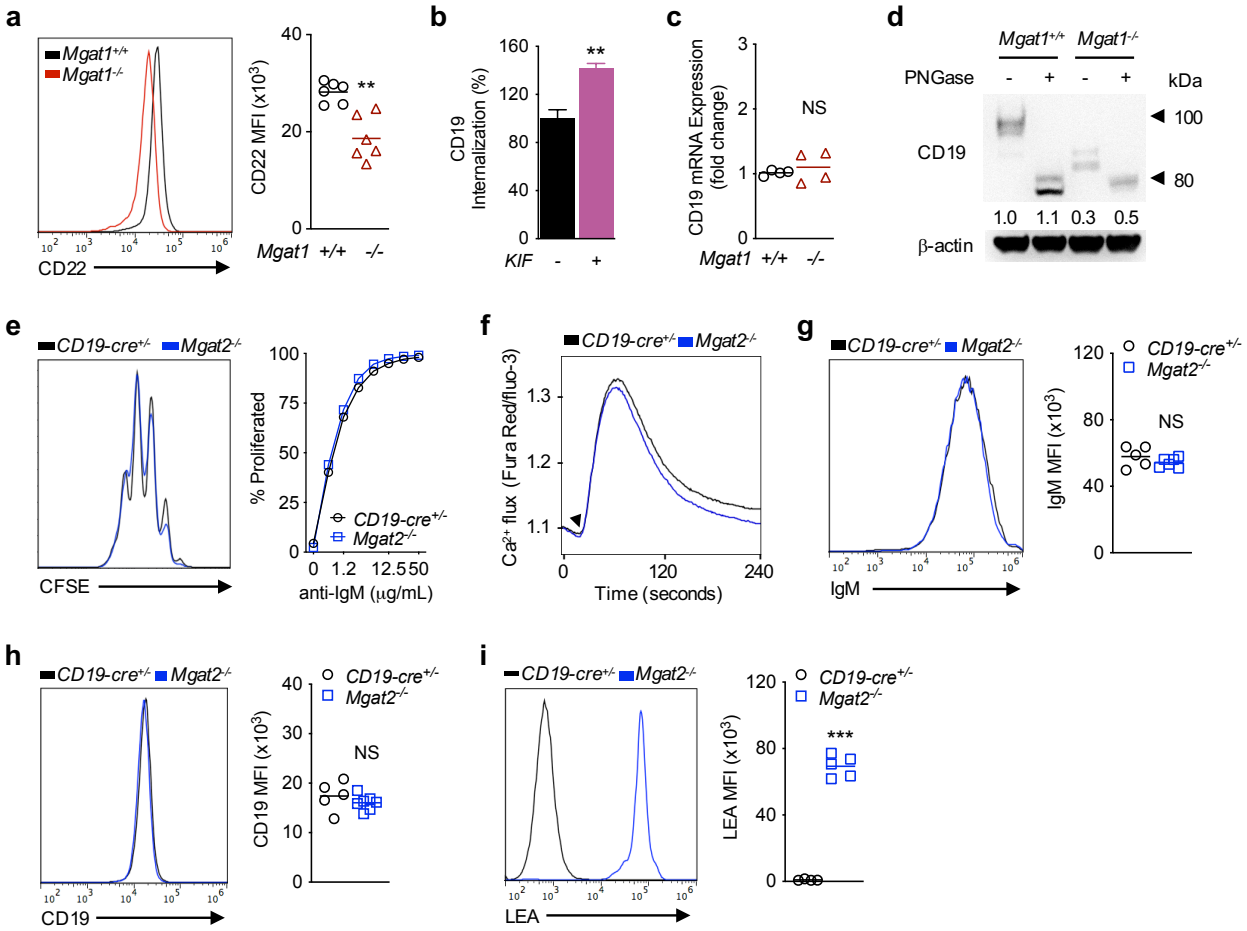

Figure 3.6. Supplement to Figure 3.5. (**a**-**c**,**e**-**i**) Flow cytometric analysis of *ex vivo* B cells for CD22 surface expression (**a**) and CD19 endocytosis rate ± KIF (**b**); anti-IgM F(ab')$_2$ induced proliferation by CFSE dilution after 2 days (**e**) and Ca^{2+} mobilization over 4 minutes (**f**); and *ex vivo* surface expression of IgM (**g**), CD19 (**h**) and LEA binding (**i**). Histogram in (**e**) represents highest anti-IgM dose. (**d**) Western blot analysis of total CD19 ± Rapid PNGase F treatment of lysates. PNGase was used to remove all N-glycans and equalize molecular weight of the protein. Data shown are mean ± s.e.m of triplicate samples (**b**,**e**) and representative of n ≥ 3 experiments. Each symbol represents one mouse, horizontal line represents the mean (**a**,**c**,**g**-**i**). Unpaired two-tailed *t*-tests with Welch's correction. NS, not significant; **p<0.01; ***p<0.001. MFI, mean fluorescence intensity.

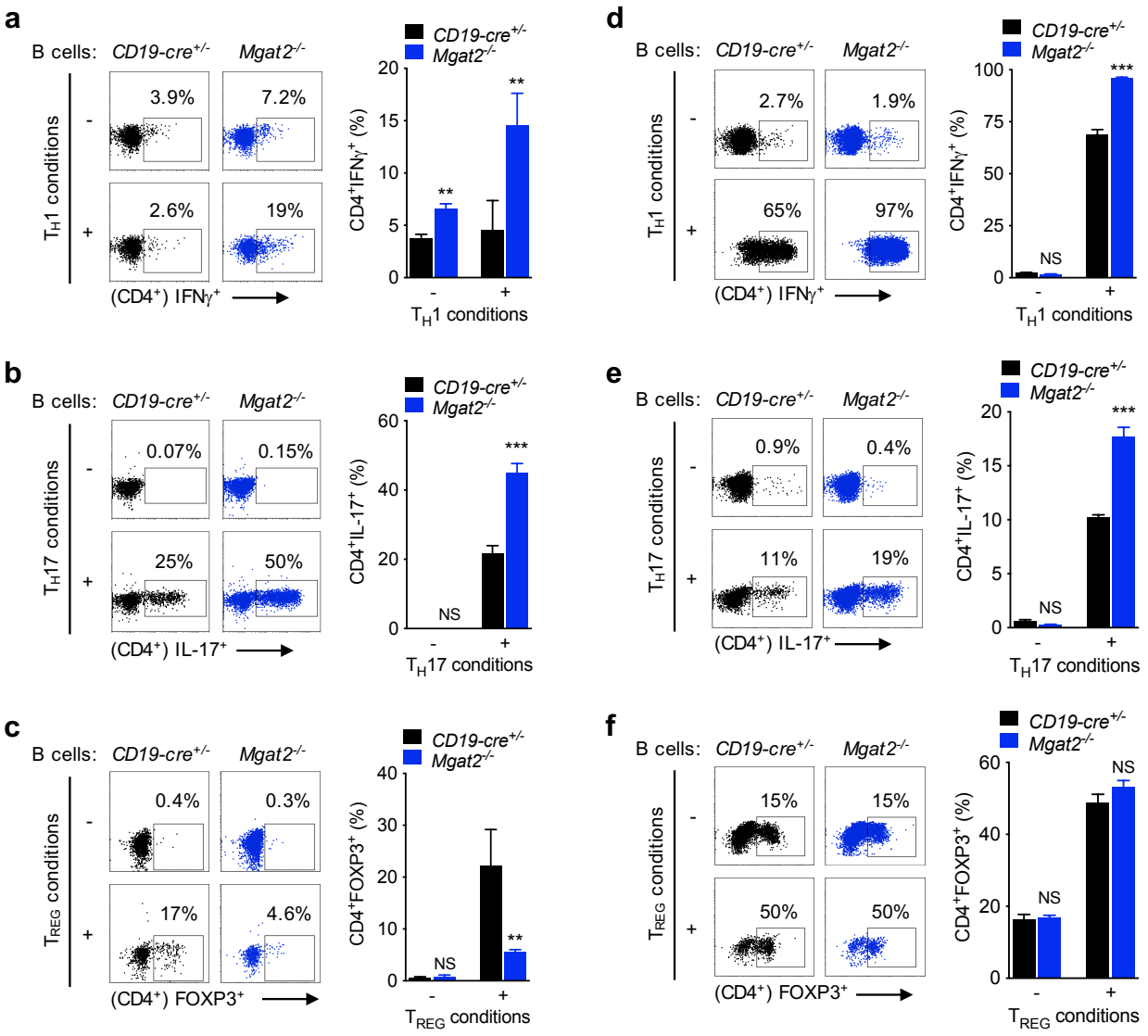

Figure 3.7. N-glycan branching deficiency in B cells promotes pro-inflammatory APC function. (**a**-**f**) LPS stimulated B cells were co-cultured with allogeneic $CD4^+$ T cells (**a**-**c**) and congenic 2D2 TCR transgenic $CD4^+$ T cells + 2.5 μg/mL $hMOG_{35-55}$ (**d**-**f**) under $T_H1/T_H17/T_{REG}$ inducing conditions. $CD4^+$ T cells were assessed by flow cytometry for intracellular staining of IFNγ after 3 days (**a**,**d**), and IL-17A (**b**,**e**) and FOXP3 (**c**,**f**) after 4 days. Data shown are mean ± s.e.m of triplicate samples and representative of n = 3 experiments. Unpaired two-tailed *t*-tests with Welch's correction. NS, not significant; **p<0.01; ***p<0.001.

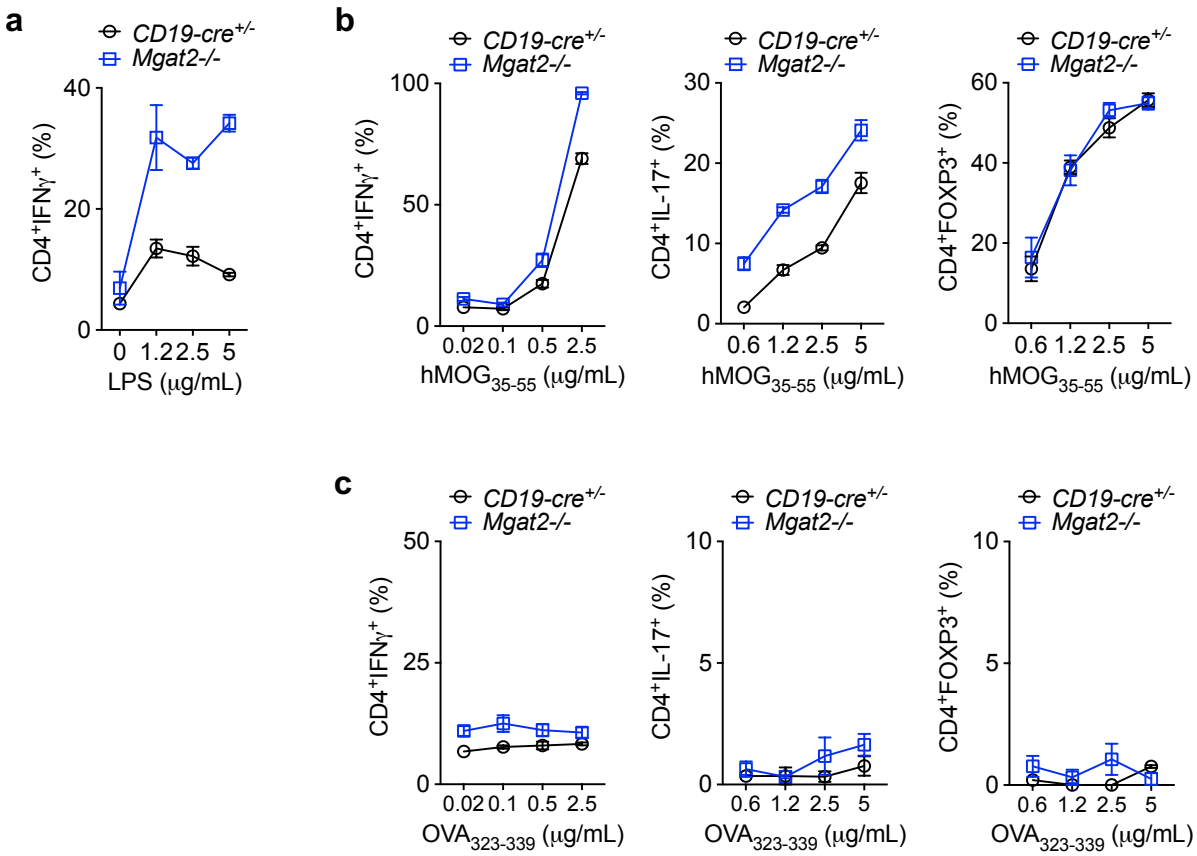

Figure 3.8. Supplement to Figure 3.7. (**a**-**c**) LPS stimulated B cells were co-cultured with allogeneic $CD4^+$ T cells under neutral conditions (**a**) and congenic 2D2 TCR^{MOG} transgenic $CD4^+$ T cells under $T_H1/T_H17/T_{REG}$ inducing conditions with indicated amounts of $hMOG_{35-55}$ (**b**) or $OVA_{323-339}$ (**c**). $CD4^+$ T cells were assessed by flow cytometry for intracellular staining of IFNγ after 3 days (**a**-**c**), and IL-17A or FOXP3 after 4 days (**b**,**c**). Data shown are mean ± s.e.m of triplicate samples and representative of n = 2 experiments.

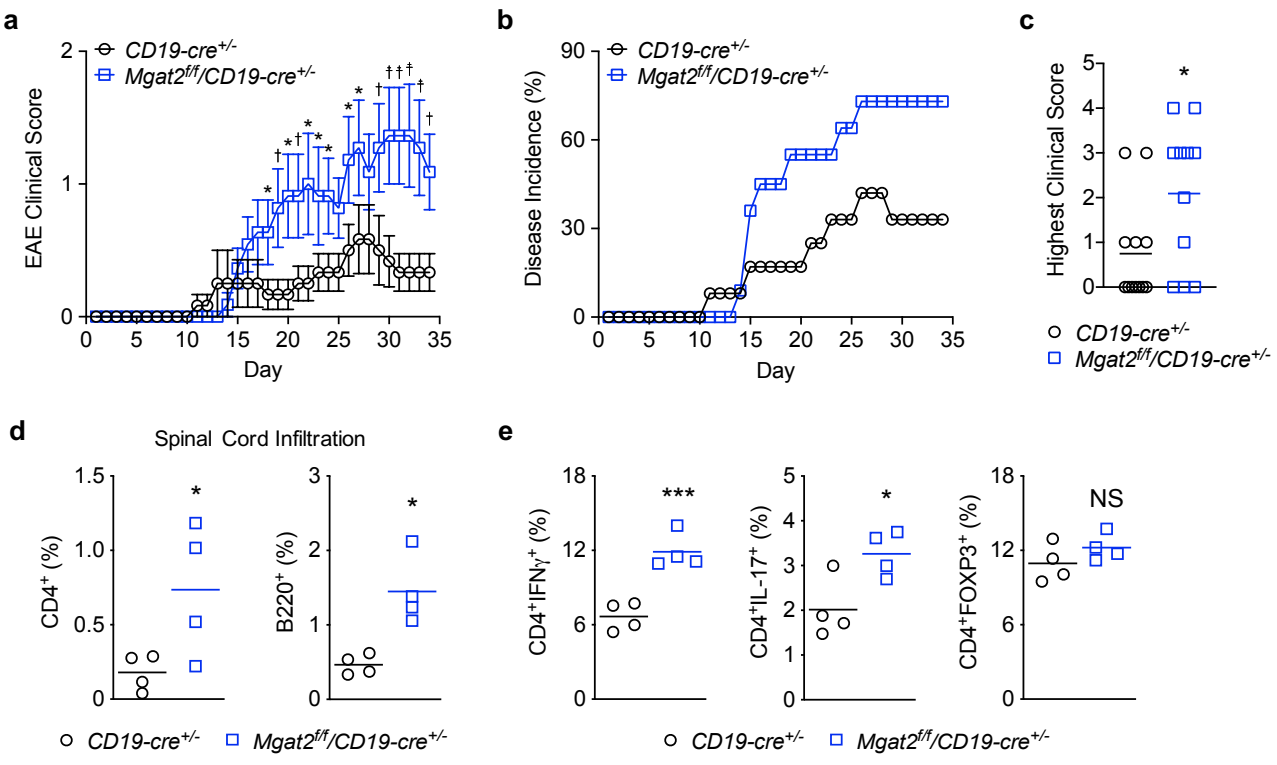

Figure 3.9. N-glycan branching deficiency in B cells promotes inflammatory demyelination. (**a**-**c**) EAE was induced in 8-12 week old mice with day 0 indicating the time of immunization and clinical score (**a**), disease incidence (**b**), and highest clinical score (**c**) monitored over 35 days (n = 12 *CD19cre*$^{+/-}$, 11 *Mgat2*$^{f/f}$*CD19cre*$^{+/-}$). (**d**,**e**) On day 35, mice induced with EAE were used for flow cytometric analysis of infiltrating CD4^{+} T cell and B220^{+} B cell in spinal cords (**d**), and *in vivo* cytokine production of splenic CD4^{+} T cells (**e**). Each symbol represents one mouse, horizontal line represents the mean, unpaired two-tailed *t*-tests with Welch's correction (**c**-**e**). NS, not significant; *p<0.05; † p<0.01; ***/ ‡ p<0.001.

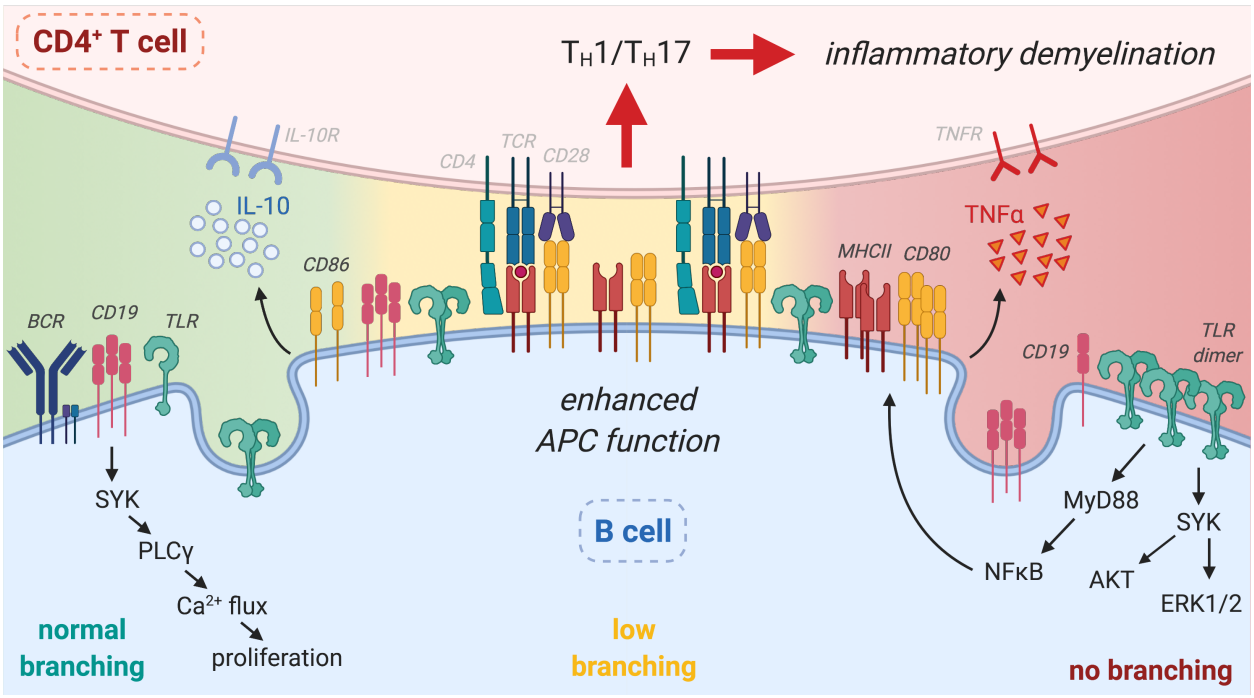

Figure 3.10. N-glycan branching decouples endocytosis of surface TLR and CD19/BCR to suppress innate and promote adaptive immunity. Branching in B cells promotes endocytic loss of surface TLR (TLR4 and TLR2) to suppress ligand-induced surface TLR signaling and downstream APC activity in B cells via reductions in MHCII, CD80 over CD86, and pro-inflammatory TNFa, which in turn inhibits B cell-dependent $CD4^+$ T_H1/T_H17 differentiation and inflammatory demyelination. Concurrently, branching promotes CD19/BCR surface-retention to drive Ca^{2+} flux and proliferation in response to BCR ligands, thereby promoting adaptive B cell function. While intermediate reductions in branching enhance surface TLR signaling, CD19/BCR surface expression/signaling is not impacted. Rather, complete absence of branched N-glycans is required to drive CD19 endocytosis and inhibit BCR responses, possibly via poly-LacNAc extension. Thus, the impact of branching on TLR/APC activity occurs over a large continuum, whereas a minimal level of branching is sufficient to promote BCR responses. Figure created with Biorender.com.

REFERENCES

1 Noseworthy, J. H., Lucchinetti, C., Rodriguez, M. & Weinshenker, B. G. Multiple sclerosis. *N Engl J Med* **343**, 938-952, doi:10.1056/NEJM200009283431307 (2000).

2 Hohlfeld, R. & Steinman, L. T Cell-Transfer Experimental Autoimmune Encephalomyelitis: Pillar of Multiple Sclerosis and Autoimmunity. *J Immunol* **198**, 3381-3383, doi:10.4049/jimmunol.1700346 (2017).

3 Ben-Nun, A. *et al.* From classic to spontaneous and humanized models of multiple sclerosis: impact on understanding pathogenesis and drug development. *J Autoimmun* **54**, 33-50, doi:10.1016/j.jaut.2014.06.004 (2014).

4 Steinman, L. Immunology of relapse and remission in multiple sclerosis. *Annu Rev Immunol* **32**, 257-281, doi:10.1146/annurev-immunol-032713-120227 (2014).

5 Kaskow, B. J. & Baecher-Allan, C. Effector T Cells in Multiple Sclerosis. *Cold Spring Harbor perspectives in medicine* **8**, doi:10.1101/cshperspect.a029025 (2018).

6 Hafler, D. A. *et al.* Risk alleles for multiple sclerosis identified by a genomewide study. *The New England journal of medicine* **357**, 851-862, doi:10.1056/NEJMoa073493 (2007).

7 Canto, E. & Oksenberg, J. R. Multiple sclerosis genetics. *Multiple sclerosis* **24**, 75-79, doi:10.1177/1352458517737371 (2018).

8 Gregory, S. G. *et al.* Interleukin 7 receptor alpha chain (IL7R) shows allelic and functional association with multiple sclerosis. *Nat Genet* **39**, 1083-1091, doi:10.1038/ng2103 (2007).

9 Lundmark, F. *et al.* Variation in interleukin 7 receptor alpha chain (IL7R) influences risk of multiple sclerosis. *Nat Genet* **39**, 1108-1113, doi:10.1038/ng2106 (2007).

10 Sawcer, S. *et al.* Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* **476**, 214-219, doi:10.1038/nature10251 (2011).

11 Hussman, J. P. *et al.* GWAS analysis implicates NF-kappaB-mediated induction of inflammatory T cells in multiple sclerosis. *Genes and immunity* **17**, 305-312, doi:10.1038/gene.2016.23 (2016).

12 Sabatino, J. J., Jr., Zamvil, S. S. & Hauser, S. L. B-Cell Therapies in Multiple Sclerosis. *Cold Spring Harbor perspectives in medicine*, doi:10.1101/cshperspect.a032037 (2018).

13 Hauser, S. L. *et al.* Ocrelizumab versus Interferon Beta-1a in Relapsing Multiple Sclerosis. *N Engl J Med* **376**, 221-234, doi:10.1056/NEJMoa1601277 (2017).

14 Hauser, S. L., Belachew, S. & Kappos, L. Ocrelizumab in Primary Progressive and Relapsing Multiple Sclerosis. *N Engl J Med* **376**, 1694, doi:10.1056/NEJMc1702076 (2017).

15 Hauser, S. L. The Charcot Lecture | beating MS: a story of B cells, with twists and turns. *Multiple sclerosis* **21**, 8-21, doi:10.1177/1352458514561911 (2015).

16 Bar-Or, A. *et al.* Abnormal B-cell cytokine responses a trigger of T-cell-mediated disease in MS? *Annals of neurology* **67**, 452-461, doi:10.1002/ana.21939 (2010).

17 Cross, A. H., Stark, J. L., Lauber, J., Ramsbottom, M. J. & Lyons, J. A. Rituximab reduces B cells and T cells in cerebrospinal fluid of multiple sclerosis patients. *J Neuroimmunol* **180**, 63-70, doi:10.1016/j.jneuroim.2006.06.029 (2006).

18 Monson, N. L. *et al.* Rituximab therapy reduces organ-specific T cell responses and ameliorates experimental autoimmune encephalomyelitis. *PLoS One* **6**, e17103, doi:10.1371/journal.pone.0017103 (2011).

19 Molnarfi, N. *et al.* MHC class II-dependent B cell APC function is required for induction of CNS autoimmunity independent of myelin-specific antibodies. *J Exp Med* **210**, 2921-2937, doi:10.1084/jem.20130699 (2013).

20 Weber, M. S. *et al.* B-cell activation influences T-cell polarization and outcome of anti-CD20 B-cell depletion in central nervous system autoimmunity. *Annals of neurology* **68**, 369-383, doi:10.1002/ana.22081 (2010).

21 Pierson, E. R., Stromnes, I. M. & Goverman, J. M. B cells promote induction of experimental autoimmune encephalomyelitis by facilitating reactivation of T cells in the central nervous system. *J Immunol* **192**, 929-939, doi:10.4049/jimmunol.1302171 (2014).

22 Iwata, Y. *et al.* Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. *Blood* **117**, 530-541, doi:10.1182/blood-2010-07-294249 (2011).

23 Okada, Y. *et al.* Signaling via toll-like receptor 4 and CD40 in B cells plays a regulatory role in the pathogenesis of multiple sclerosis through interleukin-10 production. *J Autoimmun* **88**, 103-113, doi:10.1016/j.jaut.2017.10.011 (2018).

24 Correale, J. & Farez, M. Helminth antigens modulate immune responses in cells from multiple sclerosis patients through TLR2-dependent mechanisms. *J Immunol* **183**, 5999-6012, doi:10.4049/jimmunol.0900897 (2009).

25 Fillatreau, S., Sweenie, C. H., McGeachy, M. J., Gray, D. & Anderton, S. M. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol* **3**, 944-950, doi:10.1038/ni833 (2002).

26 Demetriou, M., Granovsky, M., Quaggin, S. & Dennis, J. W. Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature* **409**, 733-739, doi:10.1038/35055582 (2001).

27 Morgan, R. *et al.* N-acetylglucosaminyltransferase V (Mgat5)-mediated N-glycosylation negatively regulates Th1 cytokine production by T cells. *J Immunol* **173**, 7200-7208 (2004).

28 Chen, I. J., Chen, H. L. & Demetriou, M. Lateral compartmentalization of T cell receptor versus CD45 by galectin-N-glycan binding and microfilaments coordinate basal and activation signaling. *The Journal of biological chemistry* **282**, 35361-35372, doi:10.1074/jbc.M706923200 (2007).

29 Lau, K. S. *et al.* Complex N-glycan number and degree of branching cooperate to regulate cell proliferation and differentiation. *Cell* **129**, 123-134, doi:10.1016/j.cell.2007.01.049 (2007).

30 Dennis, J. W., Nabi, I. R. & Demetriou, M. Metabolism, cell surface organization, and disease. *Cell* **139**, 1229-1241, doi:10.1016/j.cell.2009.12.008 (2009).

31 Grigorian, A., Torossian, S. & Demetriou, M. T-cell growth, cell surface organization, and the galectin-glycoprotein lattice. *Immunol Rev* **230**, 232-246, doi:10.1111/j.1600-065X.2009.00796.x (2009).

32 Araujo, L., Khim, P., Mkhikian, H., Mortales, C. L. & Demetriou, M. Glycolysis and glutaminolysis cooperatively control T cell function by limiting metabolite supply to N-glycosylation. *Elife* **6**, doi:10.7554/eLife.21330 (2017).

33 Mkhikian, H. *et al.* Genetics and the environment converge to dysregulate N-glycosylation in multiple sclerosis. *Nature communications* **2**, 334, doi:10.1038/ncomms1333 (2011).

34 Zhou, R. W. *et al.* N-glycosylation bidirectionally extends the boundaries of thymocyte positive selection by decoupling Lck from Ca(2)(+) signaling. *Nat Immunol* **15**, 1038-1045, doi:10.1038/ni.3007 (2014).

35 Mkhikian, H. *et al.* Genetics and the environment converge to dysregulate N-glycosylation in multiple sclerosis. *Nature communications* **2**, 334, doi:10.1038/ncomms1333 (2011).

36 Grigorian, A. *et al.* Control of T Cell-mediated autoimmunity by metabolite flux to N-glycan biosynthesis. *J Biol Chem* **282**, 20027-20035, doi:10.1074/jbc.M701890200 (2007).

37 Grigorian, A. *et al.* N-acetylglucosamine inhibits T-helper 1 (Th1) / T-helper 17 (Th17) responses and treats experimental autoimmune encephalomyelitis. *J Biol Chem*, doi:10.1074/jbc.M111.277814 (2011).

38 Lee, S. U. *et al.* N-glycan processing deficiency promotes spontaneous inflammatory demyelination and neurodegeneration. *The Journal of biological chemistry* **282**, 33725-33734, doi:10.1074/jbc.M704839200 (2007).

39 Li, C. F. *et al.* Hypomorphic MGAT5 polymorphisms promote multiple sclerosis cooperatively with MGAT1 and interleukin-2 and 7 receptor variants. *J Neuroimmunol* **256**, 71-76, doi:10.1016/j.jneuroim.2012.12.008 (2013).

40 Yu, Z., Gillen, D., Li, C. F. & Demetriou, M. Incorporating parental information into family-based association tests. *Biostatistics*, doi:10.1093/biostatistics/kxs048 (2012).

41 Yu, Z. *et al.* Family studies of type 1 diabetes reveal additive and epistatic effects between MGAT1 and three other polymorphisms. *Genes and immunity* **15**, 218-223, doi:10.1038/gene.2014.7 (2014).

42 Grigorian, A. *et al.* Pathogenesis of multiple sclerosis via environmental and genetic dysregulation of N-glycosylation. *Semin Immunopathol* **34**, 415-424, doi:10.1007/s00281-012-0307-y (2012).

43 Brynedal, B. *et al.* MGAT5 alters the severity of multiple sclerosis. *Journal of neuroimmunology* **220**, 120-124, doi:10.1016/j.jneuroim.2010.01.003 (2010).

44 Miller, S. D., Karpus, W. J. & Davidson, T. S. Experimental autoimmune encephalomyelitis in the mouse. *Curr Protoc Immunol* **Chapter 15**, Unit 15 11, doi:10.1002/0471142735.im1501s88 (2010).

45 Richner, M., Jager, S. B., Siupka, P. & Vaegter, C. B. Hydraulic Extrusion of the Spinal Cord and Isolation of Dorsal Root Ganglia in Rodents. *J Vis Exp*, doi:10.3791/55226 (2017).

46 Kawai, T. & Akira, S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* **11**, 373-384, doi:10.1038/ni.1863 (2010).

47 Leifer, C. A. & Medvedev, A. E. Molecular mechanisms of regulation of Toll-like receptor signaling. *Journal of leukocyte biology* **100**, 927-941, doi:10.1189/jlb.2MR0316-117RR (2016).

48 Siegemund, S. & Sauer, K. Balancing pro- and anti-inflammatory TLR4 signaling. *Nat Immunol* **13**, 1031-1033, doi:10.1038/ni.2452 (2012).

49 Zanoni, I. *et al.* CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. *Cell* **147**, 868-880, doi:10.1016/j.cell.2011.09.051 (2011).

50 Mkhikian, H. *et al.* Golgi self-correction generates bioequivalent glycans to preserve cellular homeostasis. *Elife* **5**, doi:10.7554/eLife.14814 (2016).

51 Schweighoffer, E., Nys, J., Vanes, L., Smithers, N. & Tybulewicz, V. L. J. TLR4 signals in B lymphocytes are transduced via the B cell antigen receptor and SYK. *J Exp Med* **214**, 1269-1280, doi:10.1084/jem.20161117 (2017).

52 Kuchroo, V. K. *et al.* B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell* **80**, 707-718 (1995).

53 Zhang, Q. & Vignali, D. A. Co-stimulatory and Co-inhibitory Pathways in Autoimmunity. *Immunity* **44**, 1034-1051, doi:10.1016/j.immuni.2016.04.017 (2016).

54 Ganley-Leal, L. M., Liang, Y., Jagannathan-Bogdan, M., Farraye, F. A. & Nikolajczyk, B. S. Differential regulation of TLR4 expression in human B cells and

monocytes. *Molecular immunology* **48**, 82-88, doi:10.1016/j.molimm.2010.09.008 (2010).

55 Mita, Y. *et al.* Toll-like receptor 4 surface expression on human monocytes and B cells is modulated by IL-2 and IL-4. *Immunol Lett* **81**, 71-75, doi:10.1016/s0165-2478(01)00328-5 (2002).

56 Noronha, A. *et al.* TILR2 and TLR4 regulate B cell responses in human inflammatory bowel disease. *Inflammatory Bowel Diseases* **14**, S8-S8 (2008).

57 Jagannathan, M. *et al.* TLR cross-talk specifically regulates cytokine production by B cells from chronic inflammatory disease patients. *J Immunol* **183**, 7461-7470, doi:10.4049/jimmunol.0901517 (2009).

58 O'Keefe, T. L., Williams, G. T., Davies, S. L. & Neuberger, M. S. Hyperresponsive B cells in CD22-deficient mice. *Science* **274**, 798-801 (1996).

59 Nitschke, L. The role of CD22 and other inhibitory co-receptors in B-cell activation. *Curr Opin Immunol* **17**, 290-297, doi:10.1016/j.coi.2005.03.005 (2005).

60 Han, S., Collins, B. E., Bengtson, P. & Paulson, J. C. Homomultimeric complexes of CD22 in B cells revealed by protein-glycan cross-linking. *Nature chemical biology* **1**, 93-97, doi:10.1038/nchembio713 (2005).

61 Collins, B. E., Smith, B. A., Bengtson, P. & Paulson, J. C. Ablation of CD22 in ligand-deficient mice restores B cell receptor signaling. *Nat Immunol* **7**, 199-206, doi:10.1038/ni1283 (2006).

62 Monroe, J. G. ITAM-mediated tonic signalling through pre-BCR and BCR complexes. *Nat Rev Immunol* **6**, 283-294, doi:10.1038/nri1808 (2006).

63 Bhatia, S., Edidin, M., Almo, S. C. & Nathenson, S. G. B7-1 and B7-2: similar costimulatory ligands with different biochemical, oligomeric and signaling properties. *Immunol Lett* **104**, 70-75, doi:10.1016/j.imlet.2005.11.019 (2006).

64 Magliozzi, R. *et al.* Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. *Brain* **130**, 1089-1104, doi:10.1093/brain/awm038 (2007).

65 Mitsdoerffer, M. & Peters, A. Tertiary Lymphoid Organs in Central Nervous System Autoimmunity. *Front Immunol* **7**, 451, doi:10.3389/fimmu.2016.00451 (2016).

Chapter 4

Conclusions and Future Directions

We have demonstrated that the galectin-glycoprotein lattice is an important regulator of B cell central tolerance and peripheral function in a cell intrinsic manner via N-glycan branching. The phenotypic consequences of N-glycan dysregulation in B cells are similar to what we've reported in T cells – on one hand, it results in the lack of a functional B cell repertoire, and on the other, it leads to aberrant B cell activation and autoimmunity. However, there are differences in how N-glycan branching regulates T and B cells worth mentioning.

When it comes to T cell proliferation, N-glycan branching deficiency leads to hyper-proliferation in a manner proportional to level of branching (i.e., *Mgat1*$^{-/-}$ > *Mgat2*$^{-/-}$ ≥ *Mgat5*$^{-/-}$).[1-3] Alternatively, GlcNAc supplementation, which further raises N-glycan branching, suppresses proliferation of T cells.[3] Hyper-proliferation of N-glycan branching deficient T cells is likely through PLCγ mediated Ca^{2+} flux, as this was shown to be enhanced.[4] For B cells, however, we observe an opposite phenotype in that complete loss of N-glycan branching due to *Mgat1*$^{-/-}$ led to reduced PLCγ activation, Ca^{2+} flux, and hypo-proliferation. Thus, N-glycan branching functions to suppress proliferation of T cells yet promote the proliferation of B cells. N-glycan branching promotes Ca^{2+} flux induced B cell proliferation by retaining CD19 at the surface, as evidenced by significant loss of surface CD19 in *Mgat1*$^{-/-}$ B cells. Notably, it is only *Mgat1*$^{-/-}$ that displayed this phenotype, as *Mgat2*$^{-/-}$ B cells have no difference in surface CD19, Ca^{2+} flux, and proliferation compared to control. *Mgat5*$^{-/-}$ B cells also proliferate similar to control.[1] This suggests poly-LacNAc extension/compensation in B cells could be more efficient than in T cells, since *Mgat2*$^{-/-}$ T cell proliferation is only comparable to that of *Mgat5*$^{-/-}$ T cells. Investigating poly-LacNAc compensation in B cells would provide

us insight in how this glycan self-correcting mechanism may differ amongst lymphocyte populations.

Loss of surface CD19 in *Mgat1*$^{-/-}$ but not *Mgat2*$^{-/-}$ B cells demonstrate how critical N-glycan branching and extension is in regulating this co-receptor. It would be worth exploring whether forcing a greater “on” rate of CD19 (e.g., by CD19 overexpression) in N-glycan branching deficient B cells could rescue the hypo-proliferative phenotype. If surface CD19 levels were made to be comparable to that of wildtype/control levels, we would expect N-glycan branching deficient B cells to become hyper-proliferative like their T cell counterparts due to enhanced BCR/TLR clustering. It would also be interesting to explore how GlcNAc supplementation to B cells would affect proliferation and other functional outcomes. Increasing N-glycan branching with GlcNAc would promote CD19 surface retention to enhance proliferation, or raise the threshold of BCR/TLR engagement to prevent receptor clustering and inhibit activation. This is likely temporally regulated and nutrient/metabolite availability dependent. Thus, various time course and metabolism studies would allow us to understand how N-glycan branching dynamically changes during the course of B cell activation, growth arrest, and differentiation.

Our data on N-glycan branching and TLRs in B cells has challenged our current model of the galectin-glycoprotein lattice. Growth-promoting receptors (e.g., TCR and receptor tyrosine kinases) typically have high numbers of N-glycans (> 5) while growth inhibitory receptors (e.g., CTLA-4) have few N-glycans (≤ 4), allowing the Golgi to globally control growth versus arrest/differentiation signaling.[5,6] As binding avidity between galectins and glycoproteins increases in proportion to LacNAc content, high N-

glycan density within a glycoprotein generates significant avidity for galectins at low levels of branching, resulting in significant surface retention under basal conditions. In contrast, low N-glycan density falls below the threshold for stable association, requiring high levels of N-glycan branching activity for maintenance at the cell surface. The growth-promoting receptor, TLR4, has a high number of N-glycan sites (10 for humans, 14 for mice), yet surface levels are barely detectable under basal conditions and when control B cells are stimulated with the TLR4 agonist, LPS. When B cells are N-glycan branching deficient, however, LPS induces a rise in TLR4 surface levels due to reduced endocytosis. Here, we have shown the galectin-glycoprotein lattice limits surface retention of growth-promoting TLR4 (and TLR2) in B cells. The current model does apply to growth-promoting CD19 (number of N-glycan sites = 5 for humans, 7 for mice), which is highly expressed and retained on the surface. Thus, we propose an alternative model of the lattice in which N-glycan branching promotes adaptive (i.e., CD19/BCR, TCR) and suppresses innate (i.e., TLR) signaling. From examining N-glycan branching levels across immune cell types, B cells, harboring both adaptive and innate growth receptors, sit between T cells and myeloid cells (Figure 4.1). Investigating how N-glycan branching regulates myeloid cells would unveil whether this proposed model stands, or if myeloid cells employ different N-glycan branching mechanisms to regulate activation and growth.

Early B Cell Development

We have demonstrated N-glycan branching to be a critical regulator of T cell and B cell central selection. A potential area of future research is determining the role of N-

glycan branching in early B cell development – at the pro- and pre- B cell stages. IL-7R signaling is essential during these stages, as it supports pro-B cell survival, the pro-B to large pre-B cell transition, and large pre-B cell proliferation.[7-9] Furthermore, galectins are suggested to play a role in early B cell development, implicating the galectin-glycoprotein lattice. Gal-3$^{-/-}$ mice have greater numbers of pro-B cells with elevated surface levels of IL-7R, suggesting Gal-3 loss led to IL-7R induced arrest at the pro-B cell stage.[10] Our group has demonstrated N-glycan branching regulates IL-7R signaling in T cells.[11] Thus, we speculate N-glycan branching may affect IL-7R signaling in pro- and pre- B cells. It would be interesting to also explore how N-glycan branching regulates other important factors in B cell development and maturation, such as Notch signaling.

Anti-inflammatory Regulatory B Cells

Like T_{REG} cells, regulatory B (B_{REG}) cells are known for their immunosuppressive role in infections, autoimmunity, organ transplantation tolerance, and cancer.[12,13] A handful of B_{REG} subsets have been identified, with one of the more prominent ones being B10 cells which are characterized as CD1d^{hi}CD5^{+} B cells that produce IL-10. We have shown N-glycan branching promotes T_{REG} induction.[14,15] Our preliminary data suggest N-glycan branching promotes the presence of B10 cells *in vivo,* evidenced by significant reductions in B10 cell percentage from *Mgat2*$^{-/-}$ and *Mgat1*$^{-/-}$ B cells (Figure 4.2). CD19 has been shown to be essential in the generation of B10 cells[16], which likely explains why *Mgat1*$^{-/-}$ B cells, being CD19 deficient, are devoid of B10 cells. However, this wouldn't explain the reduction of B10 cells in *Mgat2*$^{-/-}$ B cells as they still retain high

levels of CD19. IL-21R and CD40 signaling are also known to be important for B10 cell generation[17], so it is worth investigating whether N-glycan branching regulates these receptors for B10 cell generation, especially in *Mgat2*$^{-/-}$ B cells. Determining the role of N-glycan branching and the galectin-glycoprotein lattice in B10 cells would be novel and potentially of high impact.

Antibody Class-Switching

Producing infection fighting antibodies are the primary function of B cells, and whether N-glycan branching regulates this function is largely unknown. Immunoglobulin class switch recombination (CSR) occurs during the germinal center reaction, in which antigen engaged IgM-BCR expressing naïve B cells undergo rapid proliferation and differentiation to express class-switched IgG, IgE, or IgA receptors and antibodies with various functionalities.[18] Cytokines are important during this process, as the presence and combination of different types influence which immunoglobulin isotype the B cell switches to. Preliminary data suggests N-glycan branching plays a role in influencing B cell Ig CSR *in vitro* (Figure 4.3). Notably, N-glycan branching deficiency (*Mgat1*$^{-/-}$) enhanced CSR to a pro-inflammatory isotype, IgG3, while completely abolishing CSR to parasite fighting and allergy mediating, IgE (Figure 4.3a). The cytokine, IL-4, induces CSR to IgG1 and IgE, and preliminary data suggests surface levels of IL-4R is reduced in N-glycan branching deficient B cells (Figure 4.3b). Interestingly, reduced surface IL-4R did not affect CSR to IgG1. Further investigation is needed to delineate how N-glycan branching differentially induces IL-4R dependent IgG1 and IgE CSR, as well as

the other isotypes. It is also important and necessary to determine if this preliminary *in vitro* data have physiological relevance *in vivo* using mouse models of immunization.

There are many other areas of future research to consider when exploring how N-glycan branching and the galectin-glycoprotein lattice affects B cell homeostasis and function, such as plasma cell generation, memory B cell generation, BCR clonality, age associated B cells (i.e., B cells in aging), and single particle tracking of B cell receptors. Undoubtedly, the work to date presented in this dissertation is just the tip of the iceberg for what we have discovered in N-glycan branching regulating B cell development and functions. Sometimes considered the “boring” immune cell, the B cell has risen in prominence over the past decade as researchers have continued to uncover new things about them. I hope our continued work on N-glycan branching in B cells and T cells brings the role of glycosylation to the forefront of immunology.

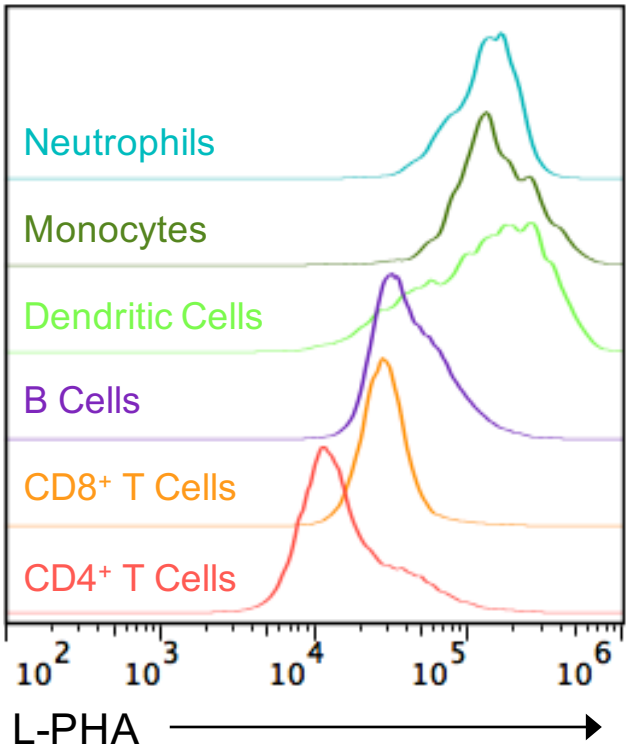

Figure 4.1. N-glycan branching levels of different immune cells. Flow cytometric analysis comparing N-glycan branching (i.e., L-PHA binding) on myeloid cells (neutrophils, monocytes, and dendritic cells) and lymphocytes (B cells, $CD8^+$ T cells, and $CD4^+$ T cells).

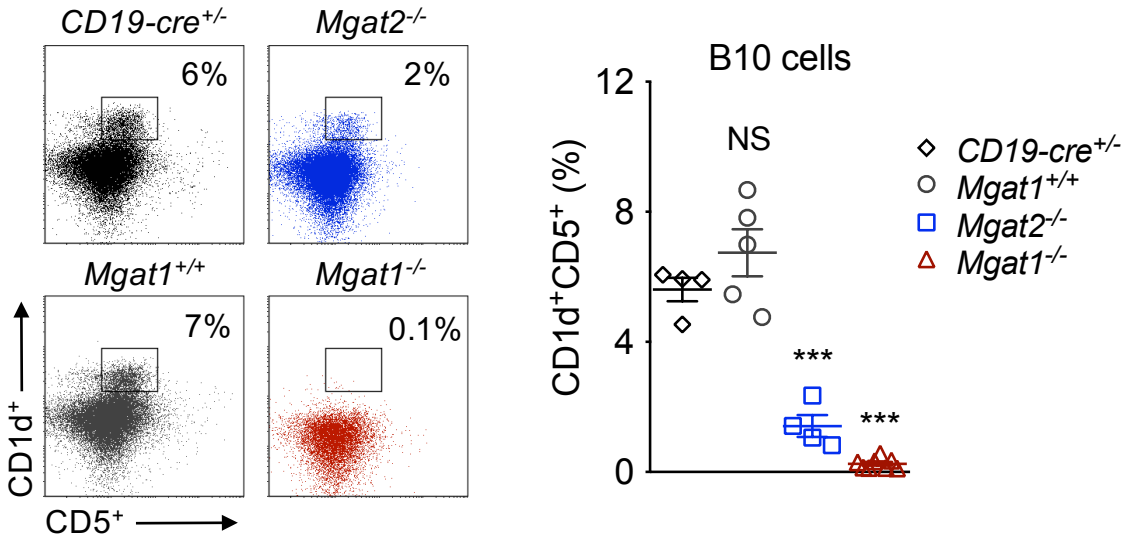

Figure 4.2. N-glycan branching affects B10 cells *in vivo*. Flow cytometric analysis of *ex vivo* splenocytes gated on B220^{+} B cells. *Mgat2*$^{-/-}$ B cells are from *Mgat2*$^{f/f}$*/CD19-cre*$^{+/-}$ mice, and *Mgat1*$^{-/-}$ B cells are from doxycycline treated *Mgat1*$^{f/f}$*/tetO-cre/ROSA26-rtTA* mice. Each dot represents one mouse, error bars indicate mean ± s.e.m. Unpaired two-tailed *t*-tests with Welch's correction. NS, not significant; ***P < 0.0005.

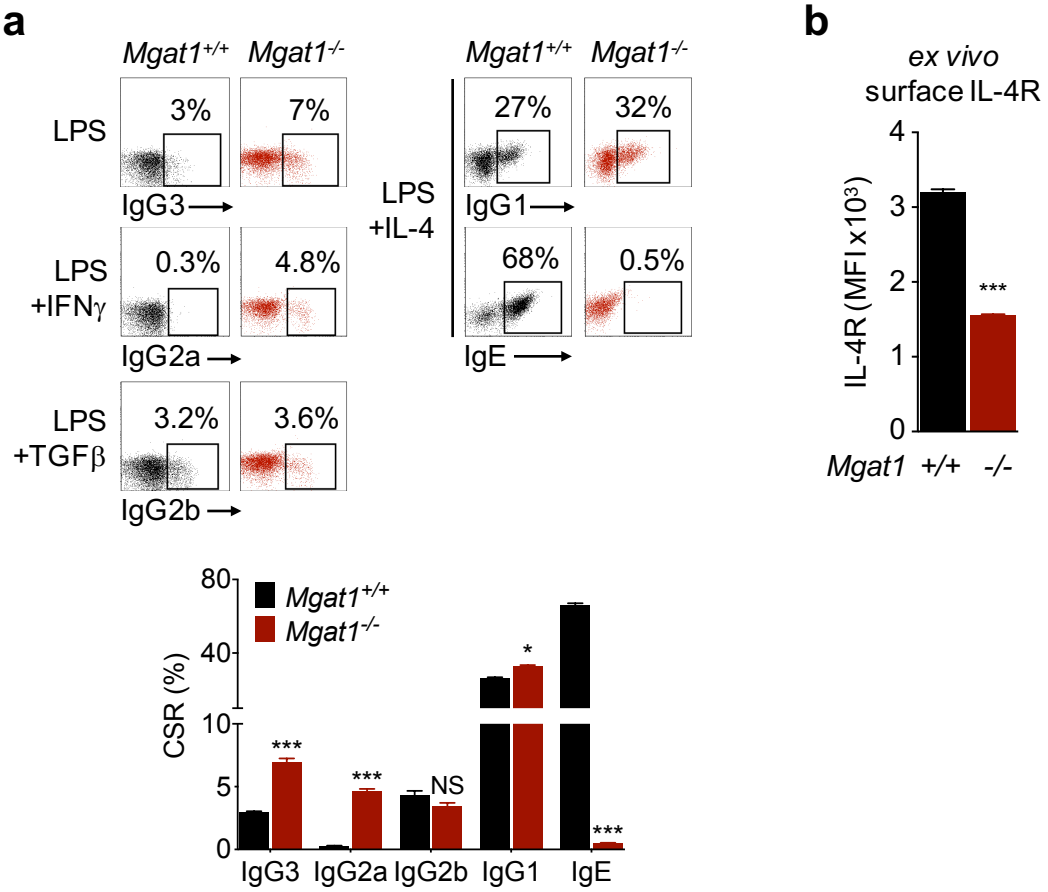

Figure 4.3. N-glycan branching regulates B cell immunoglobulin CSR *in vitro*. Flow cytometric analysis of surface staining for class-switched B cells after four days of culture in the presence of LPS ± inducing cytokines. Unpaired two-tailed *t*-tests with Welch's correction. Error bars indicate s.em. NS, not significant; ***$P < 0.0005$; **$P < 0.005$; *$P < 0.05$.

REFERENCES

1 Demetriou, M., Granovsky, M., Quaggin, S. & Dennis, J. W. Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature* **409**, 733-739, doi:10.1038/35055582 (2001).

2 Mkhikian, H. *et al.* Golgi self-correction generates bioequivalent glycans to preserve cellular homeostasis. *Elife* **5**, doi:10.7554/eLife.14814 (2016).

3 Grigorian, A. *et al.* Control of T Cell-mediated autoimmunity by metabolite flux to N-glycan biosynthesis. *J Biol Chem* **282**, 20027-20035, doi:10.1074/jbc.M701890200 (2007).

4 Zhou, R. W. *et al.* N-glycosylation bidirectionally extends the boundaries of thymocyte positive selection by decoupling Lck from Ca(2)(+) signaling. *Nat Immunol* **15**, 1038-1045, doi:10.1038/ni.3007 (2014).

5 Lau, K. S. *et al.* Complex N-glycan number and degree of branching cooperate to regulate cell proliferation and differentiation. *Cell* **129**, 123-134, doi:10.1016/j.cell.2007.01.049 (2007).

6 Dennis, J. W., Nabi, I. R. & Demetriou, M. Metabolism, cell surface organization, and disease. *Cell* **139**, 1229-1241, doi:10.1016/j.cell.2009.12.008 (2009).

7 Ochiai, K. *et al.* A self-reinforcing regulatory network triggered by limiting IL-7 activates pre-BCR signaling and differentiation. *Nat Immunol* **13**, 300-307, doi:10.1038/ni.2210 (2012).

8 von Freeden-Jeffry, U. *et al.* Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J Exp Med* **181**, 1519-1526, doi:10.1084/jem.181.4.1519 (1995).

9 Rolink, A. G., Winkler, T., Melchers, F. & Andersson, J. Precursor B cell receptor-dependent B cell proliferation and differentiation does not require the bone marrow or fetal liver environment. *J Exp Med* **191**, 23-32, doi:10.1084/jem.191.1.23 (2000).

10 de Oliveira, F. L. *et al.* Lack of galectin-3 modifies differentially Notch ligands in bone marrow and spleen stromal cells interfering with B cell differentiation. *Sci Rep* **8**, 3495, doi:10.1038/s41598-018-21409-7 (2018).

11 Grigorian, A., Mkhikian, H. & Demetriou, M. Interleukin-2, Interleukin-7, T cell-mediated autoimmunity, and N-glycosylation. *Ann N Y Acad Sci* **1253**, 49-57, doi:10.1111/j.1749-6632.2011.06391.x (2012).

12 Rosser, E. C. & Mauri, C. Regulatory B cells: origin, phenotype, and function. *Immunity* **42**, 607-612, doi:10.1016/j.immuni.2015.04.005 (2015).

13 Nouel, A., Simon, Q., Jamin, C., Pers, J. O. & Hillion, S. Regulatory B cells: an exciting target for future therapeutics in transplantation. *Front Immunol* **5**, 11, doi:10.3389/fimmu.2014.00011 (2014).

14 Araujo, L., Khim, P., Mkhikian, H., Mortales, C. L. & Demetriou, M. Glycolysis and glutaminolysis cooperatively control T cell function by limiting metabolite supply to N-glycosylation. *Elife* **6**, doi:10.7554/eLife.21330 (2017).

15 Lee, S. U. *et al.* Increasing cell permeability of N-acetylglucosamine via 6-acetylation enhances capacity to suppress T-helper 1 (TH1)/TH17 responses and autoimmunity. *PLoS One* **14**, e0214253, doi:10.1371/journal.pone.0214253 (2019).

16 Yanaba, K. *et al.* A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. *Immunity* **28**, 639-650, doi:10.1016/j.immuni.2008.03.017 (2008).

17 Yoshizaki, A. *et al.* Regulatory B cells control T-cell autoimmunity through IL-21-dependent cognate interactions. *Nature* **491**, 264-268, doi:10.1038/nature11501 (2012).

18 Mesin, L., Ersching, J. & Victora, G. D. Germinal Center B Cell Dynamics. *Immunity* **45**, 471-482, doi:10.1016/j.immuni.2016.09.001 (2016).