CHARACTERIZATION OF SWITCHED MEMORY B CELLS (SMB) IN PATIENTS WITH SECONDARY ANTIBODY DEFICIENCY DUE TO HEMATOLOGICAL MALIGNANCIES

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ABSTRACT

The global incidence of secondary antibody deficiency (SAD), an acquired immunodeficiency associated with increased risk of infections and infection-related morbidity and mortality, is on the rise. Immunoglobulin replacement therapy (IGRT) is the standard treatment for SAD associated with hematological malignancies (HM). However, SAD may be reversible in some patients and prolonged IGRT use may not be necessary. Currently, no standardized algorithm exists to guide the safe discontinuation of IGRT, and decisions largely rely on clinical judgement. This highlights the need for a reliable biomarker of humoral immune reconstitution. We hypothesized that switched memory B cells (SMB) may be associated with humoral immune reconstitution and can serve as a biomarker to guide safe IGRT discontinuation.

The primary objective of this thesis is to characterize the SMB cell proportion in patients with HM-associated SAD (HM-SAD) every 3-6 months over a one-year follow-up period. A prospective observational study was conducted involving 25 eligible adults with history of HM and receiving IGRT. Peripheral blood mononuclear cells were isolated and analyzed using spectral flow cytometry. SMB cells were defined as $CD19^+$ $CD27^+$ IgM^- IgD^- B cells and expressed as a percentage of total B lymphocytes, with normal values defined as ≥2%.

Low SMB cell frequencies were observed in 68% of participants and persisted long after disease remission despite normal total B cell frequencies in 56% of participants. No significant changes in SMB cell proportions were observed over the one-year follow-up period. Low SMB cell frequencies were not associated with age, sex, infection history, HM subtypes or duration of IGRT, but were common in participants with history of hematopoietic stem cell transplantation (83.3%) or rituximab exposure (75%). SMB cells also showed a strong positive correlation with

IgG^+ SMB cells and moderate positive correlation with IgA^+ SMB cells. These findings suggest disturbed humoral immunity characterized by sustained reductions in SMB cell proportions despite recovery of total B cells and durable disease remission. Further studies are required to determine the utility of SMB cells, alone or in combination with other immunological markers, in guiding safe IGRT discontinuation.

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ABBREVIATIONS

ANCA - Anti-Neutrophil Cytoplasmic Antibody

ALL – Acute lymphoblastic leukemia

Allo-HSCT – Allogeneic hematopoietic stem cell transplant

Auto-HSCT – Autologous hematopoietic stem cell transplant

BsAbs - Bispecific antibodies

BCMA - B-cell maturation antigen

BCR – B cell receptor

BIF – B-cell inhibitory factor

BiTE – Bispecific T cell engager

BM – Bone marrow

BSA – Bovine serum albumin

BSF-1 – B-cell stimulatory factor 1

BTK – Burton's Tyrosine Kinase

CAR-T – Chimeric Antigen Receptor T cell

CLL – Chronic lymphocytic leukemia

CML – Chronic myeloid leukemia

CMV – Cytomegalovirus

CPA – Cyclophosphamide

CIU – Clinical investigation unit

CVID – Common variable immunodeficiency

DLBCL – Diffuse large B cell lymphoma

EBV – Epstein-Barr virus

FBS – Fetal bovine serum

FDA – US Food and Drug Administration

FMO – Fluorescence minus one

GC – Germinal center

GVHD – Graft versus host disease

HL – Hodgkin's lymphoma

HM – Hematological malignancies

HSCT – Hematopoietic stem cell transplant

HSV – Herpes simplex virus

Ig – Immunoglobulins

IGRT – Immunoglobulin replacement treatment

IMiDs – Immunomodulatory drugs

IVIG – Intravenous immunoglobulin

NHL – Non-Hodgkin's lymphoma

MBCs – Memory B cells

MCL – Mantle cell lymphoma

MM – Multiple myeloma

OHRI – Ottawa Hospital Research Institute

PAD – Primary antibody deficiency

PB – Peripheral blood

PBMC – Peripheral blood mononuclear cells

PID – Primary immunodeficiency

PI – Proteasome inhibitors

RSV – Respiratory syncytial virus

SAD – Secondary antibody deficiency

SAE – Serious adverse events

SCIG – Subcutaneous immunoglobulins

SID – Secondary immunodeficiency

SMB – Switched memory B cells

scFv – Single-chain variable fragments

Tfh – T follicular helper cells

TK – Tyrosine kinase

TKI – Tyrosine kinase inhibitors

TOH – The Ottawa Hospital

VZV – Varicella-zoster virus

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CHAPTER 1: INTRODUCTION

1.1 Thesis overview

This thesis is organized into four chapters. Chapter 1 presents a comprehensive review of the existing literature on secondary antibody deficiency (SAD) in hematological malignancies (HM), the use of immunoglobulin replacement therapy (IGRT), current knowledge gaps in identifying humoral immune reconstitution, rationale for investigating switched memory B cells (SMB cells) as a biomarker of humoral immune recovery, and finally the hypothesis and objectives of this study. Chapter 2 describes the methodology employed to address the research objectives. Chapter 3 reports the study findings on all outcome measures, and Chapter 4 summarizes and discusses the implications of these results in the context of existing literature, while also highlighting the study strengths, limitations, and providing directions for future research followed by concluding remarks.

1.2 Hematological malignancies (HM)

Hematological malignancies (HM) are a diverse group of blood cancers that develop due to disruptions in hematopoiesis in the bone marrow (BM) or abnormal proliferation and differentiation of cells in the blood and the lymphatic system.[1] They are among the most common types of cancers and represent a significant global health burden.[2–4] In Canada, it was estimated that 23,600 new cases of hematological malignancies were diagnosed in 2024.[5] These accounted for approximately 10% of all cancer diagnosis and 9% of all cancer-related deaths. There is global increase in the burden of hematological malignancies driven by rapidly aging populations which will be reflected in the rise in the number of new cases and deaths despite overall declining trends in incidence and mortality.[3,4]

Hematological malignancies consist of myeloid and lymphoid neoplasms that can be classified through a hierarchical system according to cellular lineage, clinical and genetic features.[6] Some of the common subtypes are classified into lymphoma, leukemia, and multiple myeloma.[4,6,7] Each type is different in terms of clinical characteristics, treatment approaches and prognosis.[8–10]

This project focuses on B cell derived hematological malignancies such as non-Hodgkin's lymphoma, chronic lymphocytic leukemia and multiple myeloma, and their association with secondary antibody deficiency. A brief description of these HMs is presented below.

1.2.1 Lymphoma

Lymphoma is a heterogeneous group of malignant lymphoid neoplasms resulting from clonal proliferation of lymphocytes.[11] With more than 90 subtypes, they are often broadly divided into two groups: Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL).

Hodgkin's lymphoma is a relatively rare but highly curable form of lymphoma that is most commonly derived from germinal center (GC) B-lymphocytes.[12] It is characterized by the presence of the pathological Hodgkin and Reed-Sternberg cells. HL has a bimodal age distributions peaking in age group 15-34 and again in the age group >50 years of age. The Epstein-Barr virus (EBV) has been extensively studied and has shown a causal role in HL with more than 30% of HL are positive for EBV.[12]

Non-Hodgkin's lymphoma (NHL) is a neoplasm in the lymphoid tissues involving both B and T cells.[13,14] It is one of the most common HM globally and is projected to be the 5th most diagnosed cancer.[5] The average age of onset is 67 years, and the average age of mortality is 76 years. NHL encompasses over 40 major subtypes and can be classified based on prognosis into

"indolent" or "aggressive" forms. Indolent lymphomas often present with fluctuations in lymphadenopathy and include follicular lymphoma, splenic marginal zone lymphoma, and small lymphocytic lymphoma. Aggressive lymphomas, such as diffuse large B cell lymphoma (DLBCL), Burkitt lymphoma, and adult T cell lymphoma, tend to present with distinct B symptoms that may be fatal if not treated quickly.[13]

The 5-year survival rates, although varies across the globe, have improved drastically since 1975, up from 46% to 72.7% in 2016 according to US statistics.[14,15] This improvement is largely driven by novel and innovative developments in targeted therapeutics and stem cell transplants.

1.2.2 Leukemia

Leukemia, is a heterogeneous group of blood cancers that originate in the bone marrow, resulting from abnormal proliferation of hematopoietic stem cells which then flood the body with abnormal immature leukocytes "blast" cells.[16] These cells undergo clonal expansion which overtake and interfere with the function of normal cells. Based on the speed of proliferation, leukemia can be classified as "chronic" or "acute", and based on the cell of origin, it can be classified as "lymphocytic" or "myelocytic".[17,18]

Chronic Lymphocytic Leukemia (CLL) is a type of mature B cell neoplasm characterized by the proliferation and accumulation of monoclonal B lymphocytes in the peripheral blood (PB), BM and lymphoid tissues.[19] It is considered the most common leukemia in North America and accounts for approximately 30% of all leukemia, affecting mostly elderly persons with median age of 71 years at diagnosis.[20–22] CLL is classically considered to be indolent and treatment is not always necessary at diagnosis but is initiated when patients become symptomatic from the disease (around 80% of CLL patients present with asymptomatic disease).[20] The 5-year

survival rate has improved significantly in the modern novel therapy era (2015-2020) reaching over 60% with median overall survival of 7.8 years compared to 5.2 years between 2005-2014. Access to novel therapy varies across countries and consequently so do the survival rates.[23,24]

Acute Lymphoblastic Leukemia (ALL) is a disease characterized by clonal proliferation and differentiation of lymphoid progenitor cells in the BM.[25] It is the second most common acute leukemia in adults, however; it typically affects young children with only 20% of cases in adults.[25] The five-year net survival is lower in females than in males (42% vs. 51%).[26]

Clinical presentation of leukemias can be nonspecific including fatigue, swollen lymph nodes, fever, night sweats and unexplained weight loss.[27] Other symptoms specific to the type of leukemia may include petechiae, pallor, pain in joints and extremities, splenomegaly and hepatomegaly.

1.2.3 Plasma cell disease

Multiple myeloma (MM) is a type of plasma cell disease and the second most common HM, accounting for about 10% of all HM.[28] It is characterized by the expansion of malignant plasma cells, derived from post-germinal B cells, in the BM.[29,30] These cells release excess amounts of monoclonal immunoglobulins (M-proteins) in the serum or urine and result in the suppression of uninvolved (normal) immunoglobulins.[31–33] It is commonly associated with hypercalcemia, osteolytic bone disease, anemia and acute kidney injury or renal failure.[29]

MM predominantly affects the elderly where the median age at diagnosis is 69 years and this is also reflected in the steady and global increase in the incidence of MM.[28] Survival rates have significantly improved due to new treatment options, rising from 25% in the mid-1970s to nearly 61% by 2020 in the United States.[26]

Overall, despite the incredible improvements in diagnosis, treatments and consequently survival rates of these lymphoproliferative diseases, patients are often confronted with yet another medical complication: Secondary Antibody Deficiency (SAD). SAD can leave patients susceptible to recurrent, persistent and potentially life-threatening infections (bacterial, viral and fungal), which become the major cause of morbidity and mortality in many cases.

1.3 Secondary antibody deficiency (SAD)

Secondary immunodeficiency (SID) is an acquired immune condition caused by a wide spectrum of underlying diseases, such as HM, and their related treatments.[34] Secondary antibody deficiency is a type of SID that is characterized by quantitative decrease in serum IgG antibody levels (hypogammaglobulinemia), or impaired antibody function as evident by poor T cell-independent vaccine response. SAD is estimated to be 30 times more common than primary antibody deficiency (PAD), which are impairments in antibody production or function due to genetic inborn errors of immunity.[35] However, HM-associated SAD (HM-SAD) has been less extensively studied compared to PAD.[36]

SAD has been reported to impact between 22% to 85% of patients with hematological malignancies but is also prevalent in a diverse range of other conditions.[35,37–39] Around 15% of patients with NHL have hypogammaglobulinemia, which nearly doubles after receiving B cell depleting therapies like rituximab.[38] Similarly, it was also estimated that 25% of patients with newly diagnosed CLL have hypogammaglobulinemia, and another 25% will develop hypogammaglobulinemia at follow-up despite having normal IgG levels at diagnosis.[40] SAD can also affect 45-90% of MM patients and up to 65% of ALL patients.[35,41,42] The increased prevalence of SAD is primarily due to the rapid expansion of new B cell targeting therapies used for conditions including inflammatory, autoimmune, transplants and malignancies.[34,35,43–49]

As previously noted, SAD increases the risk of infections and infection-related morbidity and mortality. Studies have shown that infections are the leading cause of death in CLL and MM patients; up to 50% and 60% of deaths in MM and CLL patients, respectively, have been attributed to infections.[48,50] Therefore; assessing the patients' risk of developing SAD could allow for preventative and potentially life-saving care to be effectively administered.[42,51]

1.3.1 Diagnosis of SAD

Diagnostic and clinical criteria for SAD evaluation and management vary across regions.[51] Total serum IgG concentration is the most used indicator of antibody deficiency but assessing antibody function and the magnitude of the humoral immune response against specific pathogens is also of considerable importance.[48,52] This is necessary in cases where patients have quantitatively normal antibody levels but continue having recurrent infections. Therefore, it is usually recommended to complete a comprehensive immunological and clinical evaluation including measurement of immunoglobulins (IgG, IgA, and IgM), assessing specific antibody responses to vaccines (such as pneumococcal polysaccharide and conjugate vaccines), and thorough assessment of infection history and frequency.[45,51]

1.3.2 Mechanism of SAD in hematological malignancies

The exact mechanisms leading to SAD in patients with hematological malignancies remain under investigation. Hypogammaglobulinemia in patients with NHL is often associated with their treatments rather than the disease process itself.[42] Limited data is available on SAD in NHL patients in the literature and, therefore; requires further investigation.[45] Notably, NHL and the primary immunodeficiency (PID) called Common Variable Immunodeficiency (CVID), a condition marked by low Ig production, have been strongly associated with one another.[42,53] This links to the possibility that prolonged antibody deficiency in NHL patients may be due to an

underlying PID and may not be reversible, therefore; requiring life-long treatment for the antibody deficiency.

Similar to NHL, SAD is poorly studied in ALL patients and is often linked to the use of chemotherapeutic regimens. However, it has been reported that the disease process itself leads to qualitative defects in lymphocytes that result in hypogammaglobulinemia. [41,54]

Most patients with CLL have hypogammaglobulinemia that becomes more prominent with disease progression and associated with age.[55] Several mechanisms in CLL have been proposed to result in the qualitative and quantitative reduction of immunoglobulins. The binding of CD95 ligand on the surface of CLL cells to the CD95+ normal plasma cells in the bone marrow triggers apoptosis in the normal plasma cells reducing their numbers and their ability to produce sufficient functional antibodies.[56] Another mechanism is through the presence of dysfunctional non-neoplastic $CD5^-$ B cells which contribute to the production of dysfunctional immunoglobulins. Finally, the reduction of CD40 ligand and abnormal CD40-CD40 ligand interactions impact normal lymphocyte proliferation and differentiation including disruptions to antibody production and isotype switching.[34,57] Furthermore, dysfunctional cellular immunity is prevalent in CLL patients including modification of T helper cell function that is essential for B cell activation and differentiation, decreasing their receptor signaling and altering cytokine production.

The mechanism by which SAD occurs in MM is also multifactorial. The malignant plasma cells regulatory interference with immune functions, such as enhancing anti-inflammatory cytokine production and inhibiting immune effector cells, can disrupt the patient's ability to mount an appropriate immune response against high-risk infections with gram-positive bacteria.[58]

Immunoparesis is common in B cell malignancies and is a hallmark feature of MM affecting approximately 85-91% of newly diagnosed symptomatic MM patients.[59,60] Immune paresis is defined by the suppression of uninvolved polyclonal immunoglobulins and augments the risk of infections in these patients.[61] The overcrowding of the bone marrow with malignant cells results in the disruption of normal hematopoiesis and reduction of proliferation and differentiation of normal functional B lymphocytes. Altered cytokine activity, such as reduction of B cell stimulatory factor 1 (BSF-1) and elevation of B cell inhibitory factor (BIF), was also correlated with the suppression of normal immunoglobulins (Ig) production in MM patients.[62]

1.4 Therapies for hematological malignancies and impact on immunity

A major contributor to the development of HM-SAD is the rapid development of new therapeutics to treat HM. A vast spectrum of therapies is now available including chemotherapy and immune therapies such as immune checkpoint inhibitors, stem cell transplantation, monoclonal antibodies, and cellular therapies. Understanding how these therapies may affect immunity is essential to the evaluation of the risk of SAD and related infectious risk in HM patients.

1.4.1 Chemotherapy

Chemotherapy, along with radiation therapy and steroids, are often used to treat hematological malignancies. A limited selection of common chemotherapy agents used for HM and their impact on host immunity will be described in this section.

Cyclophosphamide (CPA) is an alkylating agent widely used in the treatment of hematological malignancies such as MM. It is often used in conjunction with other agents such as in the CHOP regimen (Cyclophosphamide, doxorubicin, vincristine, and Prednisone) to treat aggressive lymphoma.[63,64] At high doses, CPA can completely deplete hematopoietic stem cells.

CPA use is correlated with leukopenia, anemia and thrombocytopenia, all leading to a higher risk of infections and bleeding.[65]

Bendamustine is another alkylating agent approved to treat CLL, MM and NHL. It is associated with a significant high risk of opportunistic infections which were the leading cause of serious adverse events (SAE) and death post-treatment.[66]

Fludarabine is a purine analogue used to treat HM such as CLL and NHL. It causes cell-mediated immunodeficiency affecting CD4+ T cells and lasting up to 12 months after therapy.[37,67] It is associated with increasing the risk of opportunistic infections like varicella zoster virus (VZV) and cytomegalovirus (CMV).[67,68]

Cytarabine is a pyrimidine nucleoside analogue that inhibits DNA synthesis during the S-phase of the cell cycle and results in apoptosis.[69] It is used in the treatment of ALL, chronic myeloid leukemia (CML) and lymphoma and is known to be a potent immunosuppressant.[70]

1.4.2 Anti-CD20 monoclonal antibodies - Rituximab

Rituximab is a B cell targeting therapy most widely used for treating cancers, rheumatoid arthritis, multiple sclerosis, and anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis.[71–74] In 1997, it became the first approved monoclonal antibody by the US Food and Drug Administration (FDA) for cancer and continues to be one of the most widely used B cell-targeting therapies.[75] It is a human-mouse chimeric antibody of IgG1 kappa isotype that binds with high affinity to CD20 antigen present on the surface of both malignant and normal B cells to produce a rapid and severe B cell depletion.[76] CD20 is a transmembrane non-glycosylated phosphoprotein that arises during the pre-B cell stage of B cell development, and it is involved in the activation, proliferation and differentiation of B cells.[76] The mechanism of action is primarily through cell-induced apoptosis. Rituximab, therefore, does not affect the developmental stages

prior to the pre-B cell stage. CD20 is also not present on plasma or dendritic cells, therefore; theoretically, after depletion with rituximab, it would be expected that the reconstitution of normal B cell population should continue from the spared precursor cells while plasma cells continue to produce antibodies.[76] Despite its targeted action, it has been associated with SAD in 11% to 67% of adult and pediatric patients. Early combination studies of CHOP chemotherapy and rituximab (R-CHOP) showed an additive therapeutic benefit without significant added toxicity and evidence of clearing of minimal residual disease not observed with CHOP chemotherapy alone.[77] This has resulted in R-CHOP becoming one of the most common treatments for NHL.

1.4.3 Proteasome inhibitors (PI)

Proteasome inhibitors (PI), such as Bortezomib and Carfilzomib, are used to treat MM and mantle cell lymphoma (MCL) by inhibiting the activity of proteasomes.[69,78] Proteasomes are protein complexes that are responsible for the hydrolysis of proteins and preventing the accumulation of mutant proteins in the cell. When proteasome activity is inhibited, ubiquitinated proteins accumulate in MM cells, triggering the activation of an unfolded protein stress response leading to apoptosis. This process has been reported to also promote cell death in normal long-lived plasma cells by stabilizing pro-apoptotic proteins.[79] This process can decrease antibody production and secretion leading to SAD, however; studies exploring the association between PIs, SAD and infection risks in HM are very limited.[37]

1.4.4 Immunomodulatory drugs (IMiDs)

Immunomodulatory drugs can stimulate anti-tumor natural killer cells and T cells.[80] Thalidomide is a commonly used immunomodulatory medication that has antiangiogenic effects in human malignancies, induces apoptosis and modulates the microenvironment in the bone

marrow. It is also combined with dexamethasone as an induction treatment before autologous stem cell transplants. As maintenance or consolidation therapy, it suppresses residual disease and its progression, improving the overall survival in patients with MM.[81–83] Although it has an immunosuppressive effect on T cells, it does not have direct impact on infection risk.

Lenalidomide, a derivative of thalidomide, is also used in conjunction with dexamethasone and does not appear to augment the risk of infection despite its powerful influence on helper and cytotoxic T cells.[84] However, the long-term use of immunotherapy exacerbates the immunosuppressive effects of HM, thereby increasing the risk of infection-related mortality.[85]

1.4.5 Tyrosine kinase inhibitors (TKIs)

Tyrosine Kinases (TK) are enzymes that regulate cellular functions such as survival, growth, proliferation, and differentiation. They work by phosphorylating tyrosine residues on a protein. These enzymes can exhibit abnormal activity in certain cancers promoting uncontrolled cell growth and division. This makes them a very attractive target for anti-cancer agents.[86]

Three generations of tyrosine kinase inhibitors (TKI) have been approved and are used to treat various hematological malignancies including mantle cell lymphoma (MCL), CLL, and CML. Although TKIs usually have a specific anti-cancer target, they can affect other off-target kinases resulting in immunosuppressive effects and impairing B cell immunity.[87] One mechanism linked to immunosuppression is the phosphorylation of co-receptor CD19 which alters B cell proliferation. Imatinib was associated with significant reduction in peripheral blood (PB) memory B cells and low plasma IgG levels.[88] Over 50% of patients taking Bruton's tyrosine kinase (BTK) Inhibitors (such as Acalabrutinib, Zanubrutinib, and Ibrutinib), experience opportunistic infections such as pneumonia, urinary tract infections and invasive

Aspergillosis.[89,90] BTK plays an essential role in in B cell receptor signaling involved in B cell proliferation, maturation and survival, therefore; BTK inhibitors lead to B cell depletion and consequently, hypogammaglobulinemia.[91]

1.4.6 Hematopoietic stem cell transplantation (HSCT)

Hematopoietic stem cell transplantation is a major therapeutic modality used to treat HM. It delivers curative potential by allowing the restoration of cellular components of the blood compartment in the bone marrow including all immune cells.[92] HSCT can be allogeneic (allo-HSCT) or autologous (auto-HSCT) depending on the origin of the stem cells. HSCT can be used to treat ALL, MM, and aggressive lymphoma. The reconstitution of the B cell compartment may take between 9 months and up to 5 years. During this period, HSCT patients may have a defective humoral immune response and are at a higher risk of infectious complications including encapsulated bacteria such as *H. influenzae and S. pneumoniae.*[93,94] This is particularly due to the delay in the reconstitution of the memory B cells and helper T cells which are needed for mounting an effective antibody response and immunoglobulin isotype switching.[95] In addition, patients who develop graft versus host disease (GVHD), a complication following allogenic transplant, may never regain normal immune function.[38]

The increased prevalence of SAD here is primarily linked to patients with chronic GVHD presenting with hypogammaglobulinemia and significant decline in both non-class switched and class-switched memory B cells, but elevated naïve B cell counts.[96]

1.4.7 Chimeric antigen receptor T (CAR-T) cell therapy

CAR-T cell therapy is a breakthrough personalized form of immunotherapy used to treat various types of HM including NHL[97], MM[98], and B cell ALL[99]. This technique genetically engineers T cells collected from the patient's peripheral blood *in vitro* to express chimeric

antigen receptor (CAR) thereby allowing these cells to recognize specific antigens on the surface of cancer cells.[100] These modified T cells then undergo extensive expansion *in vitro* while the patients undergo lymphodepleting chemotherapy. Once the CAR-T cells are infused into the patients, they undergo rapid proliferation and exert their anti-tumor effects.

The most common targets for CAR-T cell therapy are CD19 B lymphocytes and B cell maturation antigen (BCMA). CAR-T cell therapy and the lymphodepleting chemotherapy the patient receives prior to the infusion can result in B cell aplasia and hypogammaglobulinemia that can last for 2 years, putting the patients at high risk of infections.[101–104]

1.4.8 Corticosteroids

Corticosteroids have been used in the treatment of MM, CLL, and NHL for many decades and have been integrated into novel therapeutic regimens.[105,106] Dexamethasone is one of the most frequently used effective agents that induces apoptosis in malignant MM cells although the exact mechanism remains not well defined.[107,108] Prolonged treatment with systemic corticosteroids can impact cell-mediated immunity and reduce the level of immunoglobulins in the blood, opening the patients up to opportunistic bacterial, viral and fungal infections.[42,109]

1.4.9 Bispecific T cell engagers (BiTEs)

BiTEs, such as mosunetuzumab and blinatumomab, are off-the-shelf immunotherapy for different HM such as NHL, CLL, ALL and MM.[110] They are a type of bispecific antibodies (BsAbs) engineered antibody-based molecules consisting of two different single-chain variable fragments (scFv) joined by a peptide linker.[110,111] BiTEs can bind in tandem to a specific antigen on the target tumor cell such as CD20 antigen in B cell NHL, and to effector T cells through their CD3 surface antigen.[112] This in turn activates the T cells and results in tumor cell lysis.[112] The

targeting of B cells with BiTEs leads to B cell depletion and varying degrees of hypogammaglobulinemia depending on the specific therapy used and its dosage.[37,113–115]

1.5 Infection risk and management

For many subtypes of NHL, risk of infections is often associated with hypogammaglobulinemia due to therapies than the disease itself.[116] A study by Matasar et al. reported 46.8% of patients receiving an initial treatment with mosunetuzumab, a B cell depleting bispecific T cell engager, used to treat follicular lymphoma, experienced respiratory tract infections, urinary tract infections and pneumonias.[117] Other reported infections include CMV, EBV and *Candida* sp.[118,119]

As previously noted, infections are the leading cause of morbidity and mortality in MM and CLL patients. For CLL patients, both disease-inherent immune dysfunction and the immunosuppression related to its treatment contribute to the rise in risk of infection-related morbidity and mortality that is exacerbated with advancement of the disease.[68] The disease process of CLL involves dysfunction and abnormalities in T cells[120,121], monocytes[122,123], neutrophils[124] and their complement system.[125] Hypogammaglobulinemia is a major contributor to the high risk of infections with encapsulated bacteria such as *H. influenzae* and *S. pneumoniae*. IgG serum levels below 6g/L were associated with a 5-fold increase in infection risk in CLL patients.[126] However, it has also been shown that almost all CLL patients had poor functional antibodies regardless of total IgG titers.[52] Over 75% of CLL patients who had normal IgG titers also had low specific antibodies to pneumococcus, therefore; IgG titers alone cannot identify CLL patients who are at a higher risk of infections.

Furthermore, the down-regulation of the classical complement pathways in CLL leading to the reduction in complement amounts and alterations to the function of complement receptors

CR1 and CR2 were also linked to the recurrence of severe bacterial infections in CLL patients.[127] Other factors such as T cell dysfunction, TP53 gene mutations and unmutated variable region on the immunoglobulin heavy chain may help evaluate risk of infections in newly diagnosed CLL.[128] Finally, the advancement of treatments for CLL has also expanded the spectrum of pathogens posing a high risk of infection which include *S. aureus*, mucosal candidiasis, herpes simplex virus (HSV), CMV, VZV, tuberculosis, and invasive fungal disease such as aspergillosis.[126]

Similarly, several factors are associated with higher risk of infections and related mortality in patients with acute leukemia. These factors include inherent immune defects in lymphocytes, defective cellular immunity and hypogammaglobulinemia compounded by chemotherapy leading to prolonged neutropenia, as well as severe mucositis and the use of central catheters.[54,129] Infections have been documented to occur mostly during treatment or in post-remission treatments. The type of infection varies depending on the kind of treatment received. Many studies have reported infections mostly caused by Gram-negative bacteria, but others have also seen a rise in infections caused by Gram-positive bacteria.[130] ALL patients may experience pneumonia, fungal and viral infections.[129]

Infection risk for MM patients are almost 7-fold greater than healthy controls during the first year after diagnosis.[131] Despite remarkable improvement in survival rate of patients with MM due to therapeutic advancement [132–135], around 20% of patients experience fatal outcomes to infections contributing to half of early mortality with a 3-fold risk of death compared to healthy controls.[136,137] A recent study has shown that MM patients with SID (classified as having IgG <5 g/L) had recurring respiratory infections and significantly shorter median survival time of 24 vs 66 months in MM patients with IgG ≥5 g/L. Their analysis determined that IgG < 5g/L was a

prognostic factor for MM patients.[138] They are more susceptible to developing septicemia, meningitis, pneumonia as well as viral infections such as CMV, EBV, HSV, respiratory syncytial virus (RSV) and SARS-CoV-2.[131,139] MM patients undergoing transplantation may also be at a higher risk of infections prior to engraftment due to factors such as smoking and iron overload. After transplant, infectious fatalities occur due to infections with CMV, VZV, *C. difficile* or pneumonia.[140]

There are several strategies that can be used to manage SAD and reduce infection risk in patients with HM.[35] The first approach would ideally be targeting and eliminating the underlying cause of SAD. For patients undergoing necessary cancer therapy, discontinuing treatment to mitigate the risk of developing SAD may not be feasible. Furthermore, this antibody deficiency may persist long after the treatment is completed or discontinued. Therefore, alternative measures need to be considered.

Similar to PAD, prophylactic immunization is often considered as a preventative measure for HM patients with SAD.[35] Immunization against *H. influenzae* and *S. pneumoniae* are recommended in CLL and MM patients in early stages of the disease and prior to starting any treatment.[141,142] However, evaluation of post-vaccination specific antibodies is necessary to assess level of protection and risk of infection, as well as determine the re-vaccination needs for each patient.[143] Although some patients are unable to mount an optimal antibody response due to the nature of their disease and treatment history, some benefit can still be derived from the antibodies and T cell mediated immunity.[144]

Another strategy commonly used to manage SAD is prophylactic antibiotics. There is limited evidence regarding the effectiveness of using this approach in HM patients to prevent infections or reduce infection-related mortality.[145] None the less, this is recommended for CLL

patients experiencing higher rate of recurrent severe infections.[146] It is also recommended for CLL, NHL and MM patients undergoing immunosuppressive therapies.[147,148] However, there are additional risks associated with the use of prophylactic antibiotics that must be considered including toxicity and antibiotic resistance.

Finally, Immunoglobulin replacement therapy (IGRT) has been proven to lower the risk of infections in patients with SAD due to hematological malignancies.[149–152]

1.6 Immunoglobulin replacement therapy (IGRT)

Immunoglobulin replacement therapy (IGRT) is the current mainstay treatment for SAD. Duraisingham et al, reported that the most common causes of SAD in their patients on immunoglobulin replacement treatment were the use of chemotherapy for B cell lymphoma, immunosuppressive medications, corticosteroids and rituximab.[36]

IGRT, which can be administered intravenously (IVIG) or subcutaneously (SCIG), delivers exogenous IgG antibodies to prevent recurrent infections, improve quality of life and reduce hospitalization in patients with SAD.[36,149,153,154] These products are prepared from the collective plasma of thousands of healthy blood donors ensuring that recipients are supplemented with a diverse antibody repertoires against a broad range of antigens.[155] A 2023 cross-sectional study of 140 patients with SAD revealed that the use of IGRT resulted in reductions of 82.6% in the average number of annual infections, 84.6% in emergency room visits and 83.3% in hospitalization when compared to pre-IGRT period.[156]

1.6.1 Initiation of IGRT in patients with SAD

Guidelines regarding when to initiate, continue, or discontinue therapy differ internationally, reflecting a lack of consensus on best practices.[55,150,157,158] In 2021, a European expert consensus developed recommendations for the use of IGRT in HM patients with SAD.[159]

Initiation of IGRT treatment is recommended for patients with history of recurrent or severe infections or evidence of severe hypogammaglobulinemia (IgG < 4 g/L). Furthermore, considerations of comorbidities, poor response to prophylactic antibiotics and vaccination should be considered when assessing the eligibility of a patient to start IGRT.[35] For instance, the maximum benefit was seen in patients who had poor anti-pneumococcal IgG response following immunization with Pneumovax prior to treatment in MM patients receiving IVIG prophylaxis.[160]

1.6.2 Discontinuation of IGRT and immune recovery

Unlike PAD, SAD is not always permanent and may be reversed once the underlying cause is resolved or eliminated.[94,161,162] Therefore, the use of IGRT should also be limited to the period of prevailing immunodeficiency. There is currently no formal standardized IGRT discontinuation algorithm for SAD. Some physicians have chosen the abrupt discontinuation approach at 12 months after IGRT initiation, while others chose the approach of tapering off IGRT starting at 6 months after IGRT initiation. Unfortunately, the evidence is still lacking, and more studies are needed to assess and compare the benefits and risks of either approach.[163] The current consensus showed that the average duration of IGRT for HM is 10-12 months, with the current proposed duration of 6-12 months.[159] Some physicians were stopping IGRT at 6 months and other at 12 months when their patients were free from infections. Other reported reasons for IGRT discontinuation included end of chemotherapy and recovery of normal range immunoglobulin levels.[164] It was also recommended to avoid periods on high incidence of infection when attempting IGRT discontinuation to decrease the risk of acquiring an infection during the initiation of the process.[48]

Assessments may include measuring serum immunoglobulin (IgA, IgM, and IgG) levels, vaccine response, reviewing and tracking infection history and frequency, monitoring the use of

antibiotics and assessing the patient's quality of life.[161] Ultimately, the decision will be based on the physician's clinical judgment and interpretation of the supportive laboratory parameters.[50] Therefore, specialists treating cancer patients with SAD who are not familiar with IGRT will benefit from having standardized treatment recommendations, including discontinuation.[159]

A recent retrospective study of 133 SAD patients receiving IGRT at the Ottawa Hospital between 2015 and 2019, observed that only 9% of participants discontinued IGRT. Seventy-five percent of those who discontinued IGRT met the definition of successful discontinuation (without the occurrence of severe infections requiring hospitalization and no re-initiation of IGRT within 12 months of discontinuation).[164] This study highlighted the possible inaccuracy in relying on physician's clinical judgement in predicting successful IGRT discontinuation and cautioned against initiating IGRT discontinuation in patients still receiving treatment for their HM (which was the case in all the unsuccessful discontinuations). Overall, this suggests that it is possible to safely discontinue IGRT in SAD patients, and warrants future studies to identify biomarkers of immune recovery that can better help guide safe IGRT discontinuation.

In fact, the lack of clear evidence-rooted discontinuation algorithm has a multi-dimensional impact on patients and the healthcare system. First, unnecessary or prolonged IGRT exposes patients to potential adverse events such as renal impairment and thrombotic events among others.[165–167] Second, Canada has seen a 6-8% annual increase in IGRT use, resulting in an annual cost of $143 million annually as of 2018.[168] Canada is among the top three per-capita IGRT users worldwide and remains fully dependent on US manufacturers.[169] This raises concerns regarding sustainability of the supply required to meet the rise in demand and amplifies the risk of product shortages for patients with irreversible immune deficiencies.

1.6.3 Evidence of immune reconstitution

The evaluation of humoral immune reconstitution for the purpose of IGRT discontinuation can be complex, and often involves a comprehensive evaluation of clinical, laboratory and functional indicators.

Clinical evidence should show a decrease in the number of recurrent, persistent or severe infections, particularly respiratory infections which are the most common in patients with HM-SAD.[152] This can also be reflected in the reduced utilization of prophylactic antibiotics. Laboratory evidence includes measurements of immunoglobulins IgA, IgG and IgM in the serum to check for normalization[170], and the reconstitution of peripheral B lymphocytes and the subsets impacted within that compartment following B cell depleting therapies. Many studies have indicated that B cell compartment reconstitution can be evident starting 6 months post-treatment and may take up to 2-5 years to normalize.[95] In particular, transitional and naïve B cells appear to have the fastest recovery while the recovery of memory B cells is delayed, sometimes beyond 2 years to reach age-matched proportions.[94,161,162,171,172] Finally, functional evidence can be tested by evaluating antibody response to vaccination.[173–176]

To facilitate evidence-based informed decision for safe IGRT discontinuation, the identification of a biomarker that can reliably predict humoral immune recovery and infection risk in HM patients with SAD is key. The current recommendations use serum IgG titers for the initiation and monitoring of IGRT use. Since exogenous IgG supplemented by IGRT and endogenous IgG produced by the patient cannot be clearly differentiated, a clear assessment of the patient's ability to produce functional IgG cannot be easily done.[163] Therefore, serum IgG titers cannot be used in the assessment for safe IGRT discontinuation, and alternative markers should be used. Since humoral immunity is rooted in the development and functions of B

lymphocyte, which play a key role in antibody production and creation of immune memory, it is possible that stronger evidence of humoral immune recovery can be found within the B cell compartment of patients with SAD.

1.6.4 B cell compartment and humoral immunity

B cell development begins in the bone marrow from hematopoietic stem cells through a process called hematopoiesis. These cells pass through sequential developmental stages initiating the construction and expression of their B cell receptors (BCR).[177] The BCR is a membrane bound immunoglobulin composed of two identical heavy chains (HC) and two identical light chains (LC) that can recognize an antigen. Early pro-B cells rearrange their immunoglobulin HC through the process of V(D)J recombination.[178] After successful HC recombination, the cells enter the pre-B cell stages at which they undergo rapid proliferation followed by LC VJ gene rearrangements and paring with the heavy chain.[179] These processes ensure that each cell only produces one type of heavy chain paired with one type of light chain to create a unique BCR specific to one antigen.[178,180] Once successfully completed, these immature B cells can express surface IgM type BCRs and undergo a series of tolerance tests mediated by their BCR signaling to eliminate self-reactivity. [177,181] The cells that successfully pass these tests become transitional B cells that can express IgM and/or IgD and migrate from the bone marrow to peripheral lymphoid organs where they further mature into naïve B cells.

Naïve B cells circulate through the peripheral lymphoid tissues until they encounter a specific antigen that their BCR can recognize. This triggers the activation of these cells through T-cell dependent or T-cell independent pathways. T-cell independent activation occurs when certain antigens (such as polysaccharides) cross-link the BCRs upon binding, resulting in a rapid but less-durable antibody response largely dominated by the IgM isotype.[182] In contrast, the T-

cell dependent activation of B cells occurs when B cell present antigen peptides to CD4+ T cells, particularly T follicular helper cells (Tfh), through their MCH II receptors.[183] This provides co-stimulatory signals through cytokine production and the binding of the CD40 ligand on the T cell to CD40 on the surface of the B cells. This process causes the formation of a specialized structure within the secondary lymphoid follicle called the germinal center (GC) where the activated B cells migrate to and undergo further maturation and differentiation.

In the GC, the activated B cells circulate between the light zone and the dark zone where they undergo several processes to refine their antibody specificity and function (Figure 1).[184,185] In the dark zone, the B cells become centroblasts as they undergo rounds of clonal expansion and somatic hypermutation. Somatic hypermutation, mediated by activation induced cytidine deaminase (AID), introduces mutations in the variable region of the immunoglobulin heavy and light chains of the rapidly replicating cells to increase the antibody diversity and affinity for better antigen recognition and antibody response.[186] These B cells then migrate to the light zone, becoming centrocytes after undergoing rounds of affinity maturation and class switching recombination through interactions with follicular dendritic cells and Tfh cells.[187] B cells with lower affinity BCRs either undergo apoptosis or return to the dark zone for further refinement. B cells with high affinity BCRs compete for interactions with Tfh cells to receive the needed signals to undergo class switch recombination (CSR). CSR allows the B cells to change their antibody isotypes from IgM or IgD to IgG, IgA or IgE, while preserving their antigen specificity.[185] Some centrocytes eventually differentiate into long-lived class-switched memory B (SMB) cells or antibody-secreting plasma cells. [188] Class-switched memory B cells provide rapid, robust, high affinity responses upon re-exposure to antigens and are crucial for long-term

immunity while plasma cells migrate to the bone marrow or tissues where they continue to produce high-affinity antibodies.[189]

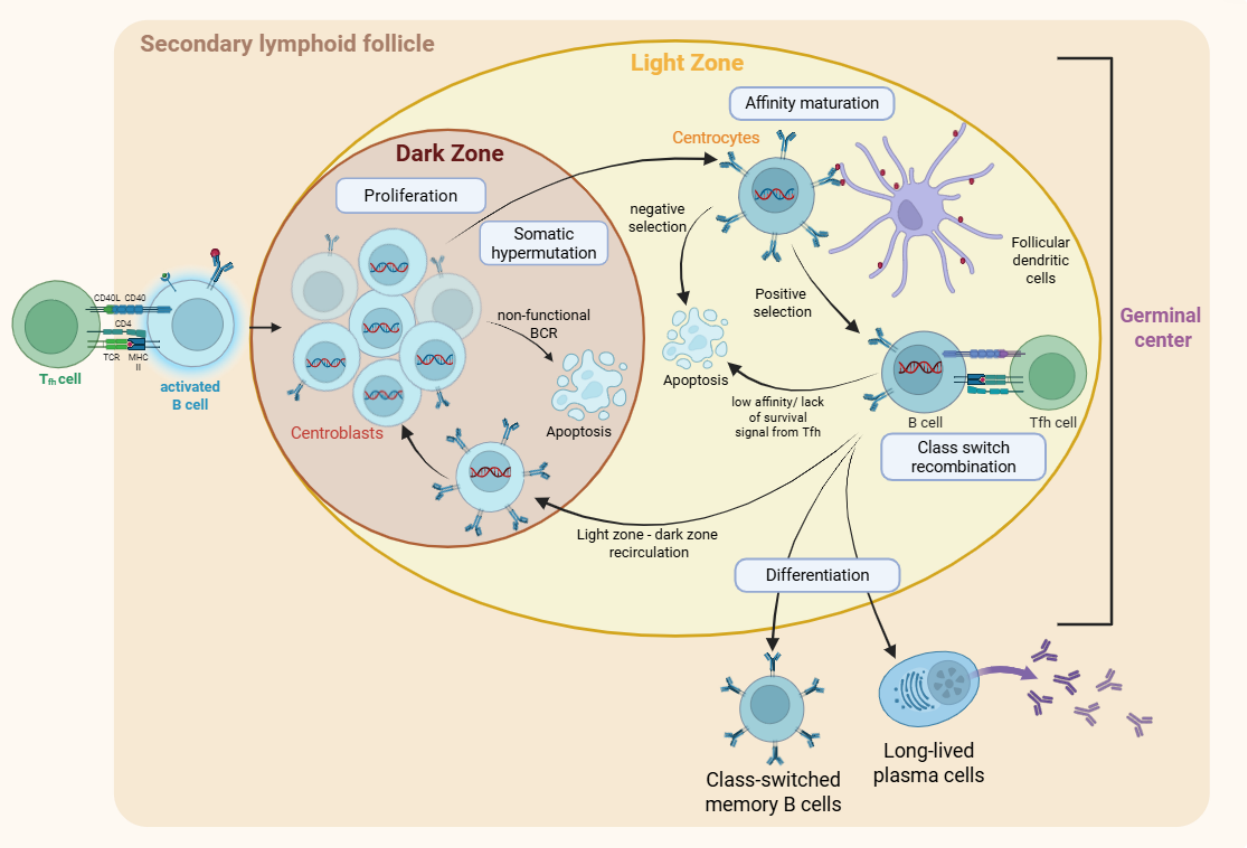


Figure 1. Schematic representation of the germinal center (GC) reaction within in the lymph node. The interaction of activated naïve B cells with follicular T helper cells leads to the formation of the GC. In the GC, the activated B cells circulate between the light zone and the dark zone. In the dark zone, centroblasts undergo rounds of proliferation and somatic hypermutation (SHM). SHM introduces mutations in the variable region of the immunoglobulin heavy and light chains to increase antibody diversity and affinity. Centroblasts then migrate to the light zone, where they become centrocytes and undergo rounds of affinity maturation and class switching recombination through interactions with follicular dendritic cells and T follicular helper cells (Tfh). Negative selection due to low affinity BCRs and lack of survival signals lead centrocytes to apoptosis or they return to the dark zone for further BCR refinement. B cells with high affinity BCRs compete for interactions with Tfh cells to receive the needed signals for class switch recombination (CSR). CSR allows the B cells to change their antibody isotypes while preserving their antigen specificity. Some centrocytes eventually exit the GC reaction by differentiating into class-switched memory B cells or long-lived antibody secreting plasma cells. The small red spheres represent antigens. BCR – B cell receptor, Tfh – T follicular helper cells.

Therefore, to assess the humoral immune recovery of patients with SAD, it is important to evaluate their long-term immunity and their capacity to produce specialized immunoglobulins in the absence of IGRT. Because SMB cells play a central role in secondary immune responses and are the precursors of the plasma cells responsible for the production of class-switched immunoglobulins, they represent a compelling B cell subset to investigate in relation to humoral immune recovery in patients with SAD.

1.7 Rationale and hypothesis:

As described in the earlier section, SMB cells are stimulated long-lived B cells that develop in the germinal centers after an initial immune response. They have undergone class switching recombination with somatic hypermutation in their immunoglobulin genes and affinity maturation of their B cell receptors (BCR), allowing them to rapidly differentiate to produce a diverse range of antibodies (IgG, IgA, and IgE) for an effective and robust secondary immune response at future antigen re-encounters. In the context of hematological malignancies and antibody deficiency, low levels of SMB cells have been associated with prolonged hypogammaglobulinemia and increased infection risk.[190–194] Furthermore, studies in primary immunodeficiency have found that patients with CVID have markedly reduced switched memory B cells proportions, lower serum IgG levels, weak response to pneumococcal vaccines and are at a higher risk of recurrent respiratory infections.[195–197] They suggested that the evaluation of SMB cells in these patients is a better predictor of clinical prognosis than the measure of serum immunoglobulins.

In addition, previously reported data showed that 43% of SAD due to non-Hodgkin's lymphoma have prolonged immunodeficiency with low SMB cells counts following treatment with rituximab, while the remaining 57% had recovery of immunodeficiency.[190] Based on these

findings, 40%-50% of SAD patients should show evidence of immune recovery reflected in their recovering SMB cells counts. Although SMB levels are not commonly measured in clinical settings for initiation or discontinuation of IGRT, their potential as a promising candidate for a biomarker of immune recovery in HM patients should be explored. Notably, SMB cells are already used to stratify patients with CVID into distinct meaningful clinical subgroups.[198–200]

We hypothesize that an increase in SMB cell proportions over time in patients with SAD due to HM will be associated with improved immune recovery marked by lower rates of moderate and severe infections. Ultimately, we aim to study the use of SMB cell proportions as a biomarker for safe IGRT discontinuation. Although not evaluated as part of this thesis, future studies will need to evaluate the performance of SMB cells as a biomarker in terms of sensitivity, specificity, reproducibility, affordability, stability, accessibility, among others.[201,202]

1.7.1 Primary objective

To begin, we will characterize the proportions of SMB cells in patients with HM-SAD.

1.7.2 Secondary objectives

We will evaluate study feasibility including recruitment rate, retention rate, and study acceptability for patients. We will explore the association of SMB cell proportions with demographic, clinical and laboratory data including as age, sex, cancer type, time since remission and cancer treatments, infection history, serum Immunoglobulin (IgG, IgA, IgM) levels and other B cell subsets.

CHAPTER 2: MATERIALS AND METHODS

2.1 Study design and approval

Switched memory B cells (SMB) as a marker for humoral immune system recovery in patients with secondary antibody deficiency due to hematological malignancies (SMB Study) is an ongoing single-arm prospective observational study at the Ottawa Hospital (TOH). Ethics approval was obtained from the Ottawa Health Science Network Research Ethics Board. Study procedures were conducted at the Clinical Investigation Unit (CIU) at the Ottawa Hospital Research Institute (OHRI). Study procedures were in compliance with the protocol and local standard operating procedures, ensuring adherence to ethical principles outlined in the Declaration of Helsinki. All participants have provided informed consent prior to the initiation of any study procedures and enrollment.

2.2 Study participants

Participant recruitment was initiated in June of 2024. Patients from the Immunoglobulin Treatment Clinic at The Ottawa Hospital with permission to contact were invited to participate in the study. Participants were screened and enrolled in accordance with the study's eligibility criteria. The inclusion criteria included adults 18 years of age and older with a history of leukemia, lymphoma or plasma cell disease. Participants must be able to speak English or French, receiving IGRT or with evidence of hypogammaglobulinemia (IgG < 6g/L) and available for ongoing follow-ups. Pregnancy was the only exclusion criterion.

2.3 Study procedures

Participants were invited to in-person baseline visit as well as follow-up visits every 3-6 months. During the baseline visits, the participants completed an acceptability questionnaire to gauge their perception of the study and to gather feedback on perceived risks, effectiveness and

convenience. This was done to identify challenges and obstacles that can be mitigated by refining study procedures. Information about the participant's general health including comorbidities, vaccination history, cancer treatment history, and 12-month history of moderate and severe infections were also collected. Moderate infections were defined as infections that require outpatient antimicrobial treatment. Whereas severe infections were defined as infections that require intravenous antimicrobial treatment and/or hospitalization. At every visit, including the baseline, the participants completed questionnaires regarding changes to their health and medications, reviewed history of infections, and questionnaires regarding their quality of life.

2.3.1 Blood processing

Whole blood samples were collected by peripheral venipuncture at every study visit and processed within 24 hours. Peripheral blood mononuclear cells (PBMCs) were isolated using density-gradient medium Lymphoprep (STEMCELL Technologies) and SepMate standard PBMC isolation protocol. PBMCs were then aliquoted and stored in -80℃ for 24-48 hours before being moved to liquid nitrogen.

2.3.2 Flow staining procedure

PBMCs were thawed and washed in RPMI medium (RPMI, 1% L-gutamine,10% fetal bovine serum [FBS]) at 37℃ before staining. PBMCs were then prepared for flow cytometry by washing in 0.2% bovine serum albumin (BSA) in phosphate-buffer saline (PBS), incubated with Horizon fixable viability Stain 510 (BD Bioscience) for 15 minutes in the dark at room temperature. Samples were stained with a surface antibody cocktail in Horizon Brilliant stain buffer (BD) and incubated for 30 minutes in the dark on ice. The following fluorochrome-conjugated surface antibodies were used; from BD Biosciences: Mouse anti-CD3 (R718, Cat# 566954), anti-CD19 allophycocyanin (APC, Cat# 561742), anti-CD21 (BV711, Cat# 563163),

anti-CD27 fluorescein isothiocyanate (FITC, Cat# 557329), anti-CD38 (BB700, clone HIT2, Cat# 566445), anti-IgD phycoerythrin (PE, Cat# 562024), anti-IgG (PE-Cy7, Cat# 561298),anti-IgM (BV605, Cat# 562977); from Thermo-Fisher: Ultracomp compensation beads (01-2222-42); from BioLegend: True-stain Monocyte Blocker (Cat# 426102); from Miltenyi Biotec: anti-IgA VioBlue (clone IS11-8E10, Cat# 130-114-005).

2.3.3 Flow cytometry

Acquisition was done on a 5-laser (355nm, 405nm, 488nm, 561nm, 640nm) spectral flow cytometer (Cytek Aurora). Instrument settings and detector configurations were optimized using SpectroFlo® software (v3.3.0, Cytek Biosciences) which allowed for real-time unmixing to quickly visualize data and statistics. Daily quality control and performance checks were performed using Cytek QC beads according to the manufacturer's guidelines. Single-stained compensation controls were prepared using UltraComp eBeads and unstained controls were prepared with PBMCs. Spillover and autofluorescence correction were reviewed and manually adjusted as needed, not exceeding 5% compensation.

2.3.4 Immunophenotyping of B cell subsets

Unmixed FCS files were exported and analyzed using FlowJo software (v10.10.0, BD Biosciences). Sequential gating strategies were applied to exclude doublets and dead cells, followed by gating on $CD19^+$ B cells. B cell subsets were identified as follows: Transitional B cells: $CD19^+$ $CD27^-$ $CD38^{++}$ IgM^+, Naïve B cells: $CD19^+$ $CD27^-$ IgD^+, Unswitched memory B cells: $CD19^+$ $CD27^+$ IgD^+, Switched memory B cells: $CD19^+$ $CD27^+$ IgD^-IgM^-, Double-negative B cells: $CD19^+$ $CD27^-$ IgD^-, and Plasmablasts: $CD19^+$ $CD27^{++}$ $CD38^{+++}$ IgM^-.

Gating thresholds were determined based on fluorescence-minus-one (FMO) controls (done for CD27, CD38, IgG and IgM antibodies). Frequency of total $CD19^+$ B lymphocytes was

calculated as a percentage from total lymphocytes, and the proportion of each B cell subset was calculated as a percentage of total B lymphocytes. Normal SMB cell proportions were considered ≥2% of total B cells as defined by EUROclass trial.[200] Final data tables (FCS statistics) were exported from FlowJo™ for downstream statistical analysis in GraphPad Prism.

2.5 Assay validation

Flow cytometry protocol optimization and validation were performed prior to patient recruitment. To determine if frozen PBMCs can be used to enumerate switched memory B cells, fresh, one-day old, and frozen PBMCs were collected from a single healthy donor (n=1) and stained using the outlined staining protocol. In total, three independent trials were performed, and the coefficient of variation (calculated as the standard deviation divided by the mean) was determined within fresh, day-old and frozen. One-way ANOVA analysis was used to determine if there were any significant differences among means.

Inter-rater reliability was assessed by comparing proportion of SMB cells of samples collected from different subjects (n=5) measured independently by 2 raters. Mann-Whitney t-test was used to determine if any significant difference existed between the raters. Intra-class coefficient (ICC) was also calculated from a 2-way ANOVA test to verify inter-rater reliability. Finally, intra-rater reliability was assessed using one-way repeated measure ANOVA for 4 replicates of 3 samples. ICC was also calculated to determine the level of agreement between the measurements from the different trials. The coefficient of variation was also calculated for all 3 samples to check intra-assay precision.

2.6 Internal test controls

A total of 8 healthy donors were included in the study, 63% are female and mean age (SD) was 34.88 (14.98) years. SMB cell proportions for the healthy donor PBMCs used for test

validation and optimization ranged from 3.64% to 28.29% from total B lymphocytes. Furthermore, we also analyzed a healthy donor sample as a control with every patient sample tested, to validate the staining protocol, and accurately distinguish operator, procedure, or reagent-induced variations from true observed disease-related differences.

2.7 Data analysis

All statistical analyses were performed using GraphPad Prism software (version 10; GraphPad Software, San Diego, CA, USA). For comparisons between two groups, an unpaired two-tailed Student's *t*-test (parametric) or Mann–Whitney *t*-test (non-parametric) was applied as appropriate. For comparisons among more than two groups, one-way analysis of variance (ANOVA) with Kruskal–Wallis test with Dunn's correction (non-parametric) was used. When analyzing two independent variables simultaneously, two-way ANOVA was used. Data are presented as median and IQR unless otherwise specified. Spearman correlation was used for correlation analysis. A p value < 0.05 was considered statistically significant. Graphs were generated using GraphPad Prism to visualize group distributions, summary statistics, and significant differences.

CHAPTER 3: RESULTS

3.1 Laboratory protocol optimization and validation

3.1.1 PBMC preparation for staining

We first wanted to determine if frozen PBMCs can be used to enumerate switched memory B cells by staining and comparing fresh, one-day old, and frozen PBMCs in 3 replicates from a one representative healthy donor (n=1). The gating strategy is shown for a representative sample in **Figure 2**. In total, three independent trials were performed. The mean (SD) frequency

of SMB cells was 6.37% (0.67) for fresh, 6.17% (0.50) for one-day old, and 5.47% (0.21) for frozen PBMCs **(Figure 3A)**. The coefficient of variation, calculated as the standard deviation divided by the mean, within fresh, day-old and frozen samples was 10.46%, 8.16% and 3.80%, respectively. Frozen samples had the lowest coefficient of variation although all three conditions were within the generally acceptable 10-15% range. One-way ANOVA analysis revealed no significant difference among the means (p= 0.1446). Overall, this indicated good comparability across fresh, day-old and frozen PBMCs. As a result, we continued working with frozen samples to assess inter-rater reliability and test-retest (intra-rater) reliability or precision.

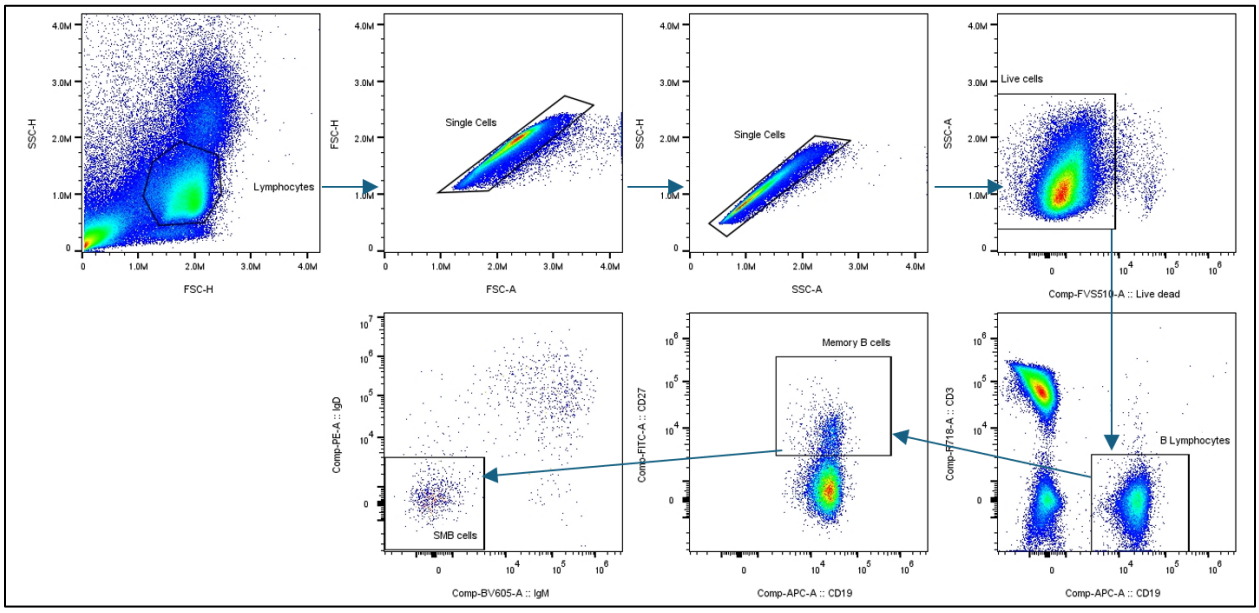


Figure 2: Flow cytometry gating strategy of a representative healthy control sample. Fresh peripheral blood mononuclear cells from healthy donors were stained and the representative scatter plots illustrating the gating strategy used to identify switch memory B cells are presented. Forward scatter (size) and side scatter (granularity) were used to select the lymphocytes, and forward scatter and side scatter height and area were used to exclude the doublets. Side scatter area and viability stain were used to select the viable cell population. Gating for $CD3^-$ and $CD19^+$ of the live cell population was used to identify B lymphocytes, followed by gating for the $CD27^+$ sub-population of memory B cells leading to the identification of the IgD^- and IgM^- SMB cells.

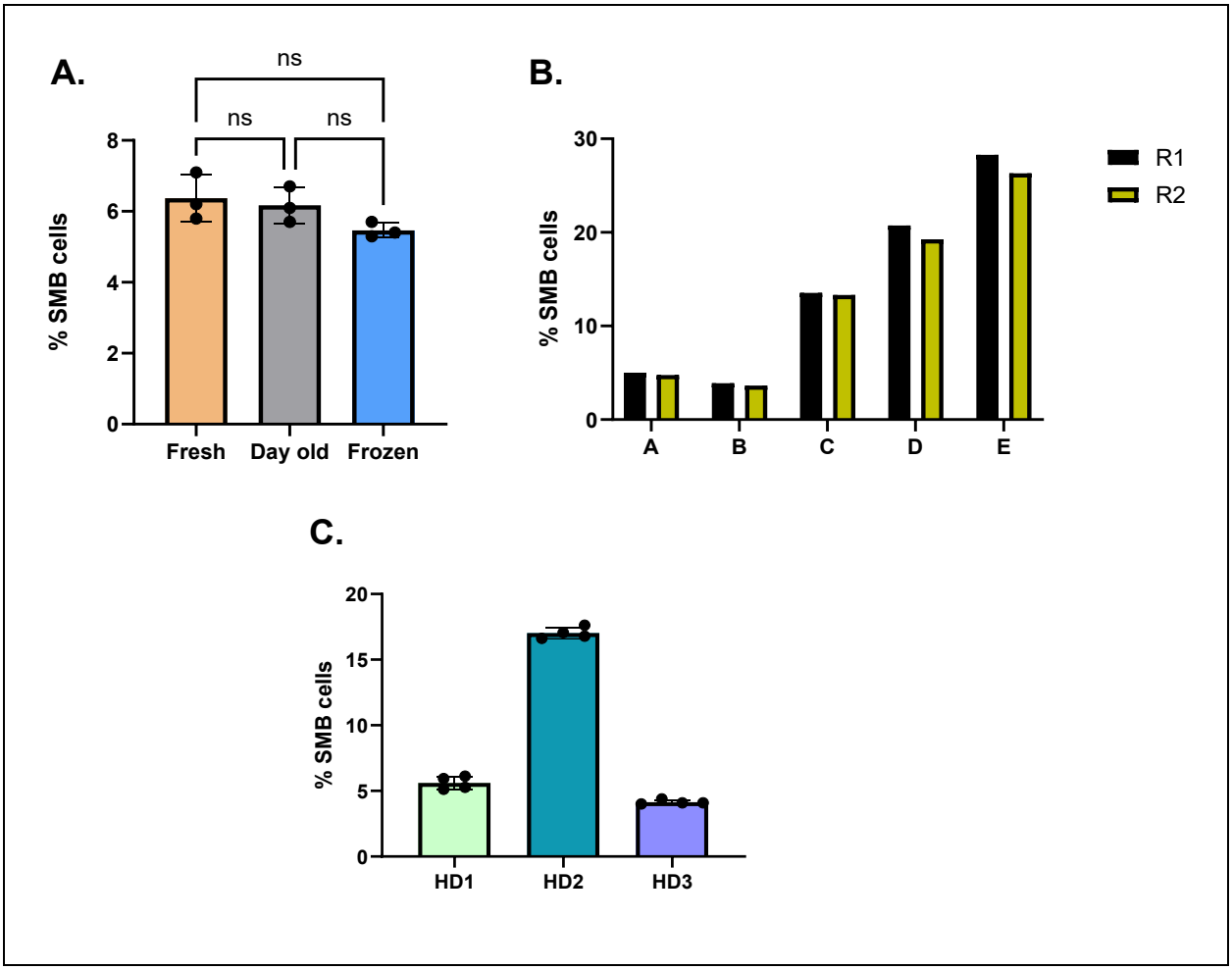


Figure 3: Laboratory protocol optimization and validation. A. Comparison of SMB cell proportions (%SMB cells) of fresh, day-old, and frozen PBMCs in a representative 3 replicates from a single healthy donor (n=1) using established flow cytometry protocol. One-way ANOVA test showed no statistical difference (ns) among the three types of samples. **B.** Comparison of SMB cell proportions of samples collected from different healthy donors (n=5) tested by 2 raters to evaluate inter-rater reliability. Mann-Whitney test showed no significant difference between raters (p=0.6905) and a good intra-class coefficient (ICC) of 0.99. **C.** One-way repeated measure ANOVA for 4 replicates of 3 different healthy control samples (n=3) to evaluate intra-rater reliability showed no significant difference between the repeated measures (p=0.5818) and had an intra-class coefficient (ICC) of 0.99. Error bars represent the standard deviation of the mean.

3.1.2 Inter-rater reliability

Inter-rater reliability was assessed by comparing SMB cell proportions of samples collected from different subjects (n=5) measured independently by 2 raters **(Figure 3B)**. Mann-Whitney t-test showed no significant difference between raters (p=0.6905). Furthermore, we calculated intra-class coefficient (ICC) using the two-way random effects model and a 2-way ANOVA test to verify inter-rater reliability. The ICC score was 0.99 indicating great reliability and agreement among the raters.

3.1.3 Intra-rater reliability

Intra-rater reliability was assessed using one-way repeated measure ANOVA for 4 replicates of 3 samples **(Figure 3C)**. There is no significant difference between the repeated measures (p=0.5818) and an ICC (using the two-way random effects model) of 0.99 showed a great agreement between the measurements from the different trials. The coefficients of variation within the 4 replicates were below 10% (8.71%, 2.57%, and 4.09%) for all 3 samples showing acceptable intra-rater reliability.

3.2 Participant recruitment outcomes

Following assay optimization and validation, recruitment commenced in June 2024. Forty-six participants from the Immunoglobulin Treatment Clinic at TOH were approached after being pre-screened for eligibility in accordance with the previously outlined inclusion and exclusion criteria. Out of the 46 patients: 13 declined to participate, 3 were undecided and remain in contact to reassess interest, 2 were excluded due to language barrier, 2 were lost-to-contact, and 1 patient was awaiting consent to be enrolled. The remaining 25 passed the screening and were enrolled in the study (**Figure 4**).

All 25 participants completed their baseline visit, 19 of which also completed their first follow-up at the 3-6 months time-point and 9 completed their second follow-up at the 9-12 months time-point.

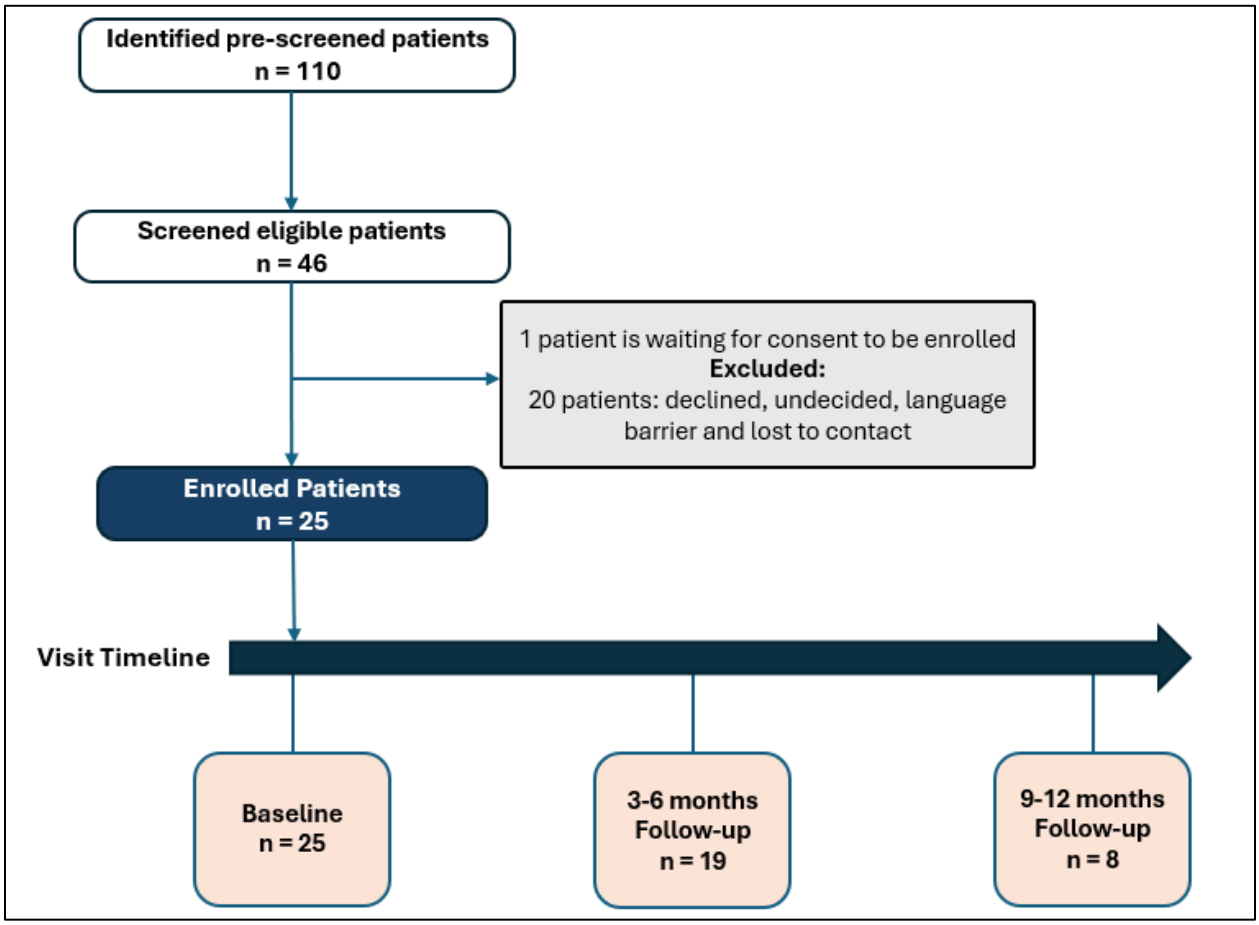


Figure 4: Consort diagram of study cohort. Eligible participants from the Immunoglobulin Treatment Clinic at The Ottawa Hospital were approached for study recruitment. Overall,110 patient charts were reviewed for pre-screening, 46 patients were screened for study eligibility, and 25 participants were successfully enrolled. Exclusion reasons for the 21 patients that were not enrolled are outlined.

3.2.1 Demographic and clinical characteristics

Twenty-five eligible participants with history of hematological malignancies categorized into Non-Hodgkin's Lymphoma (NHL, 60.0%), Chronic Lymphocytic Leukemia (CLL, 24.0%), Multiple Myeloma (MM, 8.0%), and Acute Lymphoblastic Leukemia (ALL, 8.0%) were

recruited. Several disease subtypes were found within the NHL category, including follicular lymphoma (46.7%), diffuse large B cell lymphoma (20.0%), Waldenström macroglobulinemia (13.3%), mantle cell lymphoma (6.7%), follicular mixed cell lymphoma (6.7%) and mediastinal large B cell lymphoma (6.7%). Demographic and clinical baseline characteristics are summarized in **Table 1**. The mean age (SD) of all participants was 65.2 (15.1) years old with majority of the participants being females (n= 16, 64.0%). The average age (SD) at diagnosis was 49.5 (13.8) years for NHL, 20.0 (12.7) years for ALL, 63.0 (19.8) years for MM, and 56.0 (3.2) years for CLL. All participants were on IGRT at the time of enrollment, with an average (SD) length of treatment of 5.3 (4.8) years.

Twenty participants had disease in clinical remission at the time of enrollment (100% of NHL, 100% of ALL, 50% of CLL and 0% of MM). The median (IQR) time since disease remission was 7.50 (5.25) years. The history of cancer treatment is summarized in **Table 2**. Four participants were still on cancer treatment (2 with CLL and 2 with MM) and one participant with CLL has never received cancer treatment and remained stable on active surveillance. Since enrollment, one participant had a relapse of follicular lymphoma and has been under active surveillance without any specific therapy. Another participant with MM has since stopped chemotherapy due to detrimental impact on the participant's quality of life.

Fourteen participants with disease in remission have not experienced any disease relapse since their first cancer treatment. The remaining six participants with disease in remission had 1-2 prior recurrences of their disease.

Six participants (24.0%) underwent hematopoietic stem cell transplant (HSCT) and only one of which has developed chronic GVHD requiring ongoing treatment. The median (IQR) time since the last auto-HSCT was 14.61 (9.19) years and since the last allo-HSCT was 7.72 (6.1)

years. Three participants (12.0%) underwent CAR-T therapy, one of which had previously received auto-HSCT. The median time (IQR) since last CAR-T therapy was 5.36 (1.84) years. Finally, rituximab was the most used immunotherapy in this cohort, with 20 participants (80.0%) having received it as a part of their chemotherapy regimen or for maintenance therapy afterwards. The median (IQR) time since the last rituximab dose was 6.74 (4.96) years.

Table 1: Demographic and Clinical Characteristics (n=25)

	ALL (n=2)	CLL (n=6)	NHL (n=15)	MM (n=2)
Male: Female, (n)	1:0	1:5	1:3	1:1
Mean Age in years (SD)	36.02 (11.19)	74.70 (7.19)	64.13 (13.30)	73.78 (13.83)
Mean BMI (Kg/m^2) (SD)	31.34 (9.11)	27.88 (7.55)	27.04 (4.96)	31.39 (3.59)
Mean IGRT length of treatment at enrollment, months (SD)	19.88 (9.03)	94.06 (58.39)	63.43 (59.79)	19.70 (7.73)
Race or Ethnicity n (%)*				
Black	0	0	0	0
East Asian	0	0	0	0
Indigenous	0	0	0	0
Latin American	0	0	0	0
Middle Eastern	1 (50.00)	0	1 (6.67)	0
South Asian	0	0	0	0
Southeast Asian	0	0	0	0
White	1 (50.00)	6 (100.00)	15 (100.00)	2 (100.00)
Other	0	0	2 (13.33)	1 (50.00)
Do not know	0	0	0	0
Prefer not to answer	0	0	0	0
Comorbidities n (%)				
Hypertension	1 (50.00)	1 (16.67)	3 (20.00)	2 (100.00)
Diabetes	0	0	2 (13.33)	2 (100.00)
Asthma	0	1 (16.67)	4 (26.67)	0
COPD	0	0	0	0
Bronchiectasis	0	0	2 (13.33)	0
Other chronic lung disease	0	1 (16.67)	0	0
Heart disease	0	0	4 (26.67)	1 (50.00)
Chronic kidney disease (eGRF< 60)	0	0	2 (13.33)	0
Cirrhosis	0	0	0	0

Solid organ cancer	0	4 (66.67)	2 (13.33)	1 (50.00)
Previous hematopoietic stem cell transplantation	2 (100.00)	0	3 (20.00)	1 (50.00)
Previous chimeric antigen receptor (CAR)-T cell therapy	0	0	3 (20.00)	0
Blood cancer (not in the inclusion criteria)	0	1 (16.67)	1 (6.67)	0
Chronic blood disorder (not blood cancer)	1 (50.00)	2 (33.33)	1 (6.67)	0
Chronic neurological disorder	0	0	2 (13.33)	0
Autoimmune condition	0	0	2 (13.33)	0
Obesity (BMI ≥ 30)	1 (50.00)	2 (33.33)	5 (33.33)	1 (50.00)
Depression	0	2 (33.33)	3 (20.00)	0
Clinical Blood results: [a] **Neutrophil,** 10^9/L **Lymphocyte,** 10^9/L **IgG, g · L^{-1}** **IgA, g · L^{-1}** **IgM, g · L^{-1}**	 4.55 (1.99) 1.84 (0.64) 10.85 (1.63) 0.80 (0.99) 0.75 (0.92)	 3.17 (2.52) 1.83 (1.55) 9.55 (3.60) 0.10 (0.08) 1.15 (1.23)	 2.89 (1.42) 1.10 (0.69) 9.80 (2.30) 0.10 (0.00) 0.30 (0.55)	 7.62 (3.16) 1.10 (0.45) 9.15 (0.78) 0.10 (0.00) 0.20 (0.14)
Number of participants with **moderate infections** [b] within 12 months prior to enrollment (n, %)	2 (100)	3 (50.00)	8 (53.33)	2 (100)
Number of participants with **severe infections** [c] within 12 months prior to enrollment (n, %)	0	1 (16.67)	2 (13.33)	2.00 (100)

*Participants with mixed ethnicities may report multiple ethnicities

[a]Reporting median and interquartile range (IQR).

[b]Moderate infections are defined as infections that require oral antibiotics.

[c]Severe infections are defined as infections that require hospitalization or administration of intravenous antibiotics.

Table 2: History of Cancer Treatment (n=25)

History of Cancer Treatment (n,%)	ALL (n=2)	CLL (n=6)	NHL (n=15)	MM (n=2)
HSCT	2 (100)	0	3 (20.00)	1 (50.00)
Allo-HSCT	2 (100)	0	1 (6.67)	0
Auto-HSCT	0	0	2 (13.33)	1 (50.00)
CAR-T	0	0	3 (20.00)	0
Rituximab	1 (50.00)	4 (66.7)	15 (100)	0
R-CHOP[a]	0	0	7 (46.67)	0
CHOP	1 (50.00)	0	1 (6.67)	0
Cytarabine	1 (50.00)	0	0	0
DHAP	0	0	2 (13.33)	0
R-CVP[a]	0	0	2 (13.33)	0
CVP	1 (50.00)	1 (16.67)	2 (13.33)	0
CEOP	0	0	1 (6.67)	0
FCR	0	3 (50.00)	0	0
FR	0	1 (16.67)	1 (6.67)	0
FC	0	1 (16.67)	2 (13.33)	0
Fludarabine	0	1 (16.67)	0	0
R-GDP[a]	0	0	2 (13.33)	0
DRd	0	0	0	1 (50.00)
BR	0	0	6 (40.00)	0
MPV	0	0	0	1 (50.00)
BPd	0	0	0	1 (50.00)
Acalabrutinib	0	1 (16.67)	0	0
Pembrolizumab	0	0	1 (6.67)	0
Zanubrutinib	0	1 (16.67)	0	0
Jakafi	1 (50.00)	0	0	0

[a] R- stands for rituximab

Abbreviations: ALL – acute lymphoblastic leukemia, CLL – chronic lymphocytic leukemia, NHL – non-Hodgkin's lymphoma, MM – multiple myeloma, IGRT – immunoglobulin replacement treatment, HSCT- Hematopoietic Stem Cell Transplantation, CAR-T- Chimeric Antigen Receptor T cell therapy, CHOP- Cyclophosphamide, doxorubicin, vincristine, and Prednisone, DHAP- Dexamethasone, cytarabine, and cisplatin, CVP- cyclophosphamide, vincristine sulfate, and prednisone, CEOP- Cyclophosphamide, etoposide, vincristine, and Prednisone , FCR- Fludarabine, cyclophosphamide, and rituximab , FR- Fludarabine and rituximab, FC – Fludarabine and cyclophosphamide, R-GDP- Rituximab, gemcitabine, dexamethasone and cisplatin, DRd- daratumumab, dexamethasone, and lenalidomide, BR- bendamustine and rituximab, MPV- Methotrexate, procarbazine, and vincristine, BPd - Belantamab, pomalidomide and dexamethasone.

3.2.2 Acceptability questionnaire and evaluation of study feasibility

We administered an acceptability questionnaire to each participant to evaluate the participant's perception of the study and identify areas of improvement. Summary of responses collected from 25 participants is presented in **Table 3**.

Nervousness was the most reported feeling regarding participation in the study. Around 40% of participants reported feeling nervous about potentially stopping their immunoglobulin treatment and consequently suffering from recurrent or severe infections. Many also reported feeling happy (28.00%) and excited (24.00%) to be in this study for various reasons including wanting to contribute to research and potentially being able to discontinue immunoglobulin treatment sooner than later. This also tied into the decision to join the study where the top reason reported was a desire to contribute to medical knowledge advancement (92.00%) while only 20.00% shared the desire to discontinue IGRT as the reason to join.

Furthermore, participants reported that having the study endorsed by the treating doctor (40.00%) and by their oncologist (48.00%) can help provide reassurance to potential participants by facilitating discussion of risk and benefits with a trusted healthcare provider. Using this information, we were able to approach clinicians at the Malignant Hematology clinic at TOH to promote awareness of the study for our potential participants prior to enrollment.

In addition to the acceptability questionnaire, we have also identified several barriers to recruitment early in the study. These included difficulties with long-distance travelling to attend study visits; incurring the burden of having more visits to the hospital outside of their regular follow-ups with their treating physicians, paying additional parking fees, and having additional phlebotomy done for study purposes. To mitigate these barriers, we successfully coordinated our study visits with the participant's regular appointments at the hospital so they can be done on the

same day, and we have consolidated blood draws to collect samples for the study as well as tests ordered by their treating doctors during the study visit. These adaptations have helped us maintain a recruitment rate of 2 patients per month with 100% retention and compliance rate.

Overall, these findings prove that that recruitment and sustained participation in this patient cohort is achievable and support the feasibility of conducting this study.

Table 3: Summary of responses to the acceptability questionnaires (n=25)

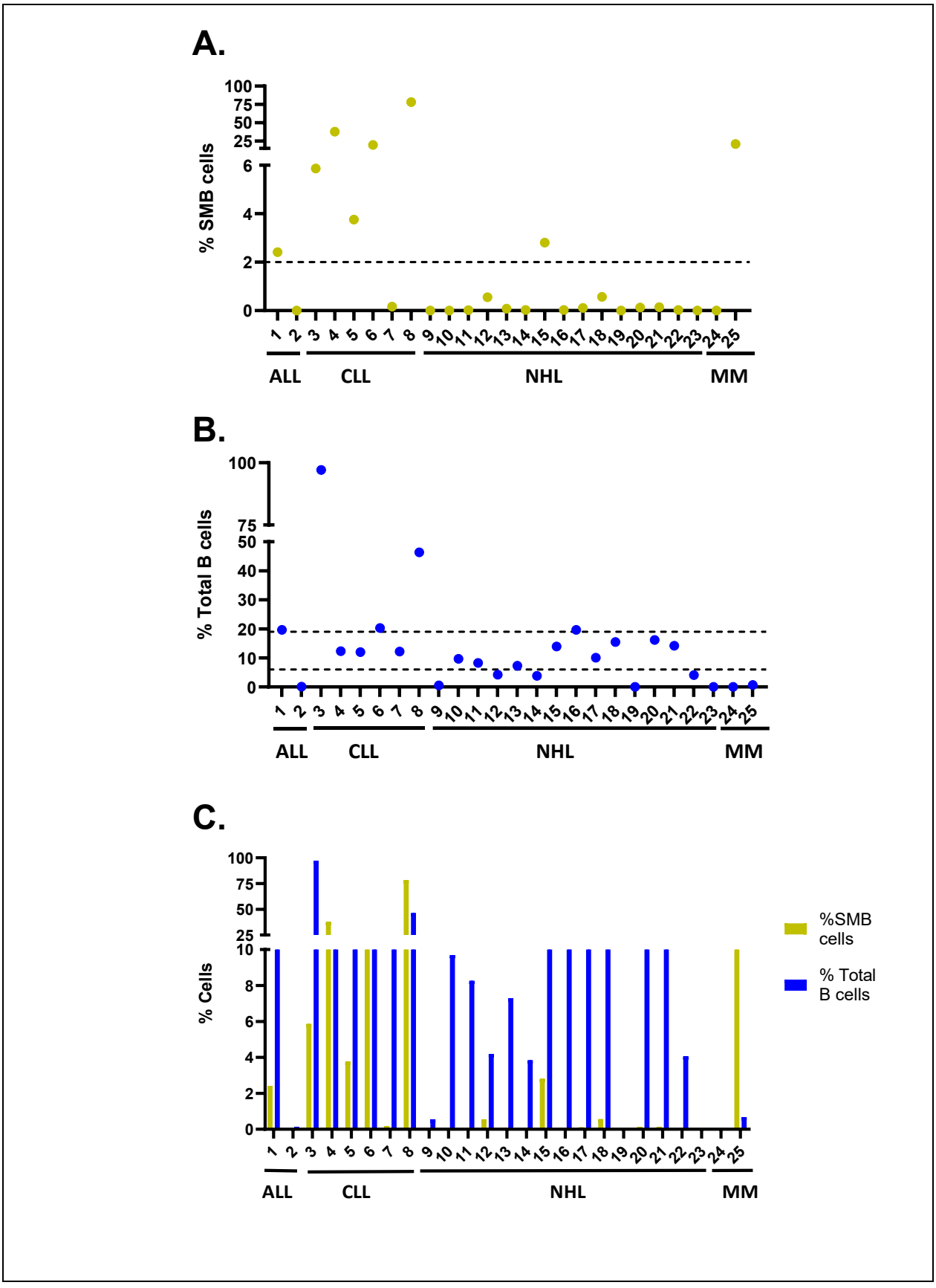
Category	Reported Response Selections
Feeling about participating in the study	• **Nervous** (40.00%) • **Anxious** (24.00%) • **Excited** (24.00%) • **Happy** (28.00%) • **Indifferent** (24.00%) • **None of the above (**4.00%)
What made you decide to join the study?	• **I trust the doctor (**44.00%) • **I trust the nurses** (32.00%) • **The coordinator explained to me well** (48.00%) • **I would like to be off immunoglobulin treatment** (20.00%) • **I have side effects with immunoglobulin treatment** (0%) • **I would like to contribute to the advancement of medical knowledge** (92.00%) • **There is a good follow-up plan in place if I am sick after stopping immunoglobulin** (36.00%) • **My caregiver encourages me to join** (12.00%) • **This study will be good for me personally** (16.00%)
Is there anything you think we can do to increase study acceptability?	• **Endorsement from my oncologist** (48.00%) • **Have the study advertised by the treating doctor** (40.00%) • **Other Suggestions** (28.00%) - The majority of suggestions were regarding study advertisements and involvement of members in their circle of care. • **Endorsement from Canadian Blood Services** (16.00%) • **Endorsement from the Ministry of Health** (16.00%) • **Have the study advertised by the IGRT program nurses** (8.00%) • **Endorsement from Leukemia Lymphoma Society** (16.00%) • **Have the study advertised by patients with similar conditions (**8.00%)

3.3 Proportion of SMB cells in patients with HM-SAD

3.3.1 Baseline assessment of SMB cells frequencies

Baseline total B cell and SMB cell frequencies were measured in 25 participants. The median (IQR) proportion of total B cells was 10.05 (11.65) % of total lymphocytes [normal range 6.0-19.0%] and the median (IQR) proportion of SMB cells was 0.13 (2.79) % of total B cells [normal threshold is considered ≥ 2%] (Individual data points shown in **Figure 5A-C**). While 56% of participants had normal total B cell proportions, only 32% had normal SMB cell

proportions. The highest proportion of SMB cells was at 78.21% from a total B cell proportion of 46.34%. This participant was diagnosed with CLL that was treated in 1997 with Fludarabine and has been in remission since.


A.
% SMB cells
100
75
50
25
6
4
2
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
ALL
CLL
NHL
MM
B.
% Total B cells
100
75
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
ALL
CLL
NHL
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% Cells
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0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
ALL
CLL
NHL
MM
%SMB cells
% Total B cells

Figure 5: SMB cells and total B cells frequencies at baseline (n=25). A. SMB cell ($CD27^+$ IgD^- IgM^-) proportions measured at baseline and the normal threshold (2%) is denoted by the dashed line. **B.** Total B cell ($CD19^+$ $CD3^-$) proportions measured at baseline and the normal range (6-19%) is denoted by the dashed line. **C.** Combined visualization of SMB cells and total B cell proportions measured at baseline. The participants were grouped in order according to their disease category as shown in the labels below the x-axis. ALL – acute lymphoblastic leukemia, CLL – chronic lymphocytic leukemia, NHL – non-Hodkin's lymphoma, and MM – multiple myeloma.

3.3.2 Longitudinal characterization of SMB cell proportions in HM-SAD

To evaluate changes in SMB cell proportions over time, total B cell and SMB cell frequencies were measured in 19 participants who completed the first follow-up (at 3-6 months after the baseline visit) and 8 participants that completed the second follow-up (at 9-12 months). No significant differences were detected between baseline, first and second follow-ups for % SMB cells and % total B cells for each participant (**Figure 6A-D**).

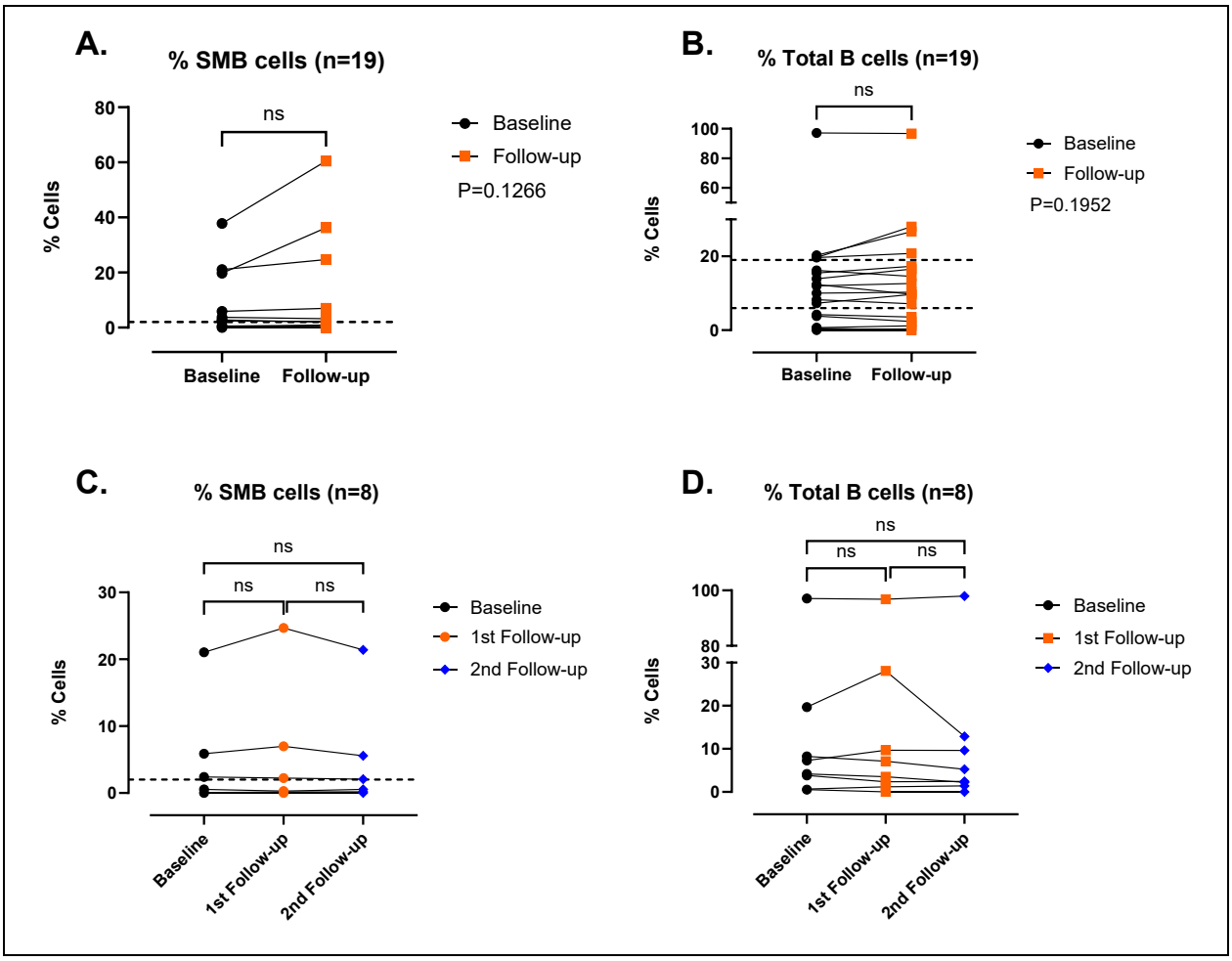

Figure 6: Longitudinal measures of total B cells and Switched memory B cells frequencies. **A**. SMB cell proportions measured at baseline and at the first follow-up (3-6 months). **B.** Total B cell proportions measured at baseline and at the first follow-up (3-6 months) (n=19). **C.** SMB cell proportions measured at baseline, at the first follow-up (3-6 months), and at the second follow-up (9-12 months) (n=8). **D.** Total B cell proportions measured at baseline, at the first follow-up (3-6 months), and at the second follow-up (9-12 months). Paired Wilcoxon test showed no significant differences between measures at baseline, first and second follow-ups for total B cell and SMB cell proportions. The normal SMB cell proportion threshold (2%) and the total B cell normal range (6-19%) are denoted by the dashed lines.

3.3.3 Rate of moderate and severe infections and SMB cell proportions

Studies have shown that low SMB cell proportions are associated with increased risk of infections and infection related morbidity and mortality.[190,191,203] We collected 12-month history

of moderate and severe infections at baseline as well as infection history at every follow-up. Baseline infection history details are summarized in **Table 4**.

At baseline, 48% of participants reported experiencing moderate infections, 24% had no infections, 20% had severe infections, and 10% had mild infections within 12 months prior to enrollment (**Figure 7**). The severe infections reported were urinary tract infections, urosepsis, pneumonia, exacerbation of bronchiectasis and septic arthritis. Some of the microorganisms identified in relation to these infections were *Escherichia coli*, Group B *Streptococcus*, *Haemophilus influenzae*, and *Klebsiella pneumoniae*. Kruskal-Wallis test showed no significant differences in SMB cell proportions among the different categories of infection severity (p=0.2290). It also showed no overall significant difference B cell proportions (p= 0.0697), however, pairwise comparison using Mann-Whitney t-test showed a significant difference between the no infections and the severe infections group (p=0.0173). Grouping participants into "no infections" (n=6) and "with Infections" (n=19) groups showed no significant difference in both SMB cells (p= 0.0690) and total B cell proportions (p= 0.2917).

Table 4: Summary of baseline infections severity and SMB cells and total B cell proportions (n=25)

	Infection Severity			
	No infections n = 6	**Mild Infections n = 2**	**Moderate Infections n = 12**	**Severe Infections n = 5**
Median % SMB cells (IQR)	0.56 (14.79)	0.00 (0.00)	0.14 (2.44)	0.03 (21.05)
Median % Total B cells (IQR)	17.58 (10.06)	5.12 (4.57)	12.07 (8.28)	0.68 (4.05)

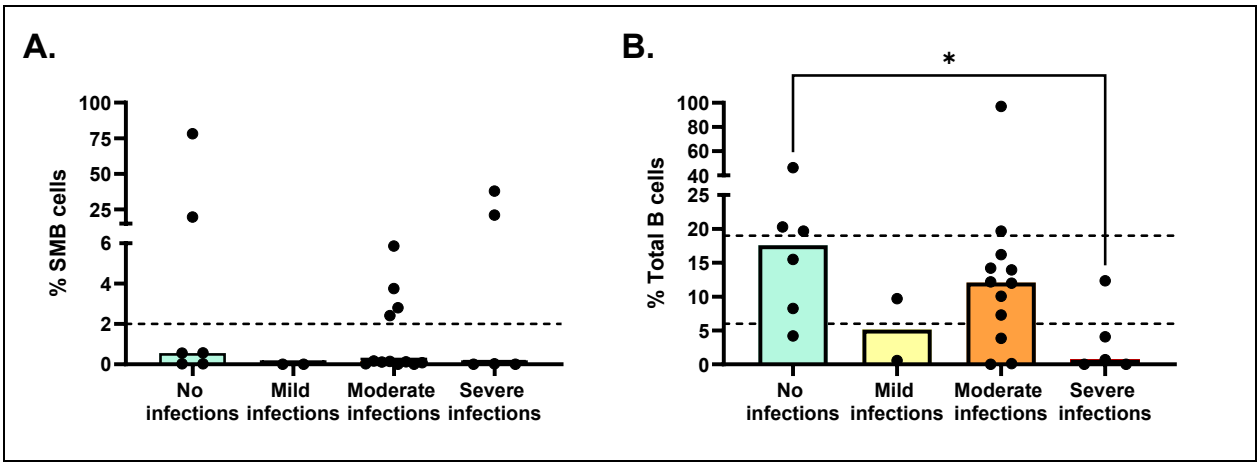

Figure 7: Baseline distribution of SMB cells and total B cell proportions across categories of infection history and severity (n=25). A. % SMB cells measured by flow cytometry at the baseline. Most participants had SMB cells percentage below the normal 2% threshold, denoted by the dashed line, and no significant differences were detected across all four categories. **B.** % total B cells measured by flow cytometry at the baseline. Most participants had total B cells percentage within the normal range (6-19%), denoted by the dashed line, the only significant difference detected using Mann-Whitney t-test was between the no-infections group and the severe infections group (*, p= 0.0173).

We previously observed no significant difference between percentages of SMB cells and total B cells at baseline and follow-up, so we compared the number of infections at baseline (12-month history prior to enrollment) to the cumulative number of infections at the second follow-up (12-months after enrollment) (**Figure 8**). Out of the eight participants who completed their second follow-up, one participant had a severe respiratory infection (positive for influenza A) requiring 12-day long hospitalization. Two participants had no infections, and the remaining five participants experienced moderate infections requiring oral antibiotics. Paired student *t*-test revealed no significant difference between the number of infections at baseline and follow-up for moderate infections (p= 0.7318) and severe infections (p= 0.6845).

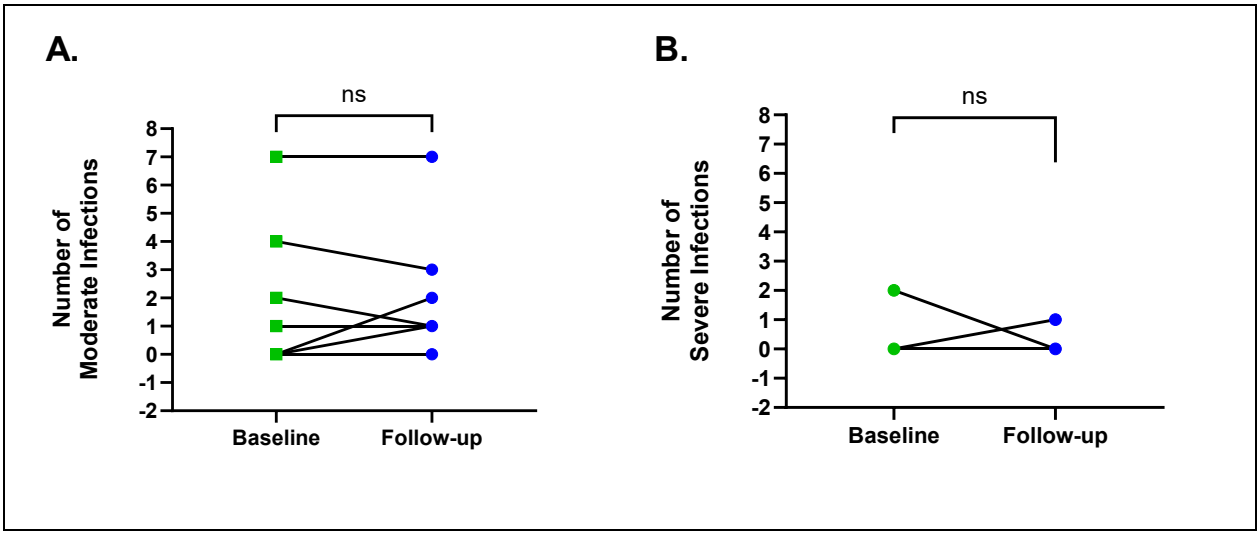

Figure 8: Plot of number of moderate and severe infections at baseline and cumulative 12-month follow-up (n=8). Individual points and lines may represent more than one participant. The number of infections reported at baseline reflects infections that occurred within 12 months prior to enrollment. **A.** Number of moderate infections reported at baseline and cumulative number of moderate infections reported between baseline and the second follow-up. Four participants reported zero infections at baseline, two of them reported zero moderate infections at follow-up while the other two reported one and two moderate infections at follow-up, respectively. **B.** Number of severe infections reported at baseline and cumulative number of severe infections reported between baseline and the second follow-up. Seven participants reported zero infections at baseline, only one of them reported one severe infection at follow-up. Paired student *t*-test revealed no significant difference between the number of infections at baseline and follow-up for moderate infections (p= 0.7318) and severe infections (p= 0.6845).

3.4 Demographic characteristics and SMB cell proportions

3.4.1 Age

Studies have shown that mature B cells, including SMB cells, significantly decrease with age and that the SMB cells decrease in elderly adults represents an intrinsic defect in immunoglobulin class-switching with age.[204] We evaluated if SMB cell proportions differ in an age-dependent manner in the study cohort. We found that age correlated moderately with SMB cell proportions (r= 0.400, p= 0.034). No significant differences were observed in SMB cell proportions (p= 0.0714) (**Figure 9A-B**) or total B cell proportions (p= 0.3110) (**Figure 10A-B**)

between participants less than 65 years (n=10) and those who were 65 years of age and older (n=15).

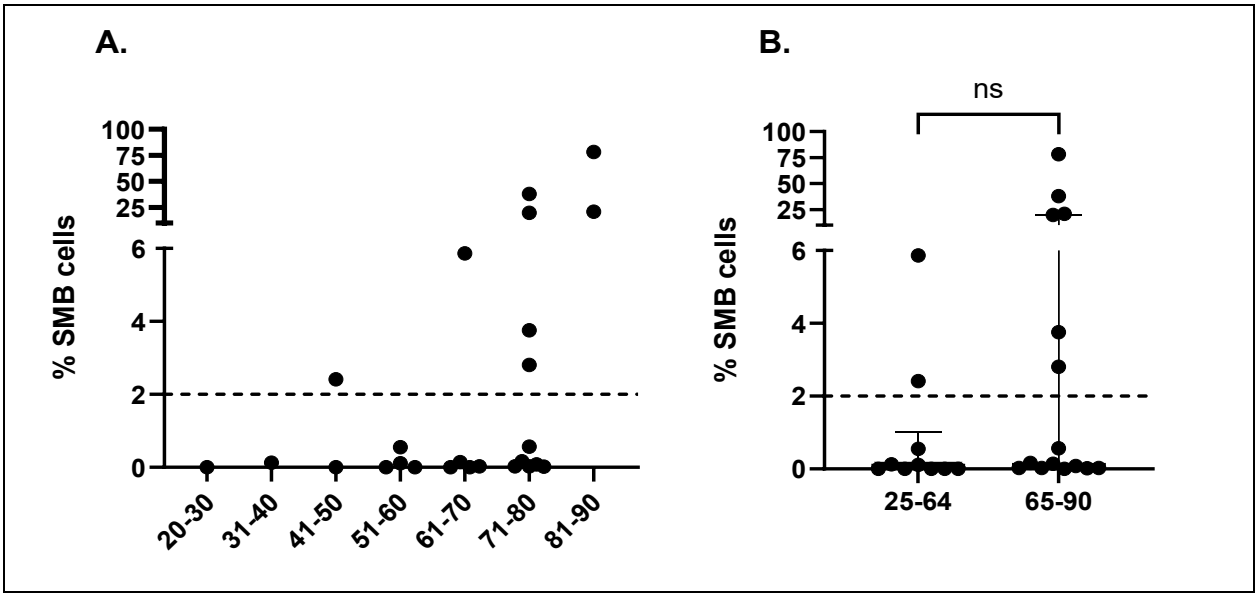

Figure 9: Distribution of SMB cell proportions at baseline, grouped by age (n=25). A. SMB cell proportions measured at baseline grouped in 10-year increments **B.** SMB cell proportions measured at baseline and categorized into age groups 25-64 and 65-90 years of age. Mann-Whitney t-test showed no significant differences between SMB cell proportions of participants in the age groups 25-64 and 65-90 years old (p= 0.0714). Error bars represent the IQR. The normal SMB cell proportion threshold (2%) is denoted by the dashed lines.

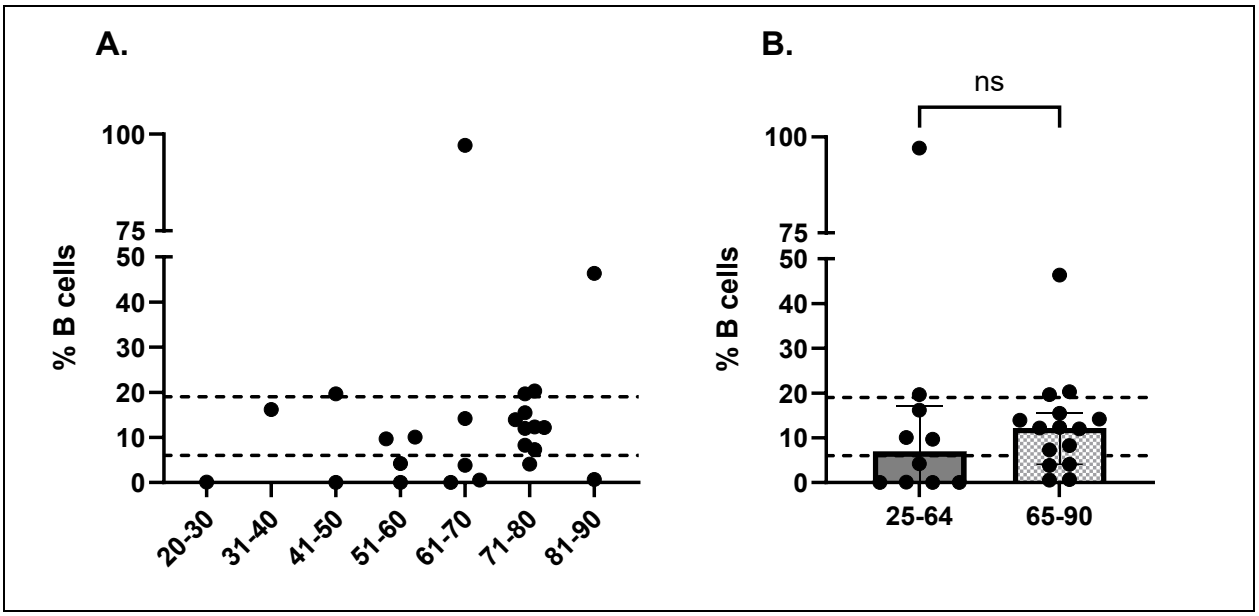

Figure 10: Distribution of total B cell proportions at baseline and grouped by age (n=25). **A**. Total B cell proportions measured at baseline and grouped in 10-year increments **B.** Total B cell proportions measured at baseline and categorized into age groups 25-64 and 65-90 years of age. Mann-Whitney t-test showed no significant differences between SMB cell proportions of participants in the age groups 25-64 and 65-90 years old (p= 0.3110). Error bars represent the IQR.

3.4.2 Sex

Sex hormones have been reported to influence immune response and B cell development and differentiation; therefore, we investigated sex differences in relation to SMB cell and total B cell proportions in our study cohort.[205] We observed no significant difference in SMB cell or total B cell proportions between male and female participants (p= 0.6247 and p= 0.3286) **(Figure 11A-B)**.

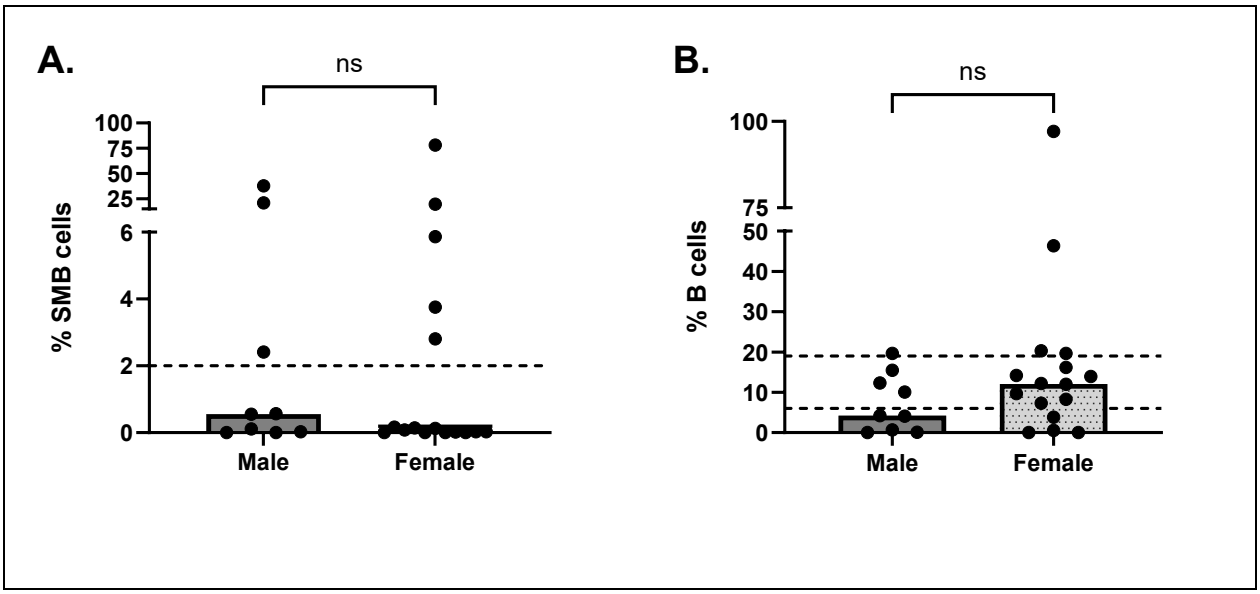

Figure 11: Distribution of SMB cell and total B cell proportions in males and females in the study cohort (n=25). A. Comparison of male (n=9) and female (n=16) median SMB cell proportions measured at baseline **B.** Comparison of male (n=9) and female (n=16) median total B cell proportions measured at baseline. Mann-Whitney t-test showed no significant differences between SMB cells and total B cell proportions in males and females (p= 0.6247 and p= 0.3286). Dashed lines indicate the normal threshold for SMB cells and normal range for total B cell proportions.

3.5 Clinical characteristics and SMB cell proportions

3.5.1 Disease category

When comparing SMB cell proportions by disease categories, we observed low SMB cell proportions in 93.33% of participants with NHL, 16.67% with CLL, 50.00% with ALL and 50.00% with MM, having median (IQR) of 0.03 (0.12) %, 12.80 (29.03) %, 1.21 (1.21) % and 10.53 (10.56) % respectively, from total B lymphocytes. The median (IQR) of total B cells for participants with NHL was 8.27 (10.13), CLL 16.31 (27.61), ALL 9.90 (9.79), and MM 0.35 (0.34) (**Figure 12A-B**). Mann-Whitney t-test showed significant differences in total B cells and SMB cells frequencies between CLL and NHL (p=0.0184 and p=0.0002) but no other significant differences were detected in comparisons between other groups. In contrast, 56% of participants

had B cell proportions within normal range (6-19% of total lymphocytes) including 60.00% of participants with NHL, 66.67% with CLL, 50.00% with ALL, and 0.00% of MM, having median (IQR) of 8.27 (10.13) %, 16.31 (27.61) %, 9.90 (9.8) %, 0.35 (0.34) %, respectively, from total lymphocytes.

CLL participants had some of the highest SMB cells and total B cell proportions. Lymphocytosis is a hallmark of CLL, and it is likely that the memory B cells captured in the current gating strategy includes monoclonal expanded IGHV-mutated CLL-B cell population. Although both MM participants have been receiving chemotherapy for the past 3-4 years, their SMB cell proportions differed drastically (0.00% and 21.05% respectively) while both had low total B cell proportions (0.01% and 0.68%, respectively).

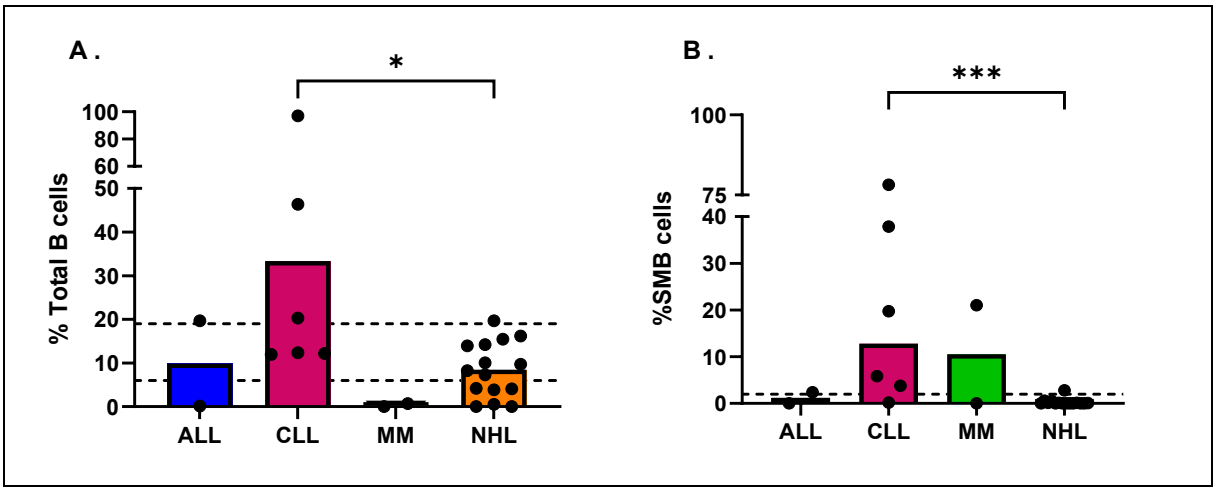

Figure 12: Measured frequencies of total B cells and Switched memory B cells in participants grouped by disease category (n=25). A. Total B cell proportions measured from total lymphocytes **B.** SMB cell proportions measured from total B cells. Mann-Whitney t-test showed significant differences in total B cells and SMB cells frequencies between CLL and NHL (p=0.0184 and p=0.0002). No other significant differences between groups were detected. Dashed lines indicate the normal threshold for SMB cells and normal range for total B cell proportions. ALL – acute lymphoblastic leukemia, CLL – chronic lymphocytic leukemia, NHL – non-Hodkin's lymphoma, and MM – multiple myeloma. The normal SMB cell proportion threshold (2%) and the total B cell normal range (6-19%) are denoted by the dashed lines.

3.5.2 Disease status

We evaluated whether there is a temporal association between the time since disease remission and SMB cell recovery. Remission was defined as the year of the last cancer treatment or the first documentation of remission by the treating physician if the last treatment details are not clear or available. Twenty participants were in remission at the beginning of the study, and the median time since disease remission was 7.50 (5.25) years. The results showed that total B cell proportions seemed to be generally within the normal range in a shorter time in remission, while SMB cell proportions remained below normal with only 20% of values ≥ 2%. (**Figure 13**).

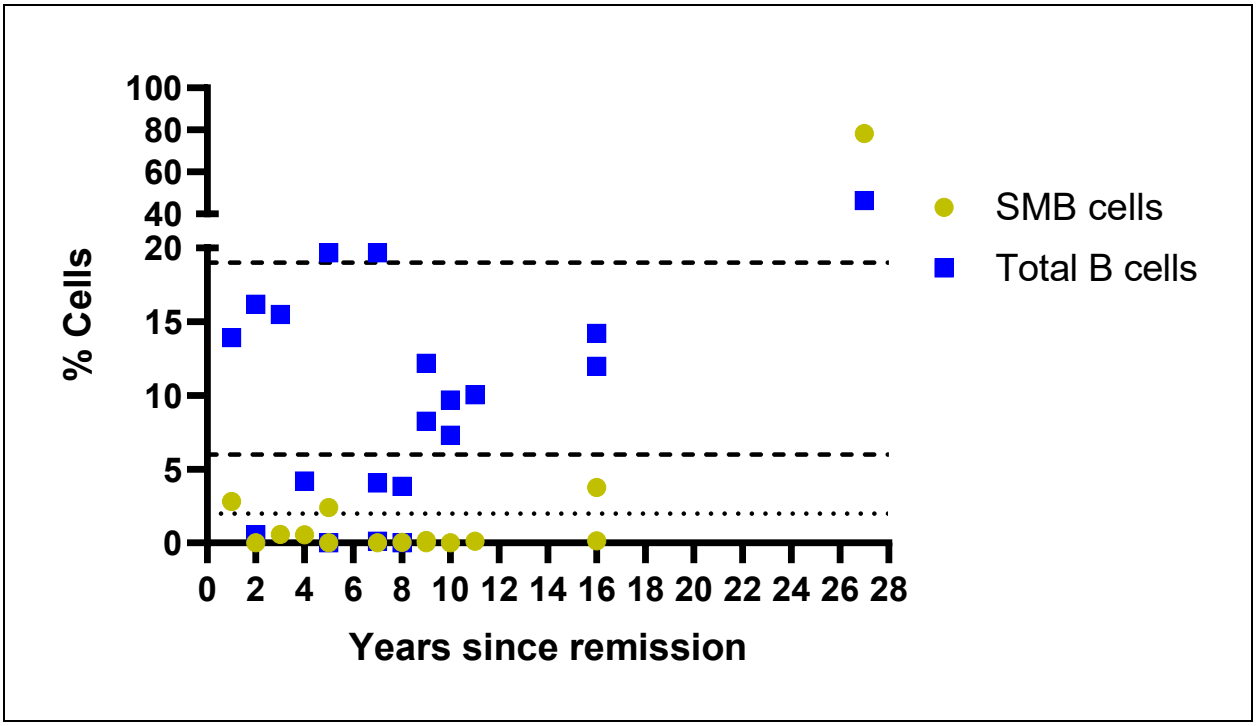

Figure 13: Measured frequencies of total B cells and Switched memory B cells in participants grouped by years since disease remission (n=20). Total B cell proportions measured from total lymphocytes and SMB cell proportions measured from total B cells at baseline. Dotted lines indicate the normal threshold for SMB cells and dashed lines mark the normal range for total B cell proportions.

3.5.3 Immunoglobulins

Serum immunoglobulin levels are important measures of patients' immune status and their ability to fight infections. They are also impacted by medical conditions such as immunodeficiencies and hematological malignancies. We calculated the median (IQR) for serum IgG, IgA, and IgM titers in our participant cohort, which were 9.70 (2.70) g/L, 0.10 (0.00) g/L and 0.30 (1.30) g/L, respectively. Two-way Anova analysis showed no significant differences among disease groups for IgG, IgA and IgM titers (p= 0.5307, 0.2395, and 0.2542, respectively) (**Figure 14**). One participant with Waldenström Macroglobulinemia had excess IgM (17.3 g/L) in alignment with the disease process. Low IgA and IgM titers were seen 88.00% and 52.00% of participants, respectively. IgG levels showed moderate positive correlation to IgA (r= 0.562, p= 0.0035) (**Figure 15**). Since IgG levels are altered by IGRT and all participants in the study are on IGRT, the relationship between SMB cell proportions and IgG titers is confounded by the treatment and the relationship with endogenous IgG cannot be isolated or accurately described here. Our data shows weak positive correlation between serum IgG titers and SMB cell proportions (r= 0.280, p= 0.176) as well as serum IgA titers and SMB cell proportions (r= 0.275, p= 0.184). Interestingly, IgM titers showed moderate positive correlation with both SMB cells and total B cell proportions (r= 0.416, p= 0.0384 and r= 0.537, p= 0.0057, respectively).

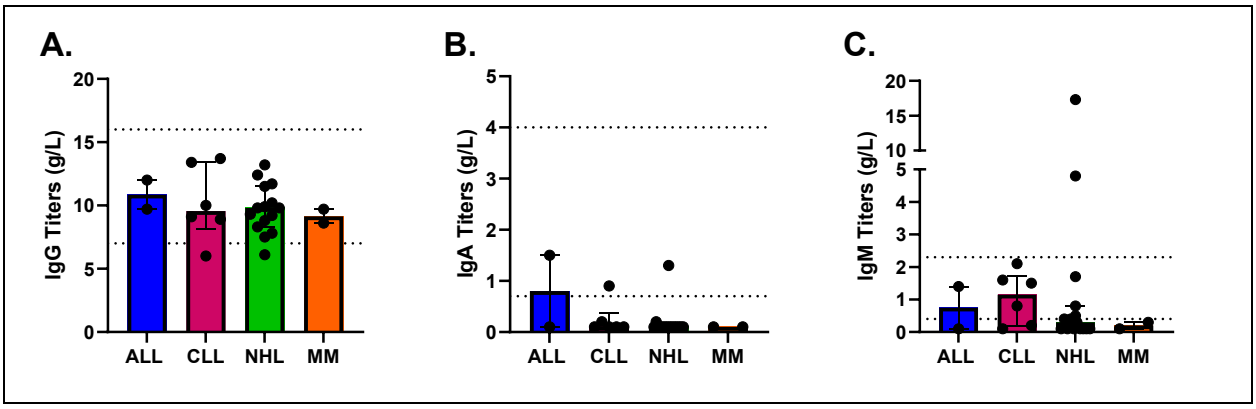

Figure 14: Serum IgG, IgM and IgA titers grouped by disease category (n=25). A. Measured serum IgG titers (g/L). **B**. Measured serum IgA titers (g/L). **C**. Measured serum IgM titers (g/L). Dotted lines indicate the normal range for IgG (7.0 – 16.0 g/L), for IgA (0.7 – 4.0 g/L) and for IgM (0.4 – 2.3 g/L). ALL – acute lymphoblastic leukemia, CLL – chronic lymphocytic leukemia, NHL – non-Hodkin's lymphoma, and MM – multiple myeloma. Error bars represent the IQR.

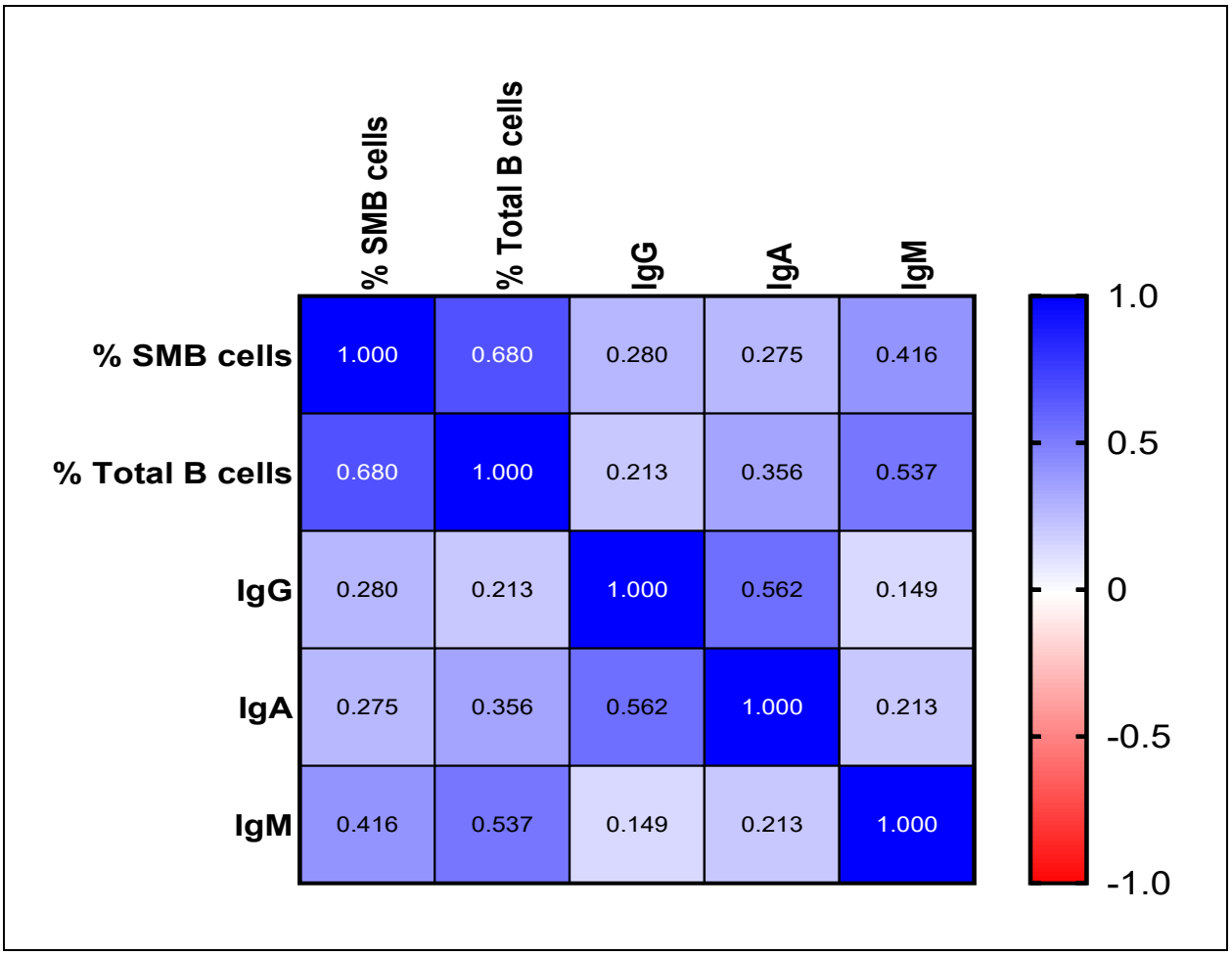

Figure 15: Heatmap of spearman *r* correlations of serum IgG, IgM and IgA titers with SMB cells and total B cell proportions (n=25). Pairwise Spearman correlation coefficients (*r*) are shown between % SMB cells, % total B cells, and serum levels of IgG, IgA, and IgM. The strength of correlation is indicated by both color intensity (blue = positive, red = negative) and the numeric value in each cell. All correlations are positive, with the strongest and expected correlation observed between SMB cells and total B cell proportions ($r = 0.680$), and IgG with IgA ($r = 0.562$). A color scale bar from -1 to 1 is included to reflect correlation magnitude and direction.

3.5.4 Time since IGRT initiation

We evaluated association between length of time the participants were on IGRT at enrollment and their baseline SMB cells and total B cell proportions. The average time participants were on IGRT was 5.32 (±4.53) years. Mann-Whitney two-tailed test showed no significant difference between participants with normal and low SMB cell proportions (<2%) in relation to time on IGRT ($p= 0.8419$) while participants with low total B cell proportions were on IGRT for a significantly lower time than those with normal total B cell proportions (n=22 due to the exclusion of 3 participants with total B cells exceeding the normal range, $p<0.0252$) (**Figure 16A-D**).

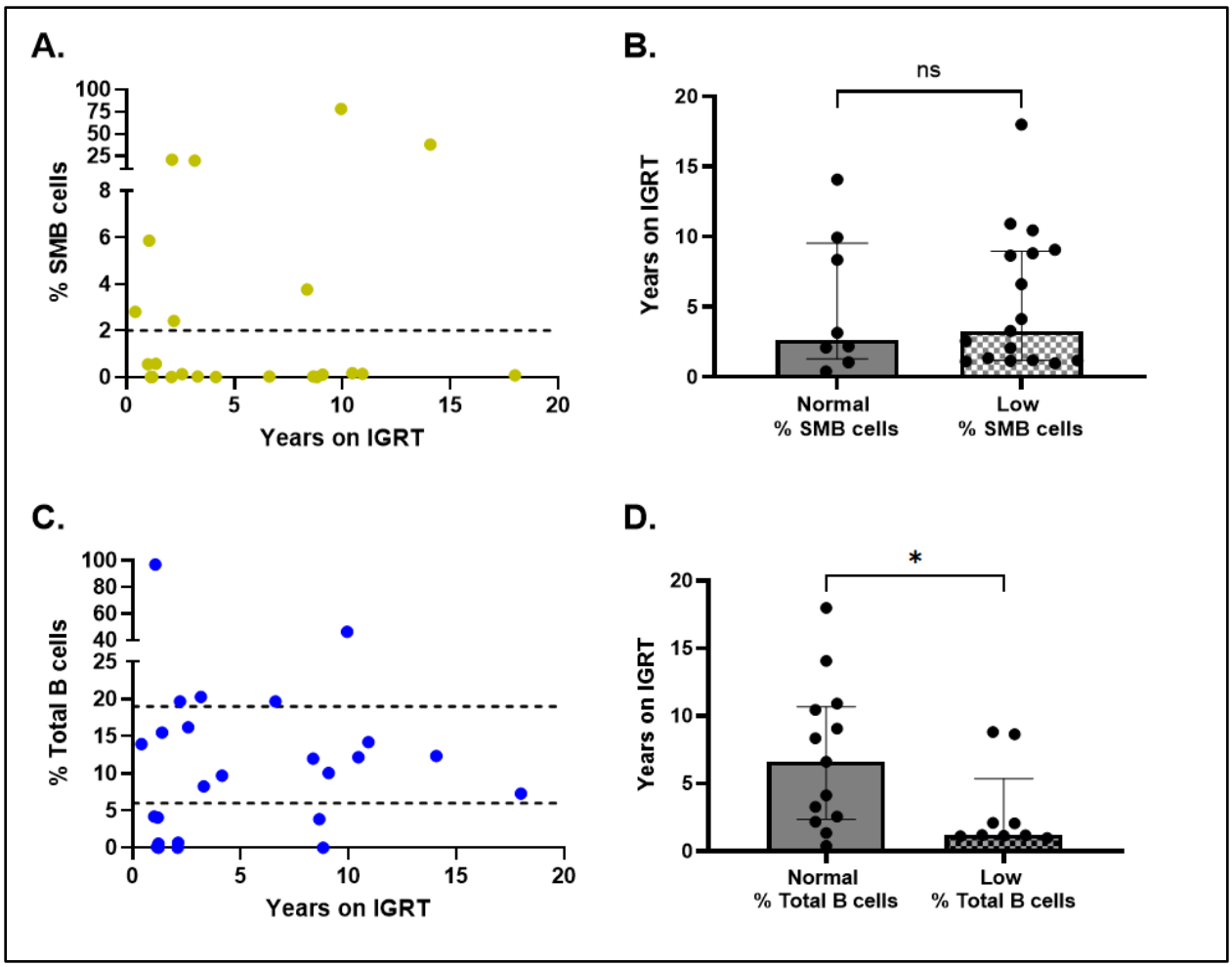

Figure 16: Duration of IGRT and SMB cells and total B cells frequencies at baseline. A. Percentage of switched memory B (SMB) cells relative to duration of immunoglobulin replacement therapy (IGRT). Each dot represents an individual patient. The dashed horizontal line at 2% denotes the threshold for normal SMB cell frequency. Most individuals have <2% SMB cells regardless of IGRT duration, suggesting limited reconstitution of this B cell subset over time. **B**. Comparison of duration of IGRT in participants with low and normal SMB cells frequencies (n=25). Mann-Whitney t-test showed no significant difference between the two groups (ns, p= 0.8419). **C.** Percentage of total B cells relative to duration of IGRT. Each dot represents an individual patient. The dashed horizontal lines denote the normal range for total B cells frequency (6.0 – 19.0%). **D**. Comparison of duration of IGRT in participants with low and normal total B cells frequencies (n=22). Mann-Whitney t-test showed significant difference between the two groups (*, p< 0.0252). Error bars represent the IQR.

3.5.5 Cancer therapeutics

Knowing the potentially curative effect of HSCT for HM and the high efficacy of B cell depleting therapy, we analyzed the SMB cell proportions of participants who received either

HSCT and/or CD-20 B cell depleting therapy, rituximab, as treatment for the HM to examine their effect on this B cell subset.

Six (24%) participants have previously undergone HSCT. Only one participant had normal SMB cell percentage while three participants showed total B cells percentage within the normal range (**Figure 17**). Both ALL participants had undergone allogenic HSCT; one participant showed recovering trends of SMB cells and total B cell proportions (2.41% and 19.69%, respectively) at 5 years post-transplant, while the other ALL participant continued to have low SMB cells and total B cell proportions at 7 years post-transplant (0.00% and 0.11%, respectively). All participants that have undergone autologous HSCT had low SMB cell proportions with only one showing recovering total B cell proportions (**Figure 18**).

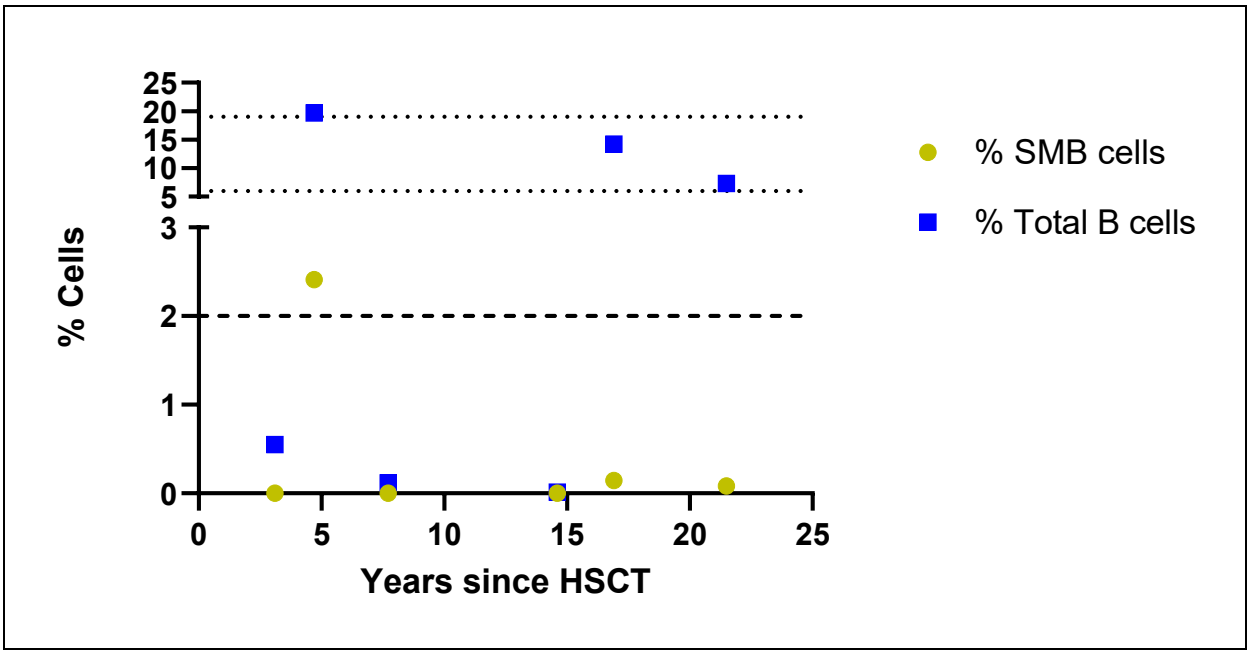

Figure 17: Percentage of total B cells and Switched memory B cells in participants grouped by years since HSCT (n=6). Each dot represents an individual patient, showing % SMB cells and % total B cells plotted against years since HSCT. The dashed horizontal line at 2% denotes the threshold for defining low SMB cell frequency and the dotted lines represent the normal range of total B cells (6-19%).

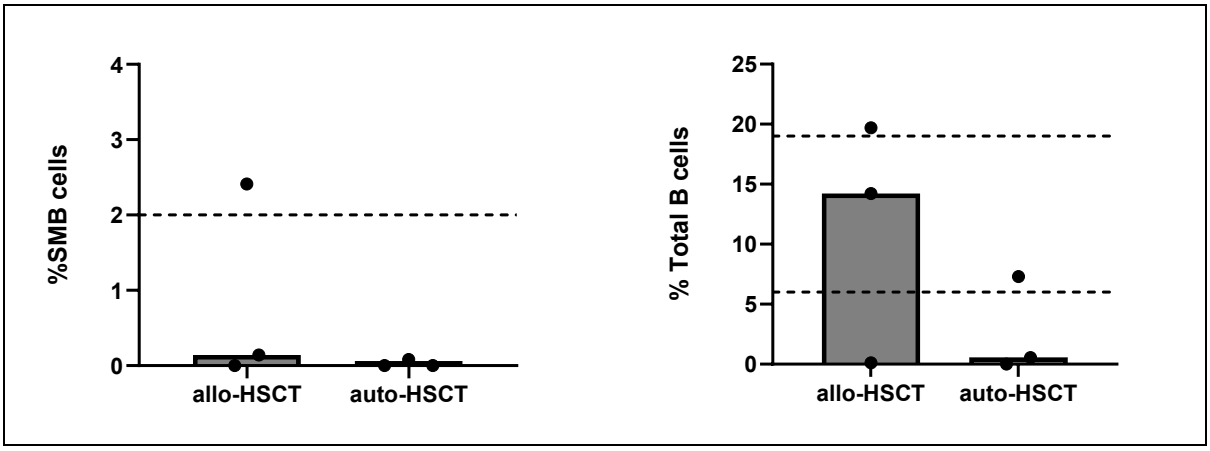

Figure 18: Percentage of total B cells and Switched memory B cells in participants grouped by type of HSCT (n=6). Each dot represents an individual patient, showing % SMB cells and % total B cells plotted against years since HSCT. **A.** Panel shows SMB cell proportions of participants that have undergone allogenic HSCT (allo-HSCT), n=3, and autologous HSCT (auto-HSCT), n=3. The dashed horizontal line at 2% denotes the threshold for normal SMB cell frequency. **B**. Total B cell proportions of participant that have undergone allogenic HSCT (allo-HSCT), n=3, and autologous HSCT (auto-HSCT), n=3. The dashed horizontal lines denote the normal range of total B cells (6-19%).

Rituximab was one of the most used B cell depleting therapies in our study cohort, with 80% of participants having received it as part of their cancer regimen or maintenance treatment. Only 5 participants (25%) showed normal SMB cells percentage while the majority (65%) of participants showed normal total B cells percentage (**Figure 19**). Two-tailed Mann-Whitney t-test showed no significant difference in the time since the last rituximab administration between participants with low and normal % SMB cells (n=20, p= 0.9328) as well as between participants with low and normal % total B cells (n=19, p=0.5789).

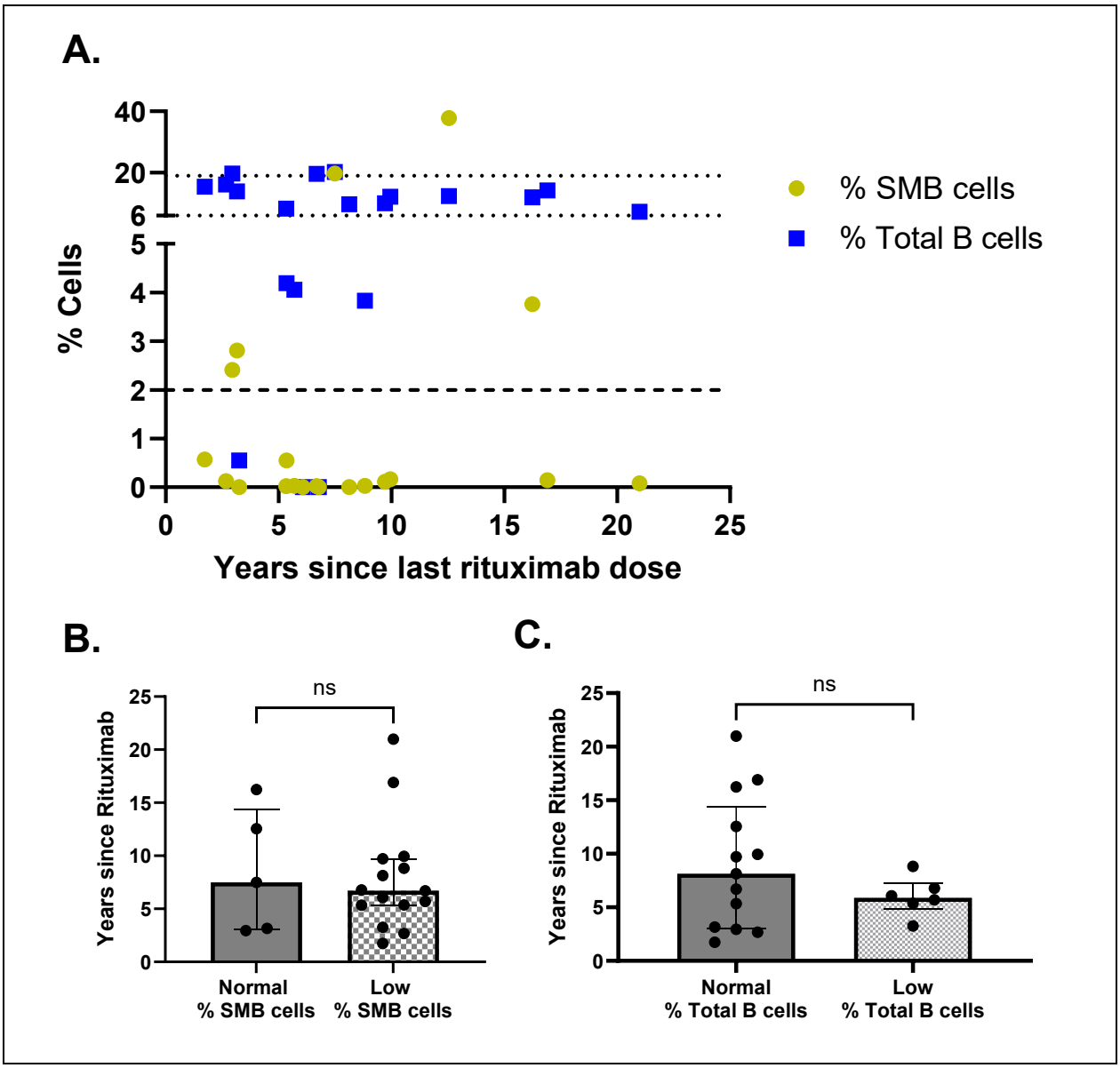

Figure 19: Measured frequencies of total B cells and switched memory B cells in participants grouped by years since last rituximab dose (n=20). A. Each dot represents an individual patient, showing % SMB cells and % total B cells plotted against years since last rituximab dose. The dashed horizontal line at 2% denotes the threshold for defining low SMB cell frequency and dotted lines represent the normal range of total B cells (6-19%). Mann-Whitney test showed no significant difference in the time since the last rituximab administration between participants with low and normal % SMB cells (n=20, p= 0.9328) **(B)** as well as between participants with low and normal % total B cells (n=19, p=0.5789) **(C)**. One participant was excluded from the total B cell analysis because their B cell percentage was greater than the normal range. Error bars represent the IQR.

3.6 B cell subsets and SMB cells in HM-SAD

Several B cell subtypes are known to be involved in autoimmune, inflammatory and malignant diseases, and represent key elements in diagnostic, prognostic and therapeutic assessments. We conducted further analysis to characterize the proportions of other B cell subsets of interest including naïve ($CD19^+$, $CD27^-$, IgD^+), transitional ($CD19^+$, $CD27^-$, $CD38^{++}$, IgM^+), unswitched memory B cells (MBCs) ($CD19^+$, $CD27^+$, IgD^+), double negative (DN) B cells ($CD19^+$, $CD27^-$, IgD^-), and plasmablasts ($CD19^+$, $CD27^{++}$, $CD38^{+++}$, IgM^-). The results are summarized in **Table 5** below.

Table 5: The frequency of other B cell subsets in each disease category

B cell subset	ALL (n=2)	CLL (n=6)	NHL (n=15)	MM (n=2)	Reference Range*
Naïve $CD27^{-}$, IgD^{+}	88.68 (0.91)	9.32 (54.52)	92.92 (11.34)	21.81 (30.84)	74.7 (17.0)
Transitional $CD27^{-}$, $CD38^{++}$, IgM^{+}	22.78 (29.43)	0.19 (1.55)	4.42 (4.71)	0.09 (0.13)	4.7 (2.9)
Unswitched MBCs $CD27^{+}$, IgD^{+}	3.89 (0.67)	11.79 (42.54)	1.61 (2.87)	13.44 (19.01)	11.7 (6.5)
Double Negative $CD27^{-}$, IgD^{+}	1.13 (0.38)	2.09 (10.36)	0.47 (0.92)	27.44 (31.90)	3.2 (2.3)
Plasmablast $CD27^{++}$, $CD38^{+++}$, IgM^{-}	0.18 (0.25)	0.04 (0.13)	0.14 (0.18)	0.00 (0.00)	1.2 (1.0)
IgA+ SMB $CD27^{+}$, IgD^{-}, IgM^{-}, IgA^{+}	0.45 (0.64)	0.001 (0.02)	0.00 (0.00)	0.38 (0.53)	9 (7.0)
IgG+ SMB $CD27^{+}$, IgD^{-}, IgM^{-}, IgG^{+}	0.57 (0.81)	0.82 (2.83)	0.00 (0.12)	8.74 (12.36)	9 (7.0)

*Reference range values are presented as median (IQR)

Studies have shown that naïve and transitional B cells are enriched in peripheral blood within 6 months after completion of chemotherapy and present evidence of the beginnings of immune reconstitution. The median (IQR) of naïve memory B cells was 88.0 (81.1) % of total B cells [normal reference is 74.7 (17) % in healthy adults reported in literature][206] (**Figure 20A**). NHL participants showed the highest naïve proportions from total B cells with median (IQR) 92.92 (11.34) %, followed by ALL participants with 88.68 (0.91) %, MM patients with 21.81 (30.84) % and CLL participants with 9.32 (54.52) %. The 6 participants with the lowest naïve B cell proportions include three who are currently receiving treatment, one CLL participant in observation, and two participants with NHL in remission for 2- and 5-years, respectively.

We measured the transitional B cell compartment in our study cohort and found the median (IQR) to be 1.96 (5.5) % [normal reference range is 4.7 (2.9) % of total B cells][206] (**Figure 20B**). The highest transitional B cell percentage of 43.59% was in an ALL participant that had undergone allo-HSCT 8 years prior. The median (IQR) of transitional B cells in the CLL group is 0.19 (1.55) %, in the NHL group 4.42 (4.71) %, in the MM group is 0.09 (0.13) %, and in the ALL group is 22.78 (29.43) %.

We also evaluated the proportion of unswitched memory B cells (MBCs), also referred to in the literature as non-switched memory, circulating marginal zone, marginal zone-like B cells. Low levels of these cells have been associated with unfavorable inflammatory status in patients with certain autoimmune diseases and common variable immunodeficiency. However, their role in hematological malignancies is not well understood. The median (IQR) for unswitched MBCs was 2.22 (7.89) % of total B cells [normal reference is 11.7 (6.5) %][206] (**Figure 20C**). The median (IQR) of unswitched MBCs was 11.79 (42.54) for CLL participants, 1.61 (2.87) % for NHL, 44 (19.01) % for MM and 3.89 (0.67) % for ALL. Overall, thirty-six percent of participants had low unswitched MBCs and were heterogenous in disease category and status.

Double negative B cells (DN B cells) have been suggested to generate autoimmunity and play an immunosuppressive role in certain cancers, but their role in hematological malignancies also remains to be explored. The median (IQR) of DN B cell proportions in the study population was 1.08 (1.50) % from total B cells [normal reference is 3.2 (2.3) %][206] (**Figure 20D**), with median (IQR) of 2.09 (10.36) % in CLL, 0.47 (0.92) % in NHL, 27.44 (31.90) % in MM, and 1.13 (0.38) % in ALL. CLL and MM participants had the highest proportions of DN B cells, and the two participants with the highest DN B cells are both currently undergoing chemotherapy.

Plasmablasts, which are immature plasma cells that can become malignant in hematological malignancies, were also measured and analyzed. Plasmablasts play an important role in immune response as the precursors of antibody secreting cells and may serve as a surrogate marker for assessing antibody responses in patients with immunodeficiency. The median (IQR) in our study cohort was 0.096 (0.22) % of total B cells [normal reference is 1.2 (1) %][206] (**Figure 20E**). When compared across disease categories, the median (IQR) was 0.04 (0.13) % in CLL, 0.14 (0.18) % in NHL, 0.00 (0.00) % in MM, and 0.18 (0.25) % in ALL.

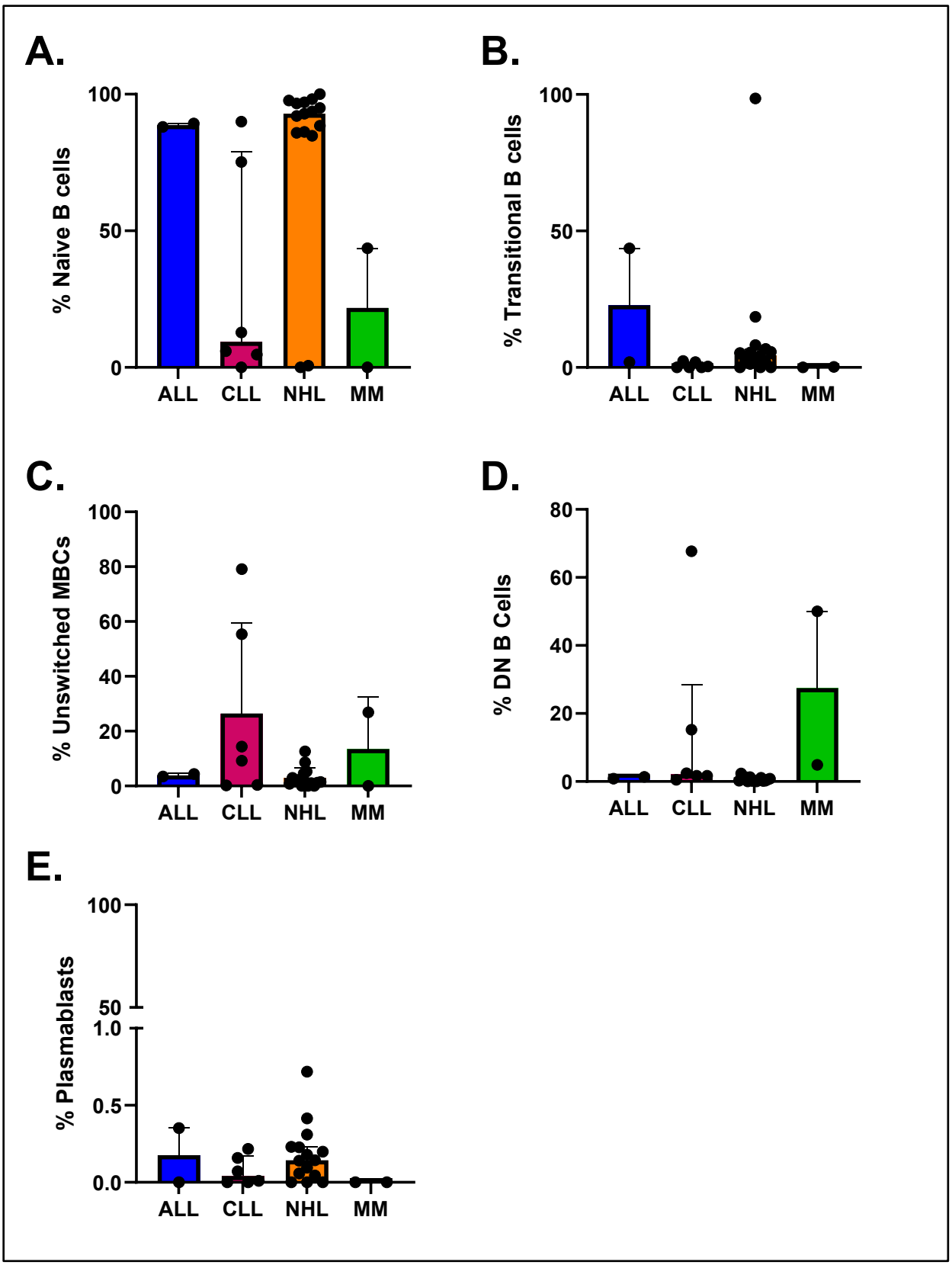
A.
% Naive B cells
100
50
0
ALL
CLL
NHL
MM
B.
% Transitional B cells
100
50
0
ALL
CLL
NHL
MM
C.
% Unswitched MBCs
100
80
60
40
20
0
ALL
CLL
NHL
MM
D.
% DN B Cells
80
60
40
20
0
ALL
CLL
NHL
MM
E.
% Plasmablasts
100
50
1.0
0.5
0.0
ALL
CLL
NHL
MM

Figure 20: Relative frequencies of different B cell subsets measured as a percentage from total B lymphocytes grouped by disease type categories (n=25). **A**. Proportions of Naïve B cell ($CD19^+$, $CD27^-$, IgD^+). **B.** Proportions of transitional ($CD19^+$, $CD27^-$, $CD38^{++}$, IgM^+). **C.** Proportions of unswitched memory B cells (MBCs) ($CD19^+$, $CD27^+$, IgD^+). **D.** Proportions of double negative (DN) B cells ($CD19^+$, $CD27^-$, IgD^-). **E.** Proportions of plasmablasts ($CD19^+$, $CD27^{++}$, $CD38^{+++}$, IgM^-). Error bars represent the IQR.

We also investigated the SMB cells compartment for IgA and IgG isotypes. The median (IQR) of IgG+ SMB cells was 0.01 (0.42) % and of IgA+ SMB cells was 0.00 (0.00) % [reference range for IgG/IgA memory B cells in healthy adults is 9 (7) %] (**Figure 21A-B**). For IgG+ SMB cells, the median (IQR) was 0.82 (2.83) % in CLL, 0.00 (0.12) % in NHL, 8.74 (12.36) % in MM, and 0.57 (0.81) % in ALL. For IgA+ SMB cells, the median (IQR) was 0.001 (0.02) % in CLL, 0.00 (0.00) % in NHL, 0.38 (0.53) % in MM, and 0.45 (0.64) % in ALL.

One CLL participant showed expansion of IgG^+ SMB cells (39.7%) while on Zanubrutinib, a BTK inhibitor that ultimately inhibits the proliferation of malignant B cells. The MM participant with normal % SMB cell has elevated serum lambda light chain and a percentage of IgG^+ SMB cells of 17.5%. Kruskal-Wallis analysis showed no significant difference in IgA^+ SMB cells or IgG+ SMB cell proportions among the 4 disease groups ($p= 0.1672$ and $p= 0.2337$, respectively).

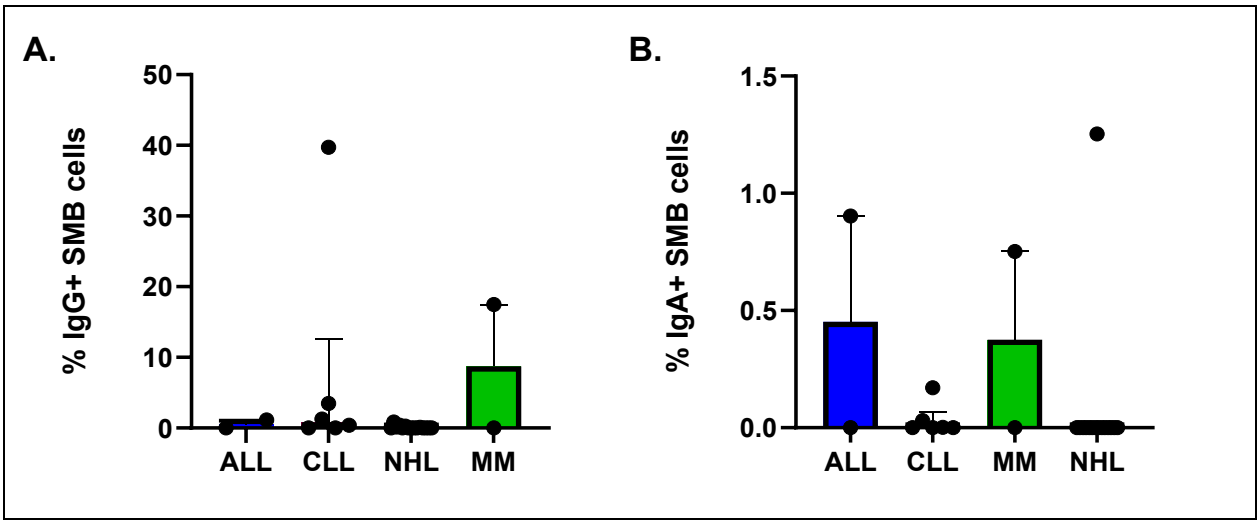

Figure 21: Frequency of IgG$^+$ and IgA$^+$ switched memory B (SMB) cells in patients with hematologic malignancies. A. Percentage of IgG$^+$ SMB cells from total B cells and **B.** Percentage of IgA$^+$ SMB cells from total B cells in patients with acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), multiple myeloma (MM), and non-Hodgkin lymphoma (NHL). Data are presented as median with individual data points shown. Error bars representing interquartile range. Kruskal-Wallis analysis showed no significant difference in IgA$^+$ SMB cells or IgG$^+$ SMB cell proportions among the 4 disease groups (p= 0.1672 and p= 0.2337, respectively).

Spearman *r* correlation analysis was conducted to evaluate associations between SMB cells and other B cells subsets as well as within the other B cells subsets (**Figure 22**). SMB cells showed strong positive correlation with IgG$^+$ SMB cells (r= 0.827, p<0.001) and moderate positive correlation with IgA$^+$ SMB cells (r= 0.630, p= 0.001). Furthermore, IgG$^+$ SMB cells had weak positive correlation with serum immunoglobulins, including IgG (r= 0.268, p= 0.195) while IgA$^+$ SMB cells had a moderate positive correlation with serum IgG levels (r= 0.489, p= 0.013) and a strong positive correlation with serum IgA levels (r= 0.698 p< 0.001). Naïve and transitional B cells had a moderately positive correlation (r= 0.660, p< 0.001). DN B cells had a moderately positive correlation with total SMB cells (r= 0.677, p< 0.001), IgG$^+$ SMB cells (r= 0.637, p= 0.001) and IgA$^+$ SMB (r=0.524, p= 0.007), and a moderately negative correlation with naïve and transitional B cells (r= -0.637, p= 0.001 and r= -0.421, p= 0.036). Finally, plasmablast

proportions showed moderate positive correlation with naïve and transitional B cells (r= 0.449, p= 0.011 and r= 0.527, p= 0.007).

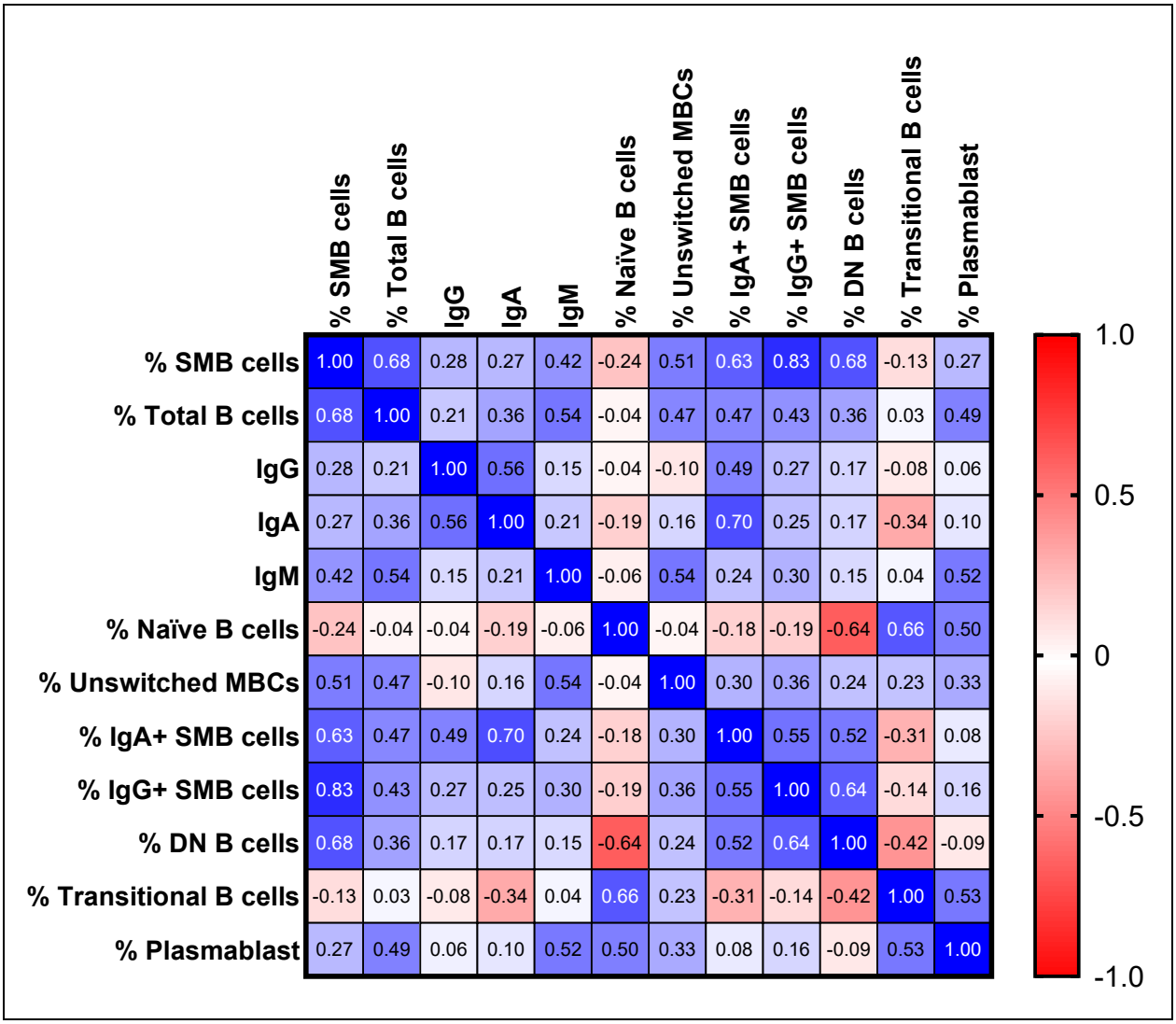

Figure 22: Heatmap of spearman *r* correlations of different B cell subsets proportions (n=25). Pairwise spearman correlation coefficients (*r*) are shown between the percentage of switched memory B (SMB) cells, total B cells, serum immunoglobulins (IgG, IgA, IgM), naïve B cells, unswitched memory B cells (MBCs), IgA+ SMB cells, IgG+ SMB cells, double negative (DN) B cells, transitional B cells, and plasmablast. The strength of correlation is indicated by both color intensity (blue = positive, red = negative) and the numeric value in each cell. A color scale bar from -1 to 1 is included to reflect correlation magnitude and direction.

CHAPTER 4: DISCUSSION

4.1 Summary of study findings

IGRT is the mainstay treatment for SAD and has been shown to effectively reduce risk of infection and related morbidity and mortality. The lack of a standardized evidence-based algorithm for safe IGRT discontinuation can lead to prolonged and potentially unnecessary use of IGRT. This underscores the need for a reliable biomarker to assess humoral immune recovery in patients with HM-SAD to support the development of a standardized IGRT discontinuation protocol. We proposed SMB cells as a promising biomarker because they are unlikely to be affected by IGRT and retain the capacity to differentiate into antibody-secreting plasma cells in response to recurrent infections. To our knowledge, SMB cells have not been well characterized in patients with HM-SAD, and this study will be the first to evaluate the feasibility of using SMB cells as a biomarker of immune recovery.

In this study, we quantified SMB cell proportions in 25 patients with HM. At baseline, our results showed that 68% of participants had low SMB cell but only 44% had low total B cell proportions. Low SMB cell frequencies were observed despite longer times since disease remission while total B cells frequencies progressed towards recovery. Infection history was not correlated to either measure at baseline or at follow-up. We also observed that frequencies of SMB cells and total B cells appear to remain stable by the 12-month follow-up. Overall, we did not identify other factors associated with low SMBs cells, with no significant differences observed based on age, sex, length of time on IGRT and disease categories. However, our data showed low % SMB cell in 83.3% and 75% of participants with history of HSCT and rituximab, respectively, regardless of time elapsed since treatment. Finally, our exploratory analysis of other B cell subsets showed a strong positive correlation between SMB cells with IgG^+ SMB cells and

moderate positive correlation with IgA^+ SMB cells. Additionally, IgG^+ SMB cells had weak positive correlation with serum immunoglobulins while IgA^+ SMB cells had a moderate positive correlation with serum IgG levels and a strong positive correlation with serum IgA levels.

Overall, this work suggests the presence of persistent immune impairment resulting in prolonged reduction of SMB cell proportions in HM-SAD patients on IGRT, despite evidence of total B cell recovery and extended duration since disease remission. Further investigation will be required to carefully evaluate the use of SMB proportions as a marker of immune recovery or perhaps a marker of ongoing immune deficiency.

4.2 Interpretation of findings

4.2.1 Characterization of SMB cells in the HM-SAD

In this study, we characterized the SMB cell frequencies in 25 participants with HM-SAD. At baseline, we found that SMB cell proportions were normal (≥2%) in only a third of the participants while the total B cell proportions appear to be within the normal range for just over half of the participants. Only one participant out of those with normal SMB cell proportion also had a percentage of total B cells below the normal range. Furthermore, both total B cells and SMB cell proportions appeared to remain stable between baseline and the second follow-ups (9-12 months). It is possible that longer follow-up duration is necessary to detect significant changes in SMB cell proportions, and to evaluate if SMB cell recovery in our study cohort is even possible. Our data suggests that SMB cell recovery is lagging compared to the overall B cell compartment recovery. The exact mechanism of the delayed or the lack of SMB cell recovery remains to be investigated and is likely multifactorial.

SMB cells are essential to the production antibodies that protect against various infections with encapsulated bacteria, which are commonly seen in patients with HM-SAD. Our

data showed no clear association between low SMB cell proportions and moderate or severe infection rate. Approximately 20% of our participants experienced severe infections during the 12 months prior to enrollment despite being on IGRT for at least 1 year. Three of these participants were still receiving treatment for their HM, while the other two have been in disease remission for 5 and 7 years, respectively. These results suggest that the SMB cell proportions alone may not predict the risk of moderate or severe infections in HM-SAD patients on IGRT. Additionally, from an alternative perspective, future analysis may also investigate the association of previous infections with specific pathogens, with SMB cell proportions. For example, CMV has been shown to impact the memory B cell compartment and consequently the specific antibody responses.[207]

4.2.2 Demographic characteristics and SMB cells

SMB cell proportions have been reported to increase with age and then decrease in the elderly.[204] We observed an increasing trend of SMB cell proportions with age but there was no significant difference detected between proportions in participants under 65 and those over 65 years of age. Given that the hematological malignancies in this study can impact SMB cell proportions and are themselves associated with age (as reflected by the younger age of the ALL group compared to other disease categories), it is difficult to isolate the effect of age alone on the SMB cell proportions in our patient cohort. Furthermore, sex differences between males and females' immune response have been well documented.[208] Peckham et al. demonstrated that the levels of SMB cells (defined as $CD19^{+}CD27^{+}IgD^{-}$) are higher in cis-females (n=19) compared to cis-males (n=11) within the 20-40 year old age group.[205] However, they found no significant sex difference between older cis-males (n=10) and age-matched post-menopausal cis-females (n=15). In our study, we did not detect any significant differences in both total B cell and SMB

cell proportions between females and males regardless of age groups. It is possible that these results are limited by the overall sample size per group (males n=9, females n=16) where only 16% of our participants were below the age of 45, and that the potential effects of age and sex are overruled by the effects of the underlying malignancies and their associated treatments.

4.2.3 Clinical characteristics, other B cell subsets and SMB cells

We then evaluated the associations between the measured SMB cell proportions in our study cohort with clinical factors such as disease categories, disease status, levels of serum immunoglobulins and history of cancer therapies.

Across disease categories, the CLL group had the highest number of participants with normal SMB cell proportions and some of the highest values of SMB cells overall. This was a stark contrast to the ubiquitous SMB cell paucity in the NHL group. While low SMB cell frequencies in the NHL group are likely due to cancer treatments based on previously reported data, one possible explanation for high SMB cell proportions seen in the CLL group is the inclusion of CLL malignant B cells within the SMB cell gating strategy in the absence of CLL markers. CLL can be categorized into 2 subtypes based on their IGVH: unmutated form originating from immature B cells subtypes, and mutated form originating from memory B cells that have undergone germinal center reactions. CLL B cells have been shown to mainly express IgM and IgD. However, in a rare subtype of the disease, both IGVH mutated and unmutated CLL B cells were capable of isotype switching to produce IgA and IgG.[209] Investigating the IGVH status, exploring the clonal diversity and measuring the proportion of CLL B cell fraction, by the addition of other markers such as CD5, CD20 and CD23 into the flow panel, may provide valuable insight on the feasibility of using SMB cells, as defined in this study, as a biomarker for immune recovery in CLL patients.[210–212]

No trends in SMB cell proportions were identified in the MM and ALL disease categories. Limited data is available in the literature on SMB cells in ALL patients, and it is often in the context of B cell immune reconstitution following cancer treatments. Both ALL participants in our study have undergone HSCT which may provide an explanation for their SMB cell proportions. Similarly, both MM participants in our study cohort were on chemotherapy at enrollment and had low total B cell proportions. However, the MM participant with the normal SMB cell and low total B cell proportions had stopped chemotherapy shortly after enrollment and was declared to be in remission soon after. The literature on SMB cells in MM is often nuanced by the selected patients' disease and treatment characteristics. Vsaianska et al. found higher levels of switched memory B cells in a cohort of 38 MM patients compared to healthy controls.[213] Another study showed the MM patients had reduced total B cells numbers primarily due to reduced memory B cells; although the ratio of switched to unswitched memory B cell was unaltered.[214] However; no direct comparisons were done with the presence and absence of SAD and further research in this area is warranted. Finally, markers such as CD38 can be used to further investigate if the SMB cell gating may include malignant multiple myeloma cells.

It has been suggested that low SMB cell levels may be a result of germinal center defects resulting in impaired ability to undergo somatic hypermutation and class switching recombination. However, the presence of normal SMB cell levels with low serum immunoglobulin levels points to a possible defect in the post-germinal center processes including SMB cell differentiation into antibody-producing plasma cells.

We then evaluated the association between serum IgA, IgG and IgM titers, which play a key role in anti-infectious immunity, and SMB cell proportions. All participants in this study

were on IGRT at the time of enrollment; therefore, our assessment of serum IgG titers in this study is limited due to the exogenous supplementation of IgG. Nearly all participants had IgG titers within the normal range and were likely controlled by IGRT dosage. Furthermore, the length of time on IGRT had no significant correlation with SMB cell proportions, however, this cannot conclusively be interpreted as a lack of interaction between IGRT and SMB cell proportions. Future studies analyzing SMB cell proportions before and after IGRT exposure will help evaluate their potential interactions.

We observed pervasively low IgA titers across all groups, while IgM levels varied. Interestingly, only serum IgM titers had a positive moderate correlation with SMB cell proportions while serum IgG and IgA had a weak positive correlation with SMB cell proportions. Considering that IgA and IgG production is dependent on the ability of SMB cells to differentiate efficiently into functional antibody-secreting plasma cells, we would have expected that IgA and IgG to have the stronger positive correlation with SMB cell proportions.[215,216] A recent study by Knight et al. demonstrated that patients with low SMB cells, regardless of diagnosis, are more likely to have low serum IgA and IgG, particularly if they have prolonged rather than transient reduction of SMB cell proportions.[217] Similar to our findings, they also observed variability in IgM levels in patients with both transient and persistent low SMB cell levels. These findings suggest the possible need for a longer follow-up period to confirm if the low SMB cell proportions observed in our study population are transient or prolonged. Given that the ultimate goal is to use this measure in a clinical setting where frequent monitoring of SMB cell proportions is likely not feasible, defining the appropriate follow-up period is of practical importance.

Low IgA is linked to increased risk of sinopulmonary and gastrointestinal infections and were found to best predict the rate of infections in CLL.[218] On the other hand , low IgM is frequently observed in MM due to immunoparesis; this was also observed in the MM patients in our study.[61,219,220] Furthermore, patients with Waldenström macroglobulinemia, a type of slow growing NHL, produce large quantities of IgM.[221] Therefore, it is proposed that IgM and IgA monitoring can be beneficial in stratifying patients at higher risk of infections despite receiving IGRT but further studies are needed to determine their predictive utility in HM-SAD alongside SMB cell proportions.[48]

Our next step was to analyze the relationship between the common cancer therapies in our study cohort with SMB cell proportions. Cancer therapies have a significant impact on the B cell compartment and shape the path to B cell recovery following treatment. All but one CLL participant in our study cohort had received treatment for their HM and 20 participants had disease in remission at enrollment. Their treatments included a variety of regimens, many of which are known to directly or indirectly cause B cell depletion, with some patients receiving multiple lines of treatments. Interestingly, low SMB cell proportions persisted despite longer times since disease remission (median of 7.5 years) with only 20% of participants showing normal values. The literature has generally reported B cell reconstitution within 2-5 years of disease remission following commonly used chemotherapies and contrast with our observations given our recorded longer remission times. Two of the key therapies we were interested in evaluating were rituximab, due to its wide-spread use, and HSCT due to its potential curative effects.

Eighty percent of our study population had the chimeric anti-CD20 monoclonal antibody treatment, rituximab, as part of their cancer treatment including maintenance dosing. Rituximab

can cause profound B cell depletion since CD20 is expressed on a wide range of B cell subsets including pre-B cells to mature B cells such as memory B cells, but not on pro-B cells.[222] We found that 75% of patients treated with rituximab showed prolonged reduction in SMB cell proportions (<2%) of whom 80% were >5 years post-treatment. The incidence of hypogammaglobulinemia following rituximab differs per disease type and was reported in 13-56% of patients.[223] It is important to note that participants in our study had received a variety of different chemotherapy treatments, many having also received multiple lines of treatment, such as CHOP (40%), DHAP (10%), CVP (30%), CEOP (5%), FCR (25%), GDP (10%), and BR (30%), in addition to HSCT and CAR-T therapy.

Although prolonged B cell depletion after rituximab treatment may be considered rare, this "persistent B cell depletion" phenomenon, as described by Efe et al.[224] appears to be common in our study participants. Only a few studies have reported this effect in diseases such as autoantibody (ANCA)-associated vasculitis and systemic lupus erythematosus, and are usually presented in limited case report series.[224–226]Another study by Irie et al, showed prolonged hypogammaglobulinemia with undetectable IgA and IgM levels and SMB cell levels below 0.5% in a patient with follicular lymphoma 6 years after treatment with rituximab.[203] It has been suggested that B cell recovery post-rituximab depends on early-stage B cell lymphopoiesis, which leads us to speculate the present of defects in these early stages as a result of cancer combination therapies or an underlying primary immunological disorder.[203,227]

HSCT requires profound immune depletion prior to transplant. However, B cell reconstitution following HSCT has not been well studied and is often reported to take up to 2 years or longer.[172,228] SMB cells were reported to appear within 3 months after HSCT but at a lower-than-normal levels and longitudinal data regarding their recovery to normal levels are very

limited.[229] We found low SMB cell proportions in five of the six participants in this study who underwent HSCT, regardless of time passed since the transplant.

Only one participant (with ALL) had normal SMB cell proportion; this patient received CVP and rituximab following the allo-HSCT transplant. The other ALL participant was diagnosed with T cell ALL and underwent CY/TBI/ATG conditioning treatment prior to the allo-HSCT without the use of rituximab. This patient also was treated for various infections including EBV viremia. The CY/TBI/ATG protocol has been reported to cause profound immunosuppression affecting both B and T cells. A study by Roll et al. found that patients who had ATG in the conditioning regime prior to HSCT had a delay in $CD27^+$ memory B cell reconstitution, including both switched and unswitched populations, that lasted beyond 1-year post-transplant.[230] Similarly, the use of TBI has been associated with reduction in both naïve and SMB cells for up to 2 years.[172] It has been suggested that that long-lasting B and T cell reconstitution defects continue to exist well beyond the reported 2 years as evident by a 30-fold increase in the risk of invasive pneumococcal infections after 10 years post-transplant.[231] A study published in 2009 showed that allo-HSCT recipients were SMB cell deficient irrespective of their time post-transplant but only defined the range of time as more than or less than 1-year post-transplant. They also showed that the SMB cell proportions correlated positively with CD4+ T cells suggesting impaired GC reactions involved in isotype switching.[232]

Similar delays in memory B cell compartment recovery have been documented in patients who underwent auto-HSCT, however; they generally normalized at 12 months post-transplant.[233] Interestingly, in our study cohort, 3 participants underwent auto-HSCT, all of which had low SMB cells and only one has shown recovery of total B cells (at 10 years post-

transplant). Analysis of this data is complicated by disease relapse in 2/3 participants post-transplant and further treatments with chemotherapy.

The heterogeneity of the chemotherapy regimens in this patient cohort made it exceptionally difficult to observe trends in relation to SMB cell proportions recovery. When analyzing the chemotherapies of patients who have normal SMB cell proportions (n=8), we noted that only one participant received R-CHOP and five previously had rituximab as part of their cancer treatment. Interestingly, participants currently on treatments such as Acalabrutinib and Zanubrutinib, and previously treated with FCR, also had normal SMB cell proportions although fludarabine is known to cause B and T cell depletion.[234,235] Furthermore, all participants who received BR were NHL patients and had SMB cell proportions below normal at remission (range 2-10 years) despite 67% of them showing total B cell recovery. A study by Suzuki et al reported persistent hypogammaglobulinemia at the 3-year follow-up in NHL patients who received BR.[236] Furthermore, serum IgA titers were below normal and only 2 participants showed normal IgM titers. In contrast to our finding, Kater et al. observed serum IgA, IgM and IgG return to above baseline levels at 24 months post BR treated CLL patients with low post-treatment infection rates.[237] These findings suggest that perhaps predicting immune reconstitution and recovery in patients with HM-SAD may require a more personalized approach stratified by treatment type among other factors such as disease category.

Our results so far have shown significant and persistent depletion of SMB cells in our cohort of patients with HM-SAD and are suggestive of possibly persistent antibody deficiency. Examining the differences in B cell subsets may present clues for the mechanism of antibody deficiency that warrants deeper investigation.[36] In our cohort, MM and CLL patients had the lowest naïve and transitional B cell proportions from total B cells, while NHL participants had

the lowest unswitched memory B cells. It has been noted that a B cell compartment reconstitution dominated by immature phenotype can be indicative of defective maturation pathways resulting in reduced memory B cell numbers.[161] Indeed, the NHL participant had the highest percentage of naïve B cells and within normal range transitional B cells, yet their switched memory, unswitched memory, and DN B cell proportions were below the normal reference range. All NHL participants have been previously treated with rituximab, 53% have received CHOP, 20% had HSCT, 20% had CAR-T and all have been in remission for > 12 months. Despite the heterogeneity within this group, the results suggest an ongoing immune dysfunction preventing B cell differentiation from naïve B cells to more mature subtypes and subsequent inability to undergo somatic hypermutation and class switching.[229] On the other hand, MM patients in our cohort had an elevated DN B cell proportion, however; both MM participants had very low total B cells. Studies suggest that DN B cell accumulation in the elderly are by-products of persistent stimulation and defective germinal center formation, which may be part of the mechanisms at play in our MM patients.[238,239]

Furthermore, both IgG+ and IgA+ SMB cells were below normal, possibly reflecting the overall low SMB cell frequencies measured in the study cohort. This was also apparent in the correlation analysis which showed a high and moderate positive correlation between overall SMB cells and IgG+ SMB and IgA+ SMB cells, respectively. The weak positive correlation between IgG^+ SMB cells and serum IgG is confounded by the ubiquitous low proportion of IgG^+ SMB cells in all disease categories apart from MM. While considering the potential GC defects leading to low SMB cell and thereby low IgG^+ SMB cell proportions, this finding can also suggest a potential impediment in the proliferation and differentiation of the present IgG^+ SMB cells and subsequent impaired production of endogenous IgG. However, it is also important to

consider that IgG^+ SMB cells are the precursors for the long-lived plasma cells that maintain serum IgG levels and mediate the rapid secondary response to infections. The proportion of IgG^+ SMB cells may not be a direct reflection of the serum concentration of IgG antibodies, but instead a measure of the immune system's capacity for future rapid production of IgG.[232,240] Furthermore, assessing the correlation of IgG^+ SMB cells with serum IgG after stopping IGRT can be helpful to tease out the effects of exogenous IgG supplementation. Meanwhile, the strong positive correlation between IgA^+ SMB cells and serum IgA suggests that the ubiquitously low serum IgA levels across our study cohort may be a result of the mechanisms leading to reduction of SMB cells or a defect in downstream IgA^+ SMB differentiation into IgA producing plasma cells.

The general positive correlation we observed between unswitched MBCs, and SMB cells, DN B cells and plasmablasts is somewhat anticipated as they arise from germinal center reactions and share common clonal lineages. However, despite their memory-like features such as class switching and antibody selection, the connection between DN B cells and switched memory B cells remains elusive.[241] We also observed an overall negative correlation between naïve B cells and switched memory B cells (IgG^+ and IgA^+) which has been observed in healthy adult controls.[242] Overall, the analysis of these B cell subsets in relation to SMB cell reconstitution did not elucidate any particular defects.

An important consideration to note is that participants who develop HM may have underlying primary immune disorders which may not be as easily identifiable and cannot be reversed as opposed to SAD. It has been suggested that the absence of immune reconstitution after therapy may be an indicator of an underlying PID.[243] Genetic testing as well as immunophenotyping and serology testing prior to delivering cancer treatment can be useful to

help identify the presence of PID in patients with HM. The identification of these patients, with hypogammaglobulinemia and persistent and recurrent infections due to underlying PID, should be considered in future studies because it may confirm the indication for life-long use of IGRT.

Finally, this thesis demonstrated the feasibility of conducting the proposed study by successfully optimizing and validating laboratory protocols, including sample preparation and flow cytometry techniques, to ensure accurate and reliable measurements. Feasibility was further supported by effective recruitment and retention of participants in this prospective study, achieved through strategies designed to reduce participant burden and foster collaboration. Findings from the acceptability questionnaire also underscored the importance of patient–physician trust and shared decision-making, particularly in clinical studies involving potential modifications to immunoglobulin replacement therapy.

4.3 Study strengths and limitations

The current literature on SMB cells in patients with hematologic malignancy–associated secondary antibody deficiency is limited. While SMB cells have been extensively studied in primary immunodeficiencies, to our knowledge, the SMB study is the first to specifically characterize SMB cell proportions in patients with HM-SAD, with the goal of evaluating their potential as markers of humoral immune reconstitution and guide safe IGRT discontinuation. The study cohort encompasses a diverse range of lymphoproliferative disorders, including patients in remission as well as those undergoing active treatment or surveillance. By employing broad eligibility criteria, the study can recruit from a larger patient population and generate a more general characterization of SMB cells in patients with hematologic malignancies. This design also enables granular subgroup analyses that may yield clinically actionable insights.

Ultimately, this SMB study seeks to provide clinicians with a reliable and measurable tool to support informed decision-making in clinical practice.

A key advantage of this prospective study design is the ability to evaluate changes in real time, in contrast to retrospective approaches. This design reduces the likelihood of missing data, while the short follow-up intervals (every 3–6 months) enable direct collection of participant-reported information on evolving health status and infection history, thereby minimizing recall bias. While most analyses presented in this thesis reflect the baseline data, the study design will allow for longitudinal monitoring of switched memory B-cell proportions over extended follow-up (>12 months) in a larger cohort. This will be valuable to address the gaps in the current literature on prolonged SMB cells depletion and immune recovery in HM-SAD.

Lastly, conducting this study through the Immunoglobulin Treatment Clinic at TOH, one of the largest immunodeficiency clinics caring for patients with SAD on IGRT in Canada, is a major advantage supporting the feasibility of recruiting enough participants to provide the necessary power for statistical analysis later.

Despite the strengths of the study, several limitations should be considered when interpreting the data presented in this thesis. First, the relatively small sample size (n=25) combined with heterogeneity in underlying diseases and prior treatments, limits the robustness and generalizability of the findings. Accordingly, the results presented here should be interpreted as exploratory and hypothesis-generating and, at most, suggestive of emerging trends. Second, future collaborations with other sites may help reduce the potential for participant selection bias, providing a more representative cohort.

Although the use of a flexible inclusion criterion enhanced feasibility and recruitment, it also introduced substantial heterogeneity related to hematologic malignancies and prior cancer

treatments. This heterogeneity introduced complex confounding variables that limit the ability to draw granular, disease-specific conclusions. Nevertheless, such diversity may enable higher-level stratification of patients with HM-SAD, which could be informative for future analyses. Additionally, grouping patients into four disease categories in the analysis may obscure important intra-group heterogeneity. At this level of analysis, it is therefore unlikely that reduced SMB cell proportions can be attributed solely to disease processes.

Another limitation is related inherently to the use of flow cytometry for B cell characterization. While flow cytometry plays a central role in the diagnosis, prognosis, and therapeutic targeting of hematological disorders, there is limited consensus in the literature regarding B-cell subset classification.[244] Different studies employ varying combinations of surface markers to define the same B-cell populations, complicating direct comparisons with existing literature. Furthermore, accurately identifying certain populations, particularly rare subsets or those with overlapping phenotypic markers, can be challenging.[245] In this study, the markers used to define naïve B cells overlap with those expressed by transitional B cells. To address this, CD38 and IgM were used to identify transitional B cells separately; however, additional markers such as CD10 and CD24 could further improve discrimination between these subsets in future studies.

4.4 Future directions

The inclusion of a control group consisting of patients with HM in remission without SAD and not on IGRT, will be useful for comparing the B cell compartments with the HM-SAD patients to identify possible clinically significant differences. Since the goal of the SMB study is to evaluate the utility of SMB cells as a biomarker for safe IGRT discontinuation, the study will continue to recruit participants and carry out the SMB cell characterization efforts in a larger

cohort. The study will utilize the measured SMB proportions as a part of a broader IGRT discontinuation screening criteria, which considers the patient's recent medical and infectious history along with other clinical measures, to determine if participants may stop IGRT during the study. This will be followed by analysis of IGRT discontinuation rate and IGRT discontinuation success rate. Based on the results obtained, a receiver operating curve (ROC) of SMB cells cut-off will be estimated as a predictor of successful IGRT discontinuation.

To determine the sample size required to adequately power this study, we considered that a retrospective study done at The Ottawa Hospital found that only 9% of the 133 patients with hematological malignancies who were receiving IGRT from 2015 to 2019 discontinued treatment during the study period.[164] The majority (75%) of patients who stopped IGRT were successful and remained free of infection. Therefore, using SMB cell criteria, we expect to yield a success rate of 90% (better than 75%) with 45% of patients stopping IGRT (based on study reporting 57% of NHL patients having recovered immunodeficiency)[190], 98 participants will be needed to yield 54 patients who discontinue IGRT and provide 80% power to demonstrate superiority to the 75% success rate.

Additionally, to reduce the participant heterogeneity and confounding variables associated with disease category, future studies should consider limiting their recruitment to a more closely related study population. For example, in our study cohort, all of the NHL participants were in remission and have received rituximab as part of their cancer treatment. The prevalence and duration of SAD in NHL patients is not well documented in the literature, therefore; this would provide a significant contribution to the data on this participant population. Another way to reduce heterogeneity would be categorizing and grouping patients according to the cancer therapies they received, such as traditional chemotherapy versus targeted treatments.

Beyond immunophenotyping, future studies should incorporate functional analyses of B cells from participants with normal SMB cell proportions to assess their capacity for differentiation and functional immunoglobulin production following appropriate stimulation. In participants with reduced SMB cell proportions, defects in the proliferation and differentiation of earlier B-cell subsets, including naïve and transitional B cells, warrant further investigation. Evaluation of antigen specific antibody response to immunization against common pathogens could be useful to assess recovery in patients receiving IGRT.[246] Furthermore, analysis of the B-cell receptor repertoire could elucidate clonal diversity and functional competence within the SMB compartment.[247,248]

Future studies should also examine the T cell compartment, particularly the CD4+ helper T cells, which may be persistently depleted post-treatment with purine analogues such as fludarabine.[249] A subset of CD4+ T cells called T follicular helper cells (Tfh) help direct B cell activation, proliferation and differentiation resulting in highly specific and long-lived antibody response. These interactions can occur at the T-B borders inside B cell follicles and GC, where we speculate the presence of GC defects resulting in impairment of processes such as somatic hypermutation and class switching. This may also potentially explain the intact presence of serum IgM in most of the study cohort. Furthermore, cumulative exposure to chemo- and immunotherapies, as well as the number of prior lines of treatment, should be considered in future analyses given their known impact on immune reconstitution.

4.5 Conclusion

In summary, this study successfully pilot-tested the feasibility of conducting a prospective clinical trial by optimizing laboratory methods for SMB cell quantification and implementing mitigation strategies that resulted in 100% participant retention during the first 12

months of enrollment. The findings reveal a prolonged depletion of SMB cells in patients with HM-SAD, persisting long after disease remission and despite apparent reconstitution of total B-cell counts—a phenomenon that has been rarely reported in the literature. To our knowledge, this is the first prospective study to characterize SMB cell reconstitution in a large, heterogeneous cohort of patients with HM-SAD receiving IGRT. These results provide novel insight into the B-cell immune reconstitution landscape in HM-SAD and lay the groundwork for identifying SMB cells as a biomarker of humoral immune recovery, representing an important step toward the development of a standardized, evidence-based algorithm for safe IGRT discontinuation.

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