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Engineering B cells for use in immunotherapy and cancer

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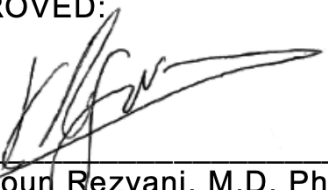
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ENGINEERING B CELLS FOR USE IN IMMUNOTHERAPY AND CANCER


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ENGINEERING B CELLS FOR USE IN IMMUNOTHERAPY AND CANCER

A

THESIS

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MD Anderson Cancer Center UTHealth

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for the Degree of

MASTER OF SCIENCE

by

Emily Louise Ensley, B.S.
Houston, Texas

December, 2020

Dedication

I would like to dedicate this thesis to my parents, Paul and Salli Ensley,
for their continuous love and support.

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I would like to thank my mentor Dr. Katy Rezvani for welcoming me into her lab and supplying me with endless support, guidance, and inspiration. She has helped me grow as a scientist by always pushing me to think critically and logically. I would also like to thank each member of my advisory committee, Dr. Vahid Afshar-Kharghan, Dr. Eric Richard Davis, Dr. Vidya Gopalakrishnan, and Dr. Michael Green for offering insightful suggestions and asking important questions that have helped guide my studies immensely. I would like to thank Dr. Rafet Basar for helping me learn how to design, conduct and manage studies. His support and encouragement has helped me become a better researcher. I would also like to thank Dr. Luis Muniz Feliciano, Dr. Pinaki Banerjee, and all other members of the Rezvani lab for the endless discussions and for allowing me to learn from them in so many valuable ways.

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ENGINEERING B CELLS FOR USE IN IMMUNOTHERAPY AND CANCER

Emily Louise Ensley, B.S.

Advisory professor: Katayoun Rezvani, M.D., Ph.D.

B cells can be divided into effector and regulatory immune cells. While effector B cells are key drivers of humoral immunity due to their ability to generate antibodies specific to pathogens, regulatory B cells (Bregs) have recently been shown to control inflammatory responses in multiple diseases through the production of anti-inflammatory cytokines, including interleukin (IL)-10, IL-35 and transforming growth factor-beta (TGF- β). *Ex vivo* expansion of B cells has been challenging due to their tendency to undergo apoptosis in culture. Thus, creating a successful expansion protocol with exceptional viability will open the door for B cells to be used directly for adoptive therapy or as a source of antibody production *ex vivo*. Furthermore, a strategy to preferentially expand Bregs may be an attractive approach to mitigate autoimmune disorders and graft-versus-host disease. The primary goal of this thesis project is to understand factors that skew B cell function and to develop a protocol for the expansion of Bregs with immunomodulatory properties for cell therapy. We hypothesized that using genetic engineering tools we can skew the function of B cells toward a suppressive phenotype and support their *ex vivo* proliferation and survival to generate a viable subset of Bregs for cell therapy. We successfully generated B cells that displayed suppressive capabilities and used mass cytometry to characterize their phenotype. However, the immunoregulatory function of B cells was transient. Interrogation of the expanded B cells at the single cell level revealed multiple markers

that could potentially be modified to maintain the suppressive capacity of the in vitro expanded B cells for future cell therapy application.

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1. Introduction

1.1 B cell development

B cells are part of the adaptive immune system. They develop in the bone marrow from hematopoietic stem cells (HSCs). HSCs develop into multipotent progenitors that further commit into cells of either the myeloid or lymphoid lineage. The common lymphoid progenitor cells have the ability to differentiate into B cells, T cells or natural killer cells. Once a cell has committed to the B cell lineage by expressing the transcription factor E2A, its development is characterized by the status of immunoglobulin heavy and light chain gene rearrangements that make up its B cell receptor (BCR) (**Figure 1**). BCRs are membrane-bound antibodies that are responsible for the antigen specificity of B cells. The process of creating a unique BCR requires recombining segments of the immunoglobulin heavy and light chains in a particular order, also known as VDJ recombination. This portion of B cell development gives rise to a virtually endless possibility of gene combinations that can be used to form BCRs. While each B cell generates a BCR that can only recognize a single antigen, the population of B cells as a whole is able to recognize a wide repertoire of antigens. This diversity in BCRs contribute to the adaptive immune response (Dorshkind 2018, Proverb 2014, Murphy 2017).

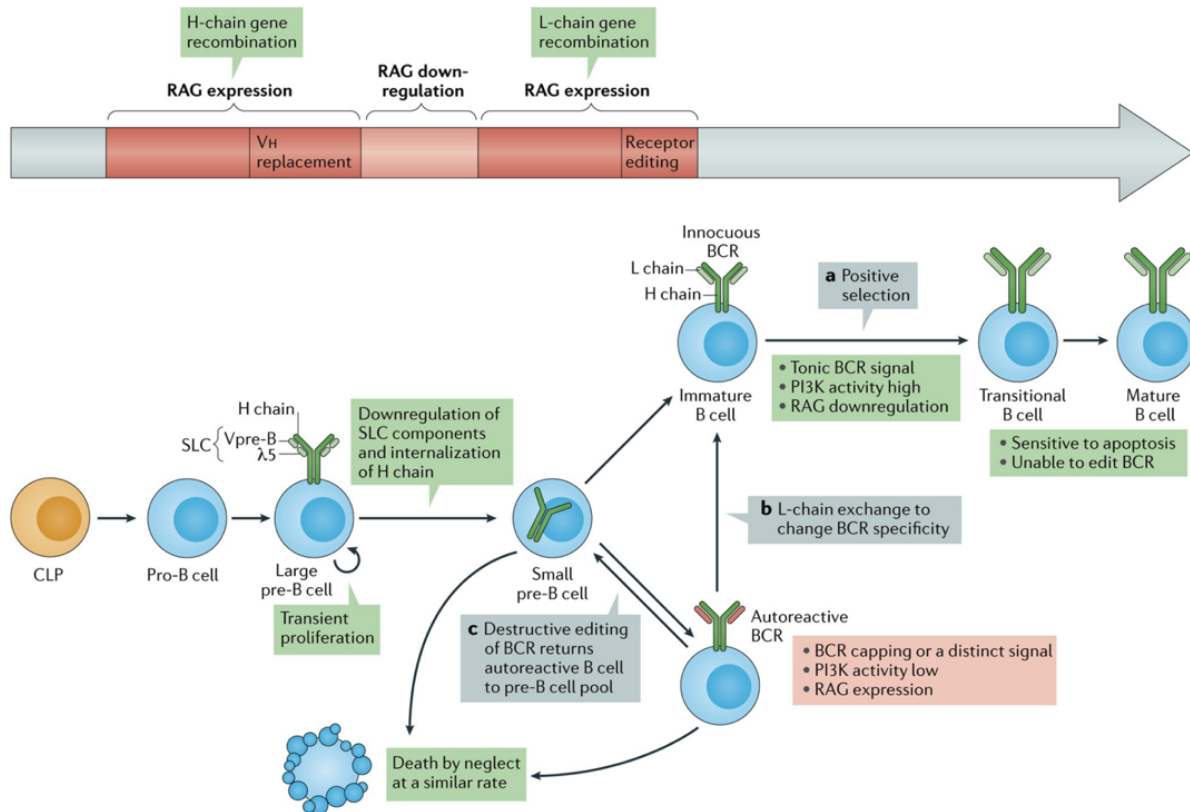


Figure 1. B cell development. The stages of B cell development are characterized based on the status of the B cell receptor (BCR) gene rearrangement, which is controlled by expression of recombination-activating genes RAG1 and RAG2. B cells with autoreactive or nonfunctional BCRs will undergo receptor editing, anergy, or apoptosis, while B cells with a functional BCRs exit the bone marrow as transitional B cells. Reproduced with permission from D. Nemazee, “Mechanisms of central tolerance for B cells,” (2017). License number: 1081954-1

B cells must pass multiple checkpoints (positive and negative selection) to ensure only B cells with functional immunoglobulins that do not react to self-antigens will survive and contribute to an immune response. Positive selection is responsible for ensuring that the newly generated B cells can recognize antigens through their newly formed BCRs. If the heavy and light chains are rearranged properly during the developmental process, the newly formed B cell receptor will provide signaling through the CD79AB immunoreceptor tyrosine-based activation motif (ITAM), which promotes B cell survival. If the B cell

receptor does not create a signal, it will undergo receptor editing. If receptor editing fails to produce a signal, the unresponsive B cell will undergo apoptosis. This process prevents non-responsive B cells from surviving and entering the periphery. While it is important for B cells to respond to antigens, it is also important that they do not respond to self-antigens. Negative selection occurs when B cells with a strong affinity for self-antigens are prevented from contributing to an immune response by undergoing receptor editing, anergy, apoptosis or clonal deletion. This process allows the establishment of self-tolerance which is necessary for preventing autoimmune disorders (Murphy 2017).

1.3 Signals driving B cell response to antigens

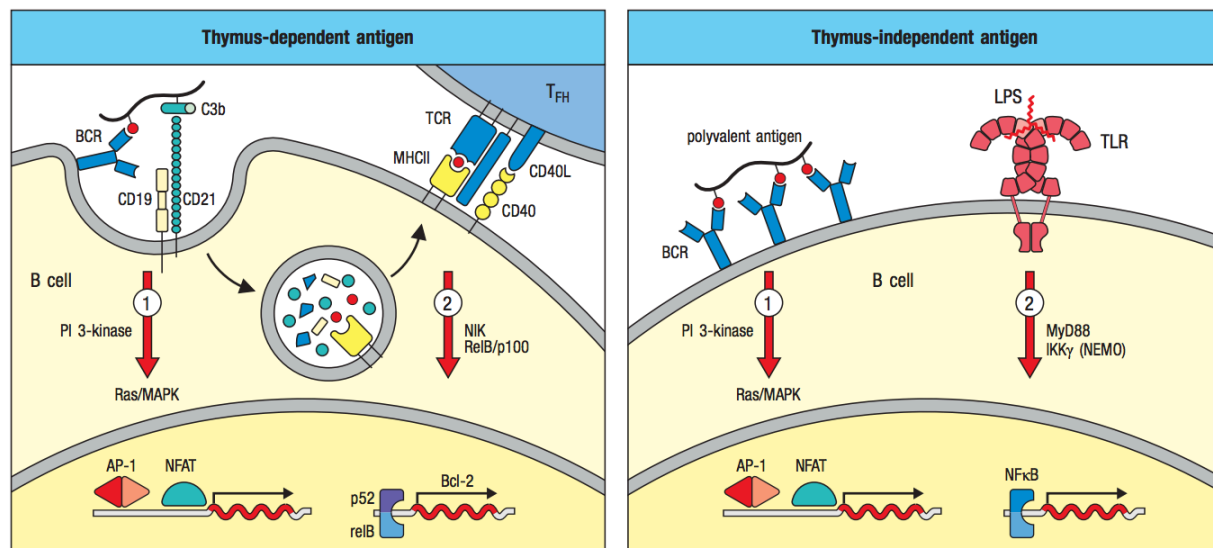


Figure 2. Activation of naïve B cells by T cell-dependent or T cell-independent antigens. B cells can be activated by T cells when antigens that produce weak BCR signaling along with T cell receptor (TCR) recognition of the same antigen is presented by major histocompatibility complex II (MHC II) in B cells and CD40 stimulation by CD40 ligand (CD154) expression on T cells. Alternatively, B cells can be activated independently from T cell help when an antigen crosslinks BCRs, resulting in a strong BCR signal as well as toll-like receptor (TLR) activation by commonly recognized

pathogen structures such as lipopolysaccharide (LPS). Reproduced with permission from K. Murphy, "Janeway's Immunobiology" (2017). PLSclear reference number: 44915

The BCR aids B cell activation by producing a signaling cascade when bound to an antigen. BCRs can also deliver the antigen to intracellular sites so it can be processed and presented to T cells through MHC class II. As a result, B cell activation involves signals provided by a T cell through T-dependent activation, or a nonprotein antigen through T-independent activation (Murphy 2017, Oleinika 2019).

T-cell dependent activation involves two signals. The first signal comes from the BCR which is enhanced by signaling cascades through CD19 and CD21 (**Figure 2**). The second set of signals are provided by T follicular helper cells. These signals include the TCR binding to MHC II on the B cell, as well as CD40 ligand (CD154) binding to CD40 on the B cell. Because protein antigens alone are not able to induce a B cell response, T-cell dependent activation allows B cells to become activated by protein antigens.

Similarly, T-independent activation also requires two signals. The first signal comes from crosslinking of the BCR by a multivalent antigen and the second signal comes from stimulation of Toll Like Receptors (TLR) by antigens expressing pathogen associated microbial patterns (PAMPs) such as GpG (TLR9 agonist) or lipopolysaccharide (LPS). This type of activation allows B cells to rapidly respond to antigens without the help of T cells (**Figure 2**, Murphy 2017, Proverb 2014).

1.3 Maturation of B cell subsets

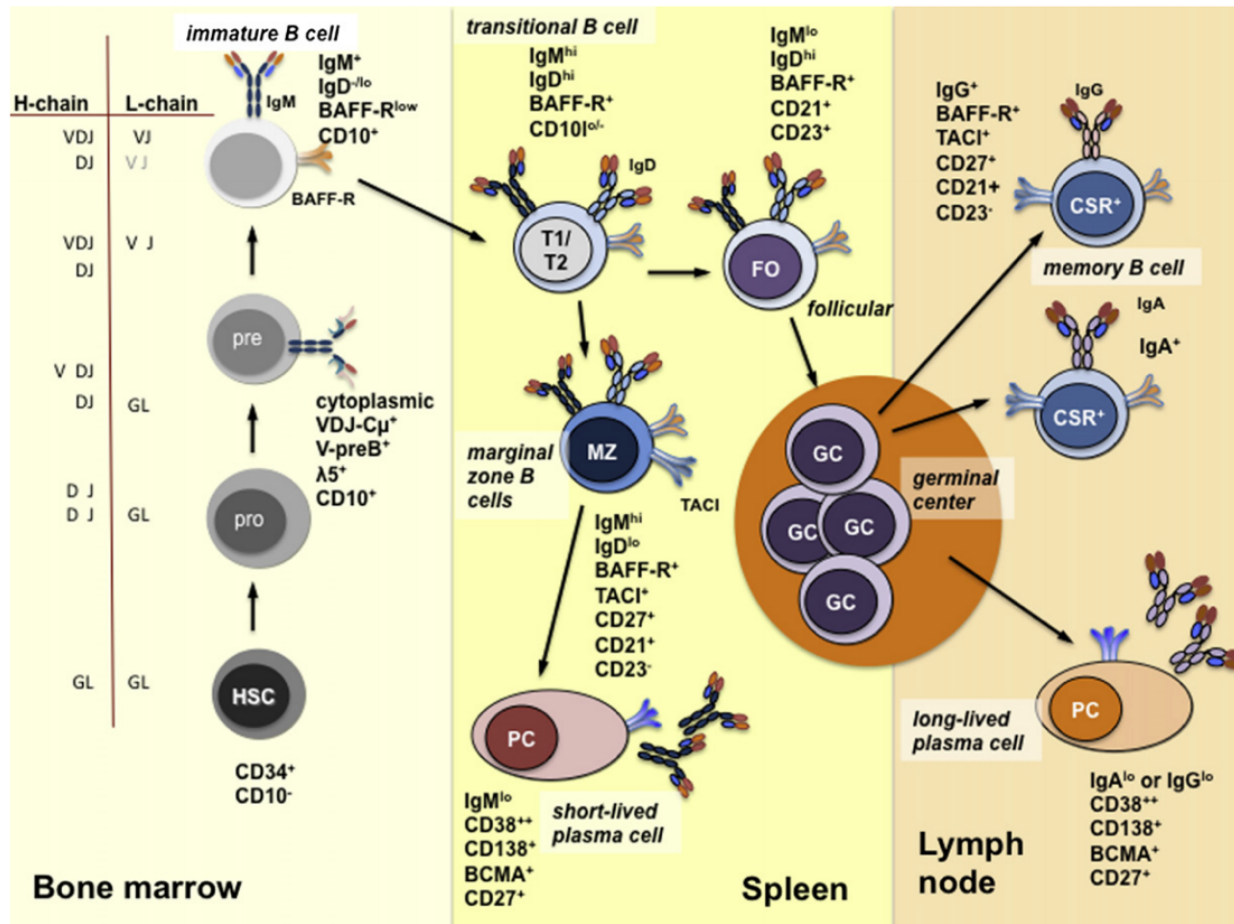


Figure 3. Differentiation of B-2 cells into various subsets. Once immature B cells exit the bone marrow, they enter the periphery and travel to secondary lymph organs as transitional B cells. In the secondary organs, they differentiate into follicular or marginal zone B cells depending on their BCR specificity. Upon exposure to antigen, marginal zone B cells will differentiate into short-lived plasma cells while follicular B cells undergo germinal center reaction and differentiate into memory B or plasma cells. Reproduced with permission from K. Pieper, "B-cell biology and development," (2013). License number: 1081970-1

As B cells mature and differentiate, they give rise to a number of functionally distinct subsets shown in **Figure 3**. The early stages of B cell maturation occur in the bone marrow, where they are initially produced, and then travel to the spleen where the final stages of maturation take place. B-1 B cells are a unique subset of B cells since they are formed in the fetal liver and are responsible for producing protective antibodies that are not in response to an encounter with a pathogen. On the other hand, B cells derived from the bone marrow are classified as B-2 cells. When B cells exit the bone marrow, they enter the periphery as transitional B cells, which are considered T1 cells when they travel to and enter the secondary lymphoid organs such as the spleen. Once T1 B cells enter the spleen, they differentiate into T2 B cells. B cell maturation is considered complete when T2 cells differentiate into either follicular or marginal zone B cells depending on the signals received (Schroeder 2017, Rawlings 2018).

Marginal zone B cells are less abundant than follicular B cells. They are found in the marginal zone of the spleen and play an important role in the early adaptive immune response. Marginal zone B cells can be activated by antigens within hours of exposure and independently of T cells to produce low affinity antibodies. Thus, they contribute to the early stages of the adaptive immune response. In contrast, follicular B cells circulate through primary and secondary lymphoid follicles of the spleen and lymph nodes and rely on T-cell dependent signals for survival. Because they require signals from T cells to be activated, it can take days to weeks for follicular B cells to begin producing antibodies. However, during this time they are able to undergo somatic hypermutation and class switch recombination, allowing them to produce a range of high affinity antibody isotypes.

Follicular B cells contribute to the late adaptive immune response and are primarily responsible for antibody responses against protein antigens. Both marginal and follicular B cell subsets rely on B cell activating factor (BAFF) signaling for survival.

MZ B cells can respond to T cell-independent antigens and will primarily protect against blood borne pathogens. Upon activation, they will turn into plasma cells and secrete IgM immunoglobulins. FO B cells can respond to T cell-dependent antigens and will form germinal centers when activated. Here, they will undergo somatic hypermutation and class switch recombination. This process gives rise to B cells that can produce higher affinity antibodies of different isotypes. FO B cells also have the opportunity to differentiate into memory B, plasmablasts, or plasma cells in germinal centers (Pieper 2013)

Plasmablasts are considered immature plasma cells. They are short lived, highly proliferative cells that are produced as an early response to infection and can rapidly secrete low affinity antibodies. Plasmablasts can be derived from follicular B cells early in the germinal center response, or directly from MZ B cells. After activation, plasmablasts have the opportunity to differentiate into plasma cells. Plasma cells can be found in the spleen, lymph nodes and bone marrow (**Figure 3**) and have a delayed response to infection as they undergo affinity maturation in the germinal center, which allows them to secrete high affinity antibodies. Plasma cells do not proliferate rapidly but can live for long periods of time and are responsible for secreting high levels of antibodies.

Memory B cells are responsible for a heightened response to pathogens encountered a second time. Memory B cells are formed after a primary immune response to an antigen and are able to produce a more rapid response to a previously recognized antigen. In the next section, the function of memory B cells is described in more detail (Murphy 2017).

Regulatory B cells (Bregs) are a B cell subset with immunomodulatory functions. Bregs can inhibit T cell responses by secreting immunosuppressive cytokines including interleukin (IL)-10, IL-35 and tumor growth factor-beta (TGF- β) and have been shown to promote regulatory T cell (Treg) formation (Flores-Borja 2013, Rosser 2015). While recent studies suggest that Bregs can differentiate from all B cell subsets in response to inflammatory signals or BCR signaling, additional studies are required to determine if Bregs arise through development or are induced under certain conditions (Rosser 2015).

1.5 B cell function in health

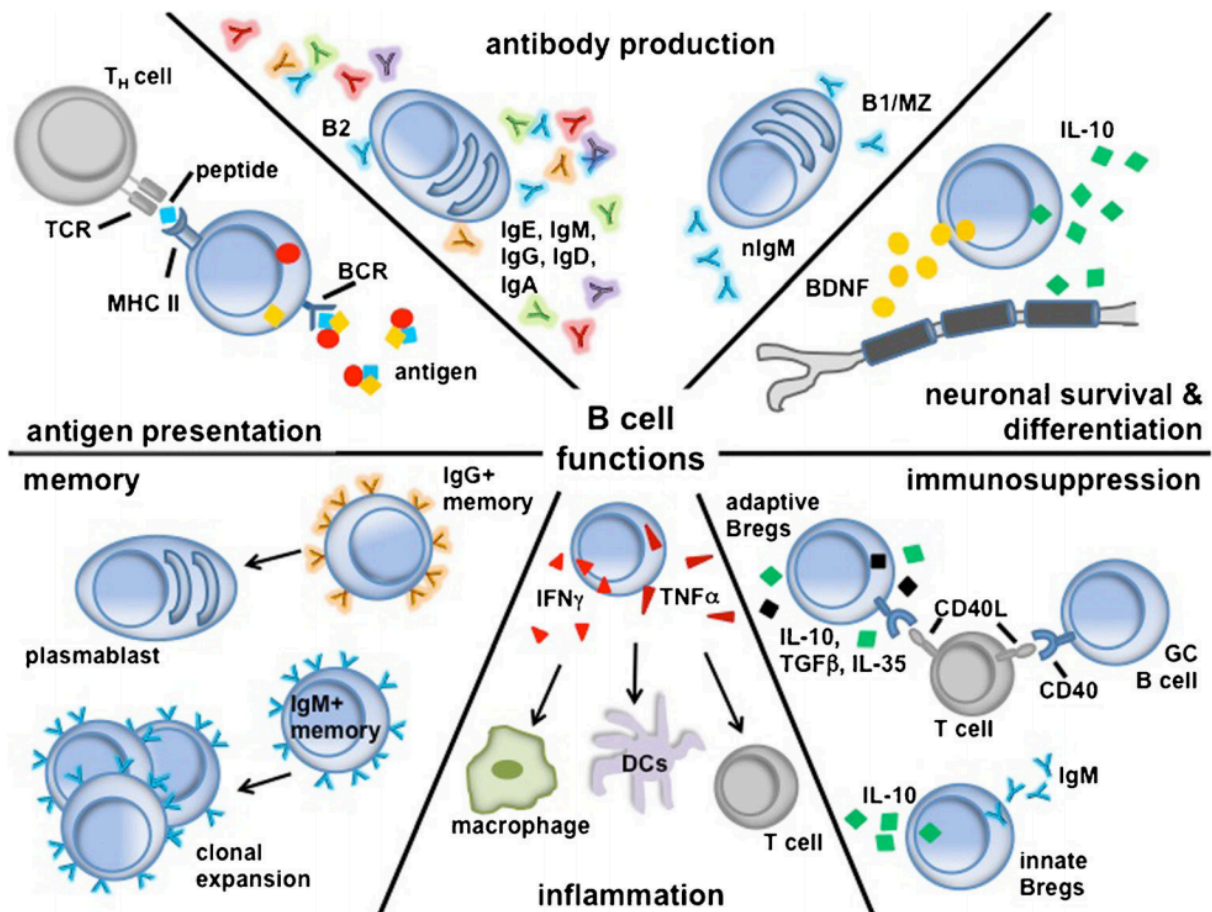


Figure 4. Role of B cells in the immune system. B cells are involved in a wide array of functions of the immune response including antibody production, inflammation, antigen presentation, memory formation, and cytokine secretion. Used with permission from U. Selvaraj, “Heterogeneity of B Cell Functions in Stroke-Related Risk, Prevention, Injury, and Repair”, (2016). License number: 1081973-1

While antibody production has historically been the most valued function of B cells, as shown in **Figure 4**, the contribution of B cells to the immune response is now recognized to include antigen presentation, memory formation, cytokine production, as well as immunomodulatory functions (Selvaraj 2016). Below I discuss in more details some of the most important roles of B cells in the immune system:

1.6 Antibody production

B cells possess multiple functions that make them a critical component to the immune system, however they are most widely valued for their ability to produce antibodies. These antibodies can act as membrane-bound surface receptors known as B cell receptors or are secreted as proteins that contribute to immunity in different ways. While each B cell is only able to produce antibodies with a single antigen specificity, the population as a whole, amounts to a wide antibody repertoire capable of recognizing an extensive number of antigens. Antibodies bind to antigens on the surface of pathogens and inhibit their function or tag them for destruction by other components of the immune system such as macrophages, NK cells and mast cells. The portion of the immune system that responds to antibodies is dependent on the antibody constant (Fc) region. The process of class switch recombination allows B cells to produce antibodies with the same variable region but different constant regions. As a result, a B cell can generate antibodies that recognize the same antigen while providing different modes of protection that are dependent on the Fc region. Once an antibody is bound to a soluble or surface antigen with its variable region, cells can use Fc receptors to bind to the Fc region of the antibody. This interaction prompts the cell to exert its effector function. For example, macrophages will perform phagocytosis, natural killer cells will secrete cytotoxic cytokines, and mast cells will release their granules, all resulting in pathogen destruction and clearance. Antibody Fc regions can also activate portions of the complement system, also resulting in pathogen destruction. The ability to secrete different isotypes allows the immune system to tailor its

response to clear different types of pathogens as effectively and efficiently as possible (Hoffman 2016).

1.7 Antigen presentation

B cells can also contribute to the adaptive immune response as antigen presenting cells. When the BCR binds to antigen, not only does it activate the B cell through a signaling cascade, but the BCR and bound antigen are internalized and the antigen is processed. This allows peptides derived from the antigen to be presented on MHC class II molecules expressed on the surface of the B cells. CD4⁺ T cells that have been activated by the same antigen can then secrete cytokines needed for B cell memory and germinal center formation, as well as plasma cell formation (Chalasani 2016, Selvaraj 2016, Murphy 2017).

1.8 Memory formation

Memory formation is another protective role of B cells. Immunological memory allows the immune system to establish a robust response to subsequent exposure to pathogens. Memory B cells can be formed from short-lived plasma cells or germinal center reactions during a primary response to an antigen (**Figure 4**). Memory B cells express Ig genes that have undergone isotype class switching and possess somatic mutations, and are able to persist in the body long-term. As a result, memory B cells are able to produce higher affinity antibodies more rapidly than the previous response. This allows memory B cells to contribute to the efficient elimination of familiar pathogens (Allen 2019, Selvaraj 2016, Murphy 2017).

1.9 Cytokine secretion and immunomodulation

B cells can also impact the immune response by secreting cytokines. Depending on their intended role, B cells can secrete either proinflammatory or anti-inflammatory cytokines (**Figure 4**). Upon activation, B cells can secrete pro-inflammatory cytokines such as interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α) (Chalasani 2016, Selvaraj 2016).

A subset of B cells named regulatory B cells (Bregs) can modulate the inflammatory response through the production of anti-inflammatory cytokines that suppress T cells and promote tolerance. Breg formation can be driven by signals provided by T cells, cytokines, and TLR stimulation produced in the microenvironment. These signals can induce Breg formation by B cell subsets from both B1 and B2 lineages. Previous research shows that Breg formation from both B1 and B2 lineages can be initiated through CD40 ligation, and that the cytokine IL-21 can initiate IL-10 production in Bregs (Lykken 2015). Bregs are able to suppress an inflammatory response and promote tolerance by secreting IL-10, IL-35 and TGF- β . Cytokine production by Bregs has been shown to inhibit formation of pathogenic cells and their inflammatory cytokine production while promoting formation of suppressive regulatory T cells (Tregs) (Selvaraj 2016).

1.10 Breg function in disease

Because regulatory B cells play a pivotal role in preventing or reducing immune-mediated inflammation, their absence or dysfunction can contribute to a wide range of diseases. Multiple groups have shown that missing or dysfunctional regulatory B cells can contribute to immune-mediated inflammatory conditions including multiple sclerosis, graft versus host disease (GvHD), systemic lupus erythematosus and rheumatoid arthritis.

Claudia Mauri and her colleagues from University College London showed that a subset of CD19⁺ CD24^{hi} CD38^{hi} B cells from rheumatoid arthritis patients were unable to convert T cells into regulatory T cells. These B cells were also unable to prevent the formation of T_H17 cells, a subset of proinflammatory T cells thought to contribute to a number of immune-mediated inflammatory diseases, including rheumatoid arthritis (Flores-Borja 2013).

In the context of systemic lupus erythematosus (SLE), Mauri and colleagues also showed that crosstalk between plasmacytoid dendritic cells and regulatory B cells is compromised in SLE due to altered STAT activation, leading to their inability to produce sufficient amounts of IL-10. This decrease in IL-10 production by regulatory B cells is thought to contribute to the pathogenesis of SLE (Menon 2016).

A study by Luisa Klotz and her team found that treatment with Fingolimod, an FDA approved drug for in multiple sclerosis (MS) that blocks Sphingosine-1-phosphate (S1P)-mediated signaling in lymphocytes, induces an increase in the percentage of regulatory B cells compared to other B cell subsets. It achieves this by limiting the trafficking of

naïve cells from the bone marrow, resulting in a higher number of regulatory B cells in circulation. This increased proportion of regulatory B cells was significantly correlated with better clinical outcomes (Grützke 2015).

A study led by Katy Rezvani and her team highlighted how Bregs can establish tolerance to the donor after hematopoietic stem cell transplant and could potentially be used to mitigate chronic graft versus host disease (Khoder 2014). Taken together, these studies provide in depth evidence on how properly functioning regulatory B cells are essential to prevent a variety of immune-mediated inflammatory diseases.

1.11 Efforts to expand B cells for cell therapy

Regulatory B cells are seen as excellent cell therapy candidates due to their immunomodulatory capabilities (Mauri 2017). A major roadblock in the development of regulatory B cell therapy is the lack of expansion protocols that support the large-scale generation of viable regulatory B cells. Several groups have published methods to expand B cells for the purpose of studying antigen specific B cell responses to vaccinations, as well as an alternative production method for generating intravenous immunoglobulin (IVIG) (Muir 2017, Néron 2012). However, the culture methods used in these studies may not translate well to the generation of regulatory B cells for adoptive therapy.

The most widely utilized culture system for B cell expansion applies feeder cells that express CD154 (CD40 ligand). This allows for the CD154 on the feeder cells to interact

with CD40 expressed on the surface of B cells, which in turn stimulates B cell proliferation (Néron 2012). This method allows for large scale expansion of B cells, however, further studies are needed to determine whether these ex vivo expanded B cells can persist long-term after infusion and in the absence of signals provided by the feeder cells. Establishing a protocol for the large scale expansion of viable B cells independent of feeder cells will open the door for B cell therapy to be used in the clinic.

1.12 Working Hypothesis

We hypothesize that using genetic engineering tools we can generate a large number of B cells with an immunosuppressive phenotype and with the potential to be used for future cell therapy.

2. Methods

2.1 Lymphocyte Isolation and B cell selection

The buffy coats used in this study were obtained from Gulf Coast Regional Blood Center, Houston, TX. The lymphocytes were isolated from peripheral blood mononuclear cells (PBMCs) from healthy donors using ficoll density-gradient separation. First, the buffy coat was diluted with phosphate-buffered saline (PBS) in a 1:1 ratio and transferred to a 50 ml falcon tube containing 15 ml of lymphoprep. The buffy coat was centrifuged at 400 g for 30 minutes, with an acceleration of 5 and a deceleration of 0. After centrifugation, the top layer containing plasma was aspirated and the white middle layer, containing lymphocytes, was removed and kept using a 5 ml pipette. The bottom layer containing erythrocytes and granulocytes was discarded. The lymphocytes were washed once with 50 ml of PBS at 400 g for 10 minutes. The lymphocytes were then washed 3 times with 20 ml PBS for 5 minutes at 400 g, or until a majority of the platelets were removed from the sample.

B cells were negatively selected from lymphocytes using magnetic beads and following the Miltenyi Biotec B cell isolation kit protocol. Briefly, B cells were incubated with the clinimacs buffer ($40\ \mu\text{l}/10^7$ B cells) and the Miltenyi Biotec antibody cocktail ($10\ \mu\text{l}/10^7$ cells) for 5 minutes at 4°C . The clinimacs buffer ($30\ \mu\text{l}/10^7$ cells) and Miltenyi Biotec microbeads ($20\ \mu\text{l}/\text{ml}$) were then added to the mixture for 10 minutes at 4°C . The B cells were selected using the clinimacs magnetic column and the separation filter. Following isolation, the B cell purity was confirmed by staining for CD19 using flow cytometry. If the purity was not above 95%, the selection process was repeated.

2.2 B cell stimulation

The purified B cells were resuspended in complete stem cell growth medium (SCGM) (CellGenix) with 10% fetal bovine serum (FBS), 1% Penicillin/streptomycin, and 1% L-Glutamine at 0.5×10^6 cells/ml. CpG ODN 2006 (3 μ g/ml, Hycuit Biotech). Anti-IgM+IgG (10 μ g/ml, Jackson ImmunoResearch) were added to the cell suspension, and 2×10^6 cells/well were plated in a twelve well plate and incubated at 37°C for 48 hours.

2.3 Transduction of activated B cells

Forty-eight hours after stimulation with CpG and anti-BCR (anti-IgM+IgG), B cells were transduced with an MMLV retroviral construct encoding for CD40 ligand (CD154) with or without IL-21. Retronectin (Takara Bio) was diluted in PBS at a 1:100 ratio and 1 ml of diluted retronectin was added to each well in a 24 well non-tissue culture plate and incubated at 37° C for 4-6 hours. The retronectin was then removed from the culture plate and the wells were washed with 1 ml of SCGM (10% FBS, 1% penicillin/streptomycin, 1% L-Glutamine) for 10 minutes at 37°C. The SCGM was then removed and 1 ml of the retroviral supernatant was added to each well. The plate was then centrifuged at 2000 g at 32°C for 2 hours. Following centrifugation, the retroviral supernatant was removed and replaced with 1 ml of fresh retroviral supernatant, as well as 0.5×10^6 B cells per well. The plate was then centrifuged at 2000 g at 32°C for 30 minutes and transferred to the CO2 incubator (37°C) for 24 hours. The next day, 1 ml of SCGM was added to each well. The transduction efficiency was determined 2-3 days later by flow cytometry analysis.

2.4 B cell transduction efficiency

To assess the transduction efficiency, 200 μ l of cell suspension was placed in a FACS tube and washed with FACS wash buffer (1% FBS in PBS) at 2000 RPM for 5 minutes. The supernatant was aspirated and the antibody cocktail (CD19 PECy7, CD40L BV711, LD AF647) (All from BD Biosciences) was added to each tube. The tubes were incubated at room temperature in the dark for 20 minutes and washed with FACS buffer at 2000 RPM for 5 minutes. IL-21 production was assessed by intracellular staining. Briefly, after surface staining, cells were permeabilized by adding 0.5 ml BD permeabilization buffer to each tube for 40 minutes at 4°C, followed by two wash steps with 2 ml of 1x BD permeabilization buffer. The IL-21 antibody (30 ng/ μ l, R&D) was added to each tube and the cells were incubated at 4°C for 35 minutes. The tubes were then washed with 2 ml of 1x BD permeabilization buffer at 2000 RPM for 5 minutes. The expression of CD19, CD40L and IL-21 was then assessed using the flow cytometer (BD Fortessa).

2.5 Assessment of B cell proliferation

B cell count and viability were recorded every two days using trypan blue and a cell cytometer. Cells were mixed with an equal volume of trypan blue and loaded onto a cell counting slide. The percentage (%) of cell viability was determined by counting the number of live and dead cells in the sample using a cytometer.

2.6 Production of CD40L and CD40L-IL21 in the retroviral supernatant (Transfection)

In order to generate the virus for B cell transduction, murine leukemia virus (MMLV) retroviral plasmids encoding CD40L or CD40L-IL21 (**Figure 5**) were cotransfected with plasmids containing packaging and envelope genes.

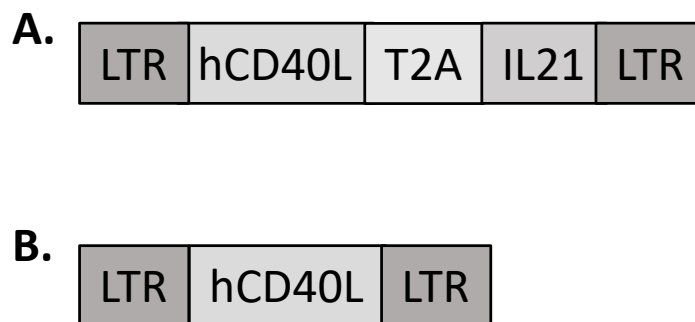


Figure 5. MMLV retroviral construct maps **A.** MMLV retroviral construct containing hCD40L and IL-21 genes. **B.** MMLV retroviral construct containing hCD40L.

One million HEK 293T cells were plated in a petri dish with 12 ml of Dulbecco's Modified Eagle Medium (DMEM). The transfection was performed 24 hours later, or when the HEK 293T cells reached at least 70% confluency. First, the media from the confluent plates were aspirated and fresh, room temperature DMEM was added and incubated at 37°C. Two thousand nanograms of helper 1 (contains gag and pol proteins), four thousand nanograms of helper 2 (contains envelope proteins), 37µl of FuGENE and 1 ml of optimum per reaction were added to a 50 ml falcon tube. One mL of this mixture was placed into an Eppendorf tube containing 6000ng of the DNA of interest (CD40L or CD40L-2A-IL21 plasmids), and incubated at room temperature for 15 minutes. The

mixture was then added dropwise to the plate. The HEK293 T-cells were incubated at 37 °C for 24 hours. After 24 hours, the supernatant was aspirated and 12 ml of SCGM was added to each plate. The supernatant was collected 48 and 72 hours post-transfection and stored at - 80°C for future use.

2.7 Assessing Transfection efficiency of HEK 293T cells

The transfection efficiency was assessed 72 hours after transfection using flow cytometry. First, 200,000 HEK 293 T cells were collected from the plate and washed with FACS buffer at 2000 RPM for 5 minutes. The buffer was aspirated, and the cells were incubated with live dead AF647 (Lifetech) and CD40L BV421 (Biolegend) antibodies for 20 minutes in the dark at room temperature followed by washings with FACS buffer. The transfection efficiency was then assessed by determining CD40L expression using a BD Fortessa flow cytometer.

2.8 Isolation of T cells from peripheral blood mononuclear cells

T cells were isolated using a magnetic negative selection kit (Miltenyi Biotech) following the manufacturer's protocol. Briefly, lymphocytes from healthy donor PBMC were incubated with clinimacs buffer (40 μ l/ 10^7 cells) and the antibody cocktail (10 μ l / 10^7 cells) at 4°C for 5 minutes. The clinimacs buffer (30 μ l / 10^7 cells) and magnetic microbeads (10 μ l / 10^7 cells) were then added to the mixture and incubated at 4°C for 10 minutes. The magnetic columns and separation filters were prewashed with 3 ml of clinimacs buffer, then the cell mixture was added. The columns were then washed three times with

cliniMACS buffer. The T-cell purity was assessed by determining the surface expression of CD3, CD4 and CD8 by flow cytometry. If the T cells had a purity of less than 95%, the selection process was repeated.

2.9 B cell proliferation assay

Magnetically selected carboxyfluorescein succinimidyl ester (CFSE)-labeled ($2\mu\text{l/ml}$; ThermoFisher) T cells (10^6 cells/mL) were resuspended in PBS and incubated at 37°C for 15 minutes. SCGM and FBS were added to the suspension in a 1:1 ratio. The cells were centrifuged for 10 minutes at 1200 RPM, then washed twice with 5 ml of complete SCGM. CFSE staining was determined by flow cytometry under the FITC channel.

2.10 B cell suppression of T cell cytokine production

CFSE-labeled T cells were activated with anti CD3/CD28 microbeads ($25\mu\text{l}/10^6$ cells) and immediately co-cultured with B cells at a ratio of 1:1, 1:2, and 1:5 in a 96 well flat bottom plate for 48 hours. Brefeldin A (BFA) ($10\mu\text{g/ml}$), phorbol myristate acetate (PMA) (50ng/ml) and ionomycin (500ng/ml) (all from Sigma-Aldrich) were added 5 hours prior to intracellular staining. After 5 hours, cells from each condition were collected and stained for the following surface markers: CD4, CD8, CD3, and CD19. The cells were then stained to assess intracellular cytokine production of $\text{TNF-}\alpha$, $\text{IFN-}\gamma$ and IL-2.

2.11 B cell suppression of T cell cytokine proliferation

First, CFSE-labeled T cells were activated with anti CD3/CD28 microbeads (25 μ l /10⁶ cells) and were immediately co cultured with B cells at a ratio of 1:1, 1:2, and 1:5 on a 96 well flat bottom plate for 72 hours. After 72 hours, the cells were collected and stained for expression of CD4, CD8, CD3, and CD19 using monoclonal antibodies.

2.12 Immunoglobulin secretion by B cells

Immunoglobulin secretion by B cells was measured by enzyme-linked immunosorbent assay (ELISA) for IgM, IgG, IgE and IgA following the manufacturer's protocol (Invitrogen).

2.13 CD40L-2A-IL21 plasmid transformation

The CD40L-2A-IL21 plasmid construct was made by Addgene. The plasmid was used to transform NEB 5 α bacteria following the high competent transformation protocol. One hundred nanograms (100ng) of plasmid DNA was added to 50 μ l of NEB5a bacteria. The bacteria were incubated on ice for 15 minutes, then heat shocked in a 42° C water bath for 45 seconds. The bacteria were placed back on ice for 5 minutes, then transferred to a tube containing 950 μ l of SOC broth. The bacteria were then placed in a 37° C incubator and shaken at 150 RPM for one hour. After the one-hour incubation, 4 ml of LB broth was added to the bacteria along with ampicillin at a concentration of 100mg/ml. The bacteria were placed in the incubator at 37° C on a shaker at 150 RPM for 6 hours. After 6 hours, the bacteria were transferred to a flask containing 500 ml LB Broth with ampicillin (100mg/ml) and incubated at 37° C on a shaker at 150 RPM overnight. The next day, the

bacteria were centrifuged, and the pellet was stored in -80°C for maxiprep preparation (plasmid isolation).

2.14 CD40L-2A-IL21 plasmid cloning

A maxiprep selection kit (ThermoFisher) was used to extract the plasmid DNA from the bacterial pellet formed during the plasmid transformation procedure following manufacturer's directions. The DNA concentration was quantified by nanodrop (ThermoFisher) and stored at -20°C for further use.

2.16 B cell culture

B cells (0.5×10^6 cells/ml) were cultured in complete SCGM media supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-Glutamine, along with different combinations of the following cytokines: IL-2 (50 IU/ml), IL-4 (4 ng/ml), and IL-21 (30 ng/ μ l).

2.17 Flow cytometry

Table 1. List of antibodies used for surface staining in functional and proliferation assays

Antibody	Fluorophore	Volume per test	Company
LD	BV 405	0.5 μ l	Lifetech
CD19	PE Cy7	1 μ l	BD Biosciences
CD4	APC	2 μ l	E Biosciences
CD8	PerCP Cy5.5	2 μ l	Biolegend
CD3	APC Cy7	1 μ l	Biolegend

Two hundred thousand (200,000) B cells were collected, transferred into a FACS tube in 2ml of FACS buffer (1% FBS in PBS) and centrifuged at 2000 rpm at 19 °C for 5 minutes. After centrifugation, the buffer was aspirated from the tubes and the antibodies shown in **Table 1** were added to each tube for 20 minutes in the dark at room temperature. Cells were then washed and fixed in 1.8% paraformaldehyde. The tubes were then stored in the dark at 4°C until acquired on the Fortessa flow cytometer.

Table 2. List of antibodies used for intracellular staining

Antibody	Fluorophore	Volume per test	Company
IL-2	PE	2.5 μ l	BD Biosciences
TNF- α	AF700	2.5 μ l	Biolegend
IFN- γ	V450	2.5 μ l	BD Biosciences

Immediately after surface staining, 0.5 ml of BD cytofix/cytoperm was added to each sample at 4° C for 40 minutes. During the incubation period, 1x wash buffer was prepared by diluting the 10x BD wash buffer with deionized water. When the incubation period was

over, 1 ml of wash buffer was added to each sample and the cells were centrifuged at 2000 rpm for 5 minutes. The wash buffer was then aspirated and 2 ml of 1x wash buffer was added to each sample. The samples were centrifuged at 2000 RPM for 5 minutes, the supernatant was aspirated and an antibody master mix containing the antibodies in **Table 2** was added to each sample. The cells were incubated at 4⁰C for 30 minutes, Cells were then washed and fixed in 1.8% paraformaldehyde. The tubes were then stored in the dark at 4⁰C until acquired on the Fortessa flow cytometer.

2.18 Mass Cytometry

Table 3. List of antibodies used for mass cytometry

Metal tag	Antibody	Metal tag	Antibody
89Y	CD45	161Dy	BAFF
141Pr	CD138	162Dy	CD79b
142Nd	CD19	163Dy	CD1d
143Nd	CD5	164Dy	CD23
144Nd	HLA ABC	165Ho	CD40
145Nd	CD45RB	166Er	CD24
146Nd	IgD	167Er	CD38
147Sm	CD20	168Er	CD21
148Nd	PDL-1	169Tm	CD45RA
149Sm	CD25	170Er	HLA-DR
150Nd	CD73	171Yb	CD9
151Eu	IGM	172Yb	PDL-2
152Sm	CD95	173Yb	CXCR4
153Eu	CXCR5	174Yb	PD1
154Sm	CD86	175Lu	CD85j
155Gd	CD27	176Yb	CD22
156Gd	CXCR3	209Bi	CD11b
158Gd	CD10	In115	CD80
159Tb	CD11c	Pt194	CD3
160Gd	CD39	Pt198	LD

Sample preparation and acquisition

A protocol developed by the Rezvani lab was used for this project (Li 2019). Briefly, frozen B cell samples were thawed and stained with cisplatin for two minutes. The samples were then stained with the antibody panel shown in **Table 3** and shaken at 145 RPM for one hour. Iridium was diluted in 1.8% PFA at a ratio of 1:1000 and added to each sample. The samples were incubated for 20 minutes in the dark at room temperature. Following

incubation, the samples were filtered and stored at 4°C until they were acquired using a Helios instrument (fluidigm) with the Helios 6.5.358 acquisition software (fluidigm) at 300 events/second.

Data analysis

Using Fluidigm normalization software 2, the mass cytometry data were normalized based on EQ™ four element signal shift over time. Cytobank was used to perform Vi-SNE analysis.

3. Results

To develop a protocol for B cell expansion, we first stimulated peripheral blood (PB) B cells with CpG ODN 2006 (3 μ g/ml), and anti-IgM+IgG (10 μ g/ml) and cytokines IL-2 (50 IU/ml), IL-4 (4ng/ml) and/or IL-21 (30 ng/ml) for 48 hours prior to genetically engineering these cells with retroviral constructs expressing either CD40L or CD40L and IL-21 (CD40L-2A-IL21) (**Figure 6A**). B cells transduced with CD40L-2A-IL21 B cells secrete IL-21 autonomously and expand to the same extent as B cells expressing CD40L alone supplemented with exogenous IL-21 (**Figure 6B-E**). We also tested the effects of IL-2 and IL-4 supplementation on B cell proliferation since they have been previously shown to be involved in Breg skewing (Lund 2008). We found that a combination of IL-2 and IL-4 supplementation significantly increased B cell proliferation when compared to the supplementation of IL-2 or IL-4 separately. (**Figure 6B-E**). Taken together, our data suggest that the combination of CD40L and IL-21 successfully drive B cell proliferation in vitro and that the addition of IL-2 and IL-4 further increases this fold expansion.

Figure 6

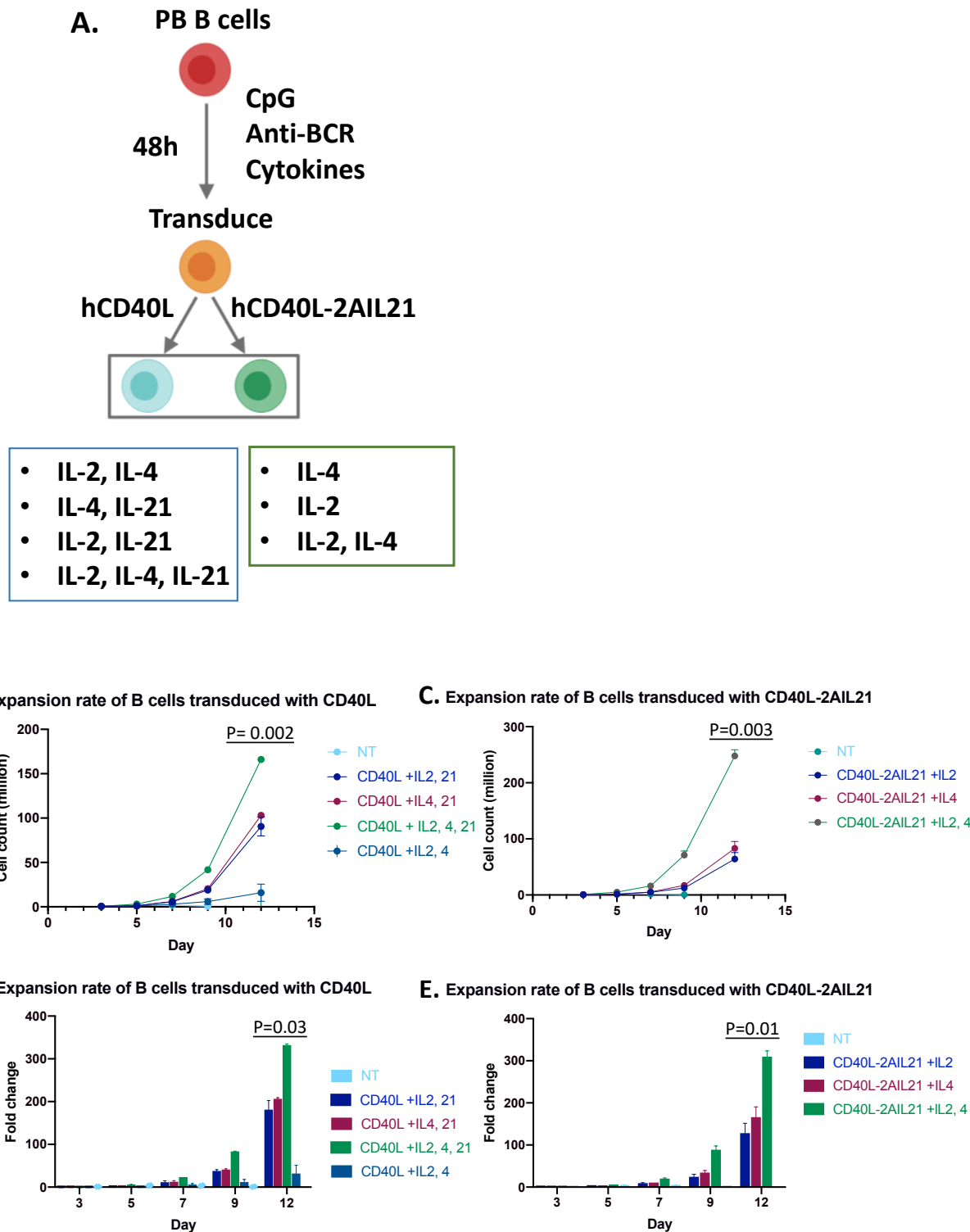


Figure 6. B cells transduced with CD40L or CD40L-IL21 and supplemented with cytokines undergo rapid expansion. A. Schematic diagram for the expansion conditions tested. **B.** Expansion rate (cell count) of B cells transduced with CD40L and supplemented with exogenous IL-2 and/or IL-4. **C.** Expansion rate (cell count) of B cells transduced with CD40L-2A-IL-21 and supplemented with exogenous IL-2 and/or IL-4. **D.** Expansion rate (fold change) of B cells transduced with CD40L and supplemented with exogenous IL-2 and/or IL-4. **E.** Expansion rate (fold change) of B cells transduced with CD40L-2A-IL-21 and supplemented with exogenous IL-2 and/or IL-4.

Next, we determined if B cells generated using the expansion methods described in **Figure 6** can suppress T cell function. To answer this question, CD40L-2A-IL21-expressing B cells were cocultured with activated T cells in the presence of IL-2, IL-4 or IL-2/IL4 for 48 hours followed by assessment of T cell cytokine production. Resting T cells that were not activated with CD3/CD28 microbeads served as a negative control. Indeed, we observed a significant decrease in IFN- γ and TNF- α production by activated T cells when cocultured with B cells (**Figure 7A-C**). Importantly, this suppressive capacity was not dependent on IL-2, IL-4 or combined IL-2/IL-4 supplementation (**Figure 7A-C**). These findings indicate that B cells transduced with the CD40L-2A-IL21 construct can suppress T cell function independently from IL-2 or IL-4 cytokines.

Figure 7

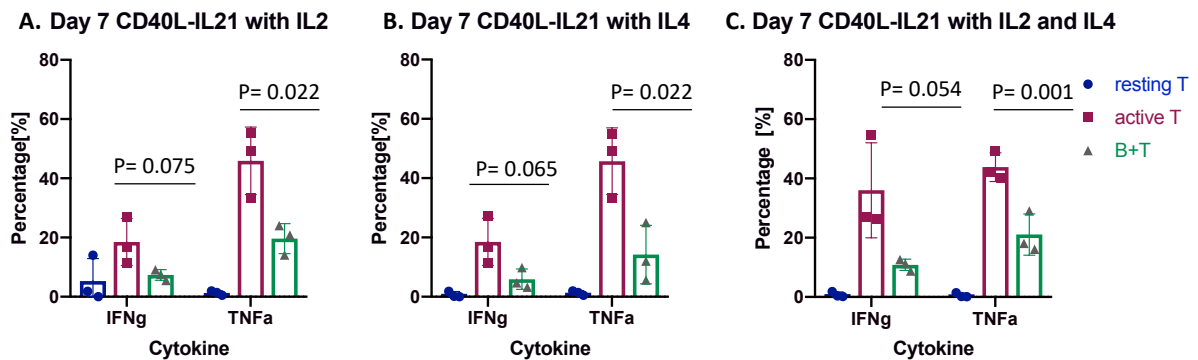


Figure 7. T cell cytokine production is reduced when co-cultured with B cells expanded in conditions supplemented with IL-2, IL-4, or IL-2/IL4. B Cells were expanded by CpG/Anti-BCR stimulation for 48 hours, followed by transduction with CD40L-IL21. Following transduction, the B cells were cultured in complete SCGM media and supplemented with IL-2 alone, IL-4 alone, or IL-2 and IL-4 combined **A.** IFN- γ and TNF- α production by T cells cocultured with B cells transduced with CD40L-2A-IL21 construct and supplemented with IL-2. **B.** IFN- γ and TNF- α production by T cells cocultured with B cells transduced with CD40L-2A-IL21 construct and supplemented with IL-4. **C.** IFN- γ and TNF- α production by T cells cocultured with B cells transduced with CD40L-2A-IL21 construct and supplemented with IL-2 and IL-4. Resting T cells were not activated with CD3/CD28 microbeads and were used as negative control. Activated T cells were activated with CD3/CD28 microbeads and were used as positive control.

To examine whether B cells expressing CD40L or CD40L-2A-IL21 could suppress T cell proliferation, we labeled T cells with CFSE, activated them with CD3/CD28 beads and cultured them with or without Bregs for 72 hours. Non-activated (resting) T-cells were used as negative control. As shown in **Figure 8A-C**, B cells cocultured with activated T cells significantly reduced T cell proliferation compared to activated T cells cultured alone. The addition of IL-2 or IL-4 did not increase the suppressive activity of B cells further. These findings support an immunomodulatory effect for B cells stably expressing CD40L or CD40L-21.

Figure 8

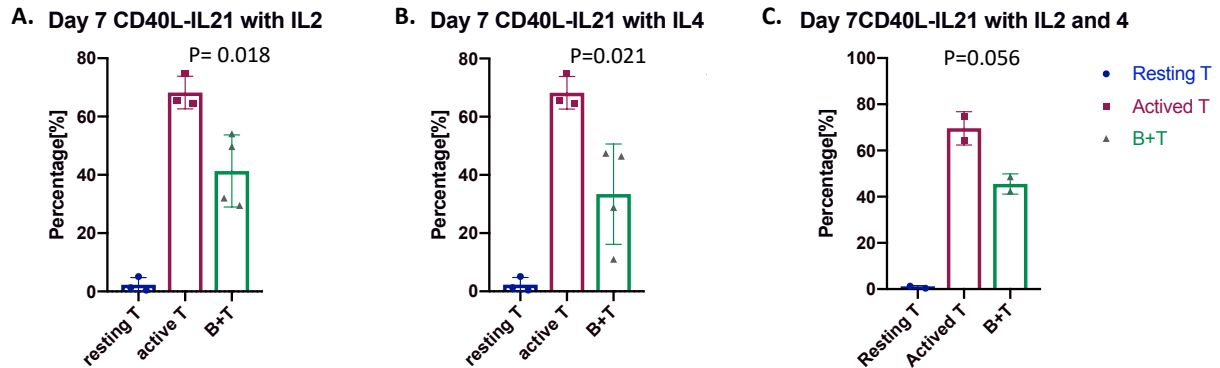


Figure 8. CD40L-IL21 transduced B cells expanded in conditions supplemented with IL-2, IL-4, or IL-2 and IL-4 suppress T-cell function. B Cells were expanded by CpG/Anti-BCR stimulation for 48 hours, followed by transduction with CD40L-IL21. Following transduction, the B cells were cultured in complete SCGM media and supplemented with IL-2 alone, IL-4 alone, or IL-2 and IL-4 combined. **A.** Percent proliferation of T cells when cocultured with CD40L-IL21 transduced B cells expanded with IL-2. **B.** Percent proliferation of T cells when cocultured with CD40L-IL21 transduced B cells that were expanded with IL-4. **C.** Percent proliferation of T cells when cocultured with CD40L-IL21 transduced that were expanded with the combination of IL-2 and IL-4.

Next, we examined whether the suppression of T cells by B cells is dependent on the B:T cell ratios (1:1, 1:2, 1:5) or the length of culture (up to 10 days) . The addition of B cells to activated T cells at a 1:1 ratio resulted in a significant decrease in IFN- γ , TNF- α and IL-2 production (**Figure 9A-C**). However, B cells were unable to suppress T cell cytokine production at B:T cell ratios of 1:2 or 1:5 (**Figure 9A-C**). We also observed that the B cell suppressive capacity was transient and was lost over time **Figure 9A-C**).

Figure 9

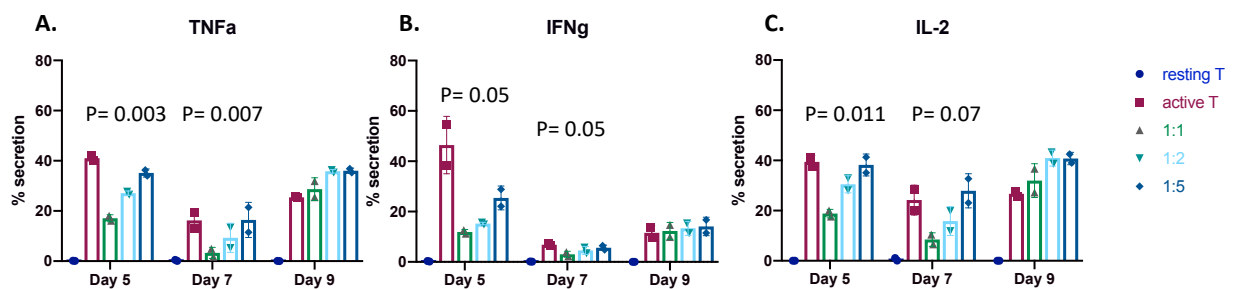


Figure 9. CD40L-IL21 transduced B cells expanded in conditions supplemented with IL-2 and IL-4 suppress T-cell cytokine production. B cells were expanded by activation with CpG and Anti-BCR, followed by transduction with CD40L-IL21 and cultured in SCGM with IL-2 and IL-4. A. TNF- α production by T cells cultured with B cells at different ratios over time. B. IFN- γ production by T cells cultured with B cells at different ratios over time. C. IL-2 production by T cells cultured with B cells at different ratios over time.

We next investigated if the B:T cell ratio or length of culture also affected T cell proliferation. As shown in **Figure 10**, we cocultured B cells with CFSE-labeled T cells at B:T cell ratios of 1:1, 1:2 and 1:5 and observed that B cells are only able to suppress T

cell proliferation when cocultured at a 1:1 ratio. However, B cells were unable to suppress T cell proliferation at B:T cell ratios of 1:2 or 1:5. Moreover, B cells lost their ability to suppress T cell proliferation as they over time in culture (Day 5 vs Day 9) indicating that B cells are capable of suppressing T cell proliferation, an effect that is diminished over time.

Figure 10

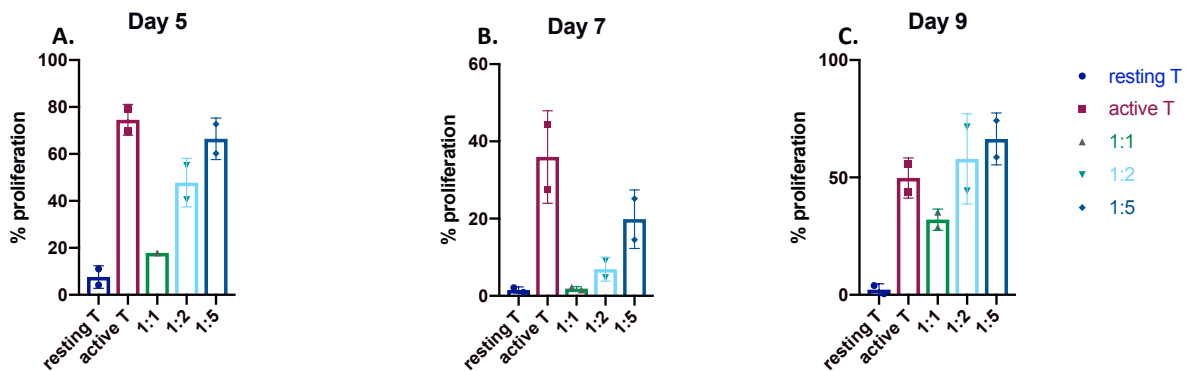


Figure 10. CD40L-IL21 transduced B cells expanded in conditions supplemented with IL-2 and IL-4 suppress T-cell proliferation. B cells were expanded by activation with CpG and Anti-BCR, followed by transduction with CD40L-IL21 and cultured in SCGM with IL-2 and IL-4. A-C Percentage of proliferation of CFSE labeled T cells that were cultured with B cells at different ratios at day 5 (**A**), day 7 (**B**) and day 9 (**C**) after B cell transduction. Resting T cells (dark blue) were not activated with CD3/CD28 microbeads and were used as the negative control. T cells (red) activated with CD3/CD38 microbeads were used as positive control.

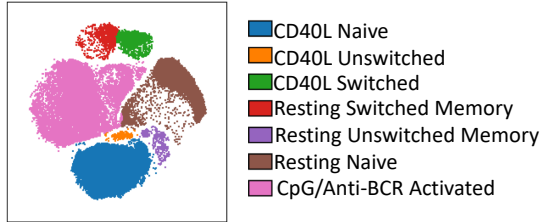
To gain a deeper understanding of the changes in the phenotype of B cells upon activation, we used cytometry by time-of-flight (CytoF) and a panel of 40 antibodies against markers of B cell activation, survival, homing and differentiation (**Table 3**). Using viSNE, a t-distributed stochastic neighbor embedding (tSNE) algorithm, we next analyzed

and displayed high-dimensional data on two-dimensional maps (**Figure 11**). Activation of Nontransduced B cells with CpG and anti-IgM for 48 hours resulted in downregulation of CD1d and CD24, CD79b, and IgD (**Figure 11 B**). CD1d is a surface molecule found on B cells that can present nonpeptide antigens to invariant natural killer T (iNKT) cells and has previously been shown to be downregulated in highly proliferative B cells (Chaudhry 2014). Activation of the surface molecules CD24 and IgD has been previously shown to promote B cell apoptosis (Suzuki 2001 and Yang 2018). Therefore, downregulation of these markers may decrease the likelihood of B cells undergoing apoptosis. CD79b is involved in mediating BCR signaling, and it is unclear as to why it would be downregulated upon BCR activation. However, it could be a negative feedback mechanism described by Grundström and colleagues, which argue that this downregulation can aid in higher BCR affinity (Verma-Gaur 2012). We also found that following activation with CpG and anti-IgM for 48 hours, B cells upregulated CD39, CD85j, CD86, CD25 and PD-L1 (**Figure 11B**). CD39 is an ectoenzyme involved in 5-Amp mediated signaling and has been previously associated with Breg function and phenotype (Figueiro 2016). This suggests that upregulation of CD39 may indicate an increase in B cell immunomodulatory function. In addition, CD85j is an inhibitory molecule that has been shown to regulate antibody production (Merlo 2005). An increase in CD85j expression may influence the isotype and number of antibodies produced by these cells, however more studies are needed to confirm this phenomenon. CD86 is a costimulatory molecule expressed by B cells and promotes B cell proliferation (Suvas 2002). Upregulation of CD86 contributes to the proliferative state of activated B cells. Both CD25 and PD-L1 have previously been shown to play a role in Breg formation, indicating that activation with CpG and anti-IgM may lead

to a higher percentage of regulatory B cells when compared to activation by CD40L and IL-2. This experiment highlights the heterogeneity of B cell populations found in PBMCs and the influence of B cell activation in driving an immunoregulatory phenotype.

Figure 11

A



B

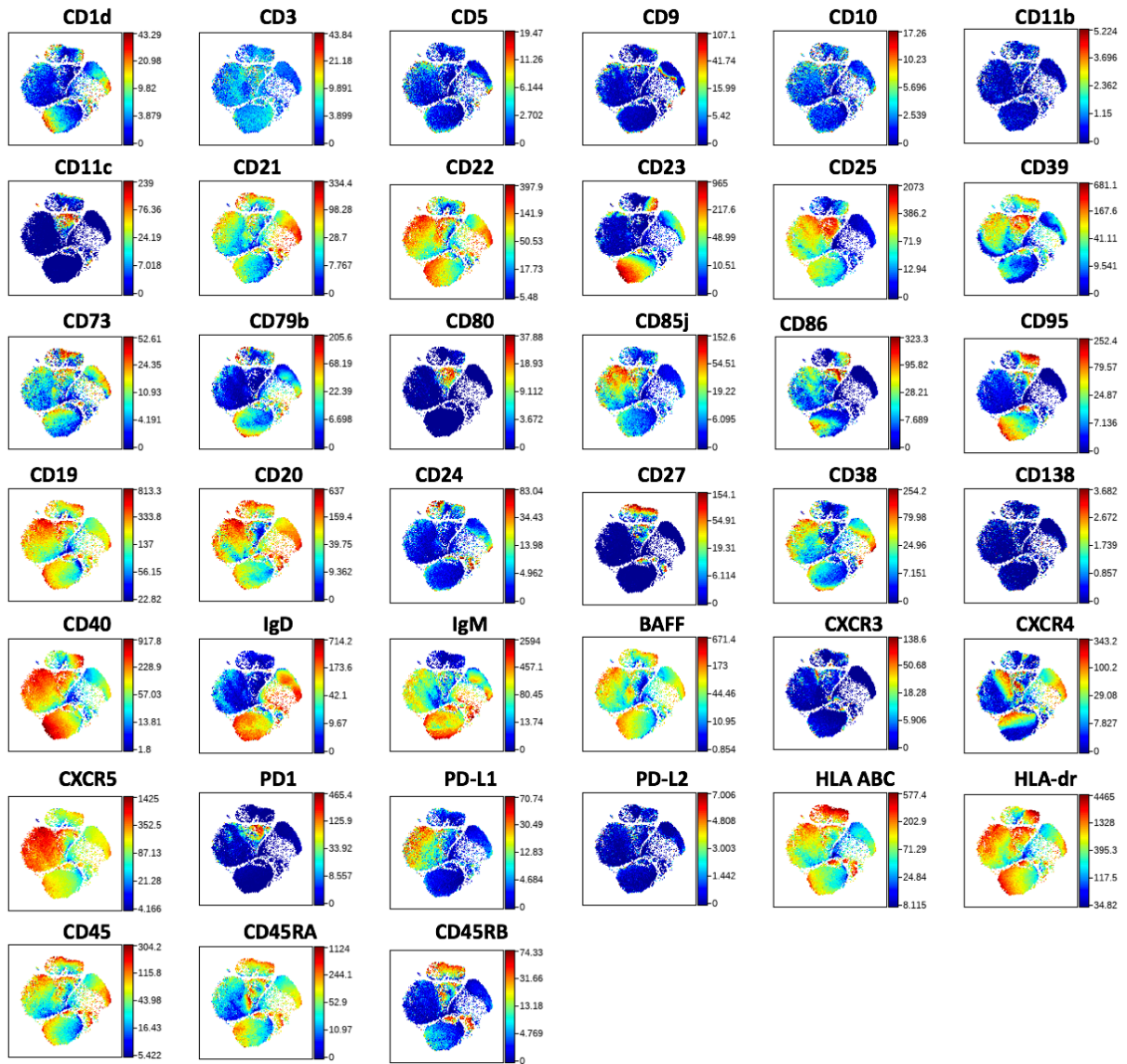
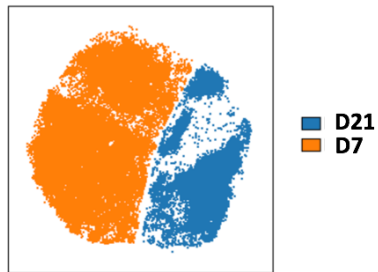


Figure 11. B cells gain CD25 and PD-L1 expression following activation with CpG/Anti-BCR B cells were isolated from PBMCs and activated with either CD40L or CpG/anti-BCR for 48 hours. **A.** Displays the location of each B cell subset on the t-SNE map. **B.** Represents Individual t-SNE maps displaying the surface expression of 39 different B cell markers.

Since expanded B cells lose their capacity to suppress T cell cytotoxicity and proliferation in culture over time, we next examined changes in their surface expression at different culture timepoints by mass cytometry (**Figure 12**). We compared the phenotype of B cells at a timepoint when they are known to be suppressive (day 7) and once they have lost their suppressive abilities (day 21) (**Figure 12 A**). We then performed a *t*-SNE analysis in order to demonstrate the differences in surface marker expression between the two timepoints. This analysis allowed us to visualize high-parameter data on the single cell level. We most notably found that while CD73, PD-L1 and CD25 are expressed on day 7 of culture when the B cells are suppressive, the expression of these markers is lost after 21 days of culture (**Figure 12 B**). These data suggest that CD73, PD-L1 and CD25 may play an important role in the suppressive capacity of our B cells and their loss overtime may contribute to the transient suppressive capacity of B cells generated with this protocol.

Figure 12

A



B

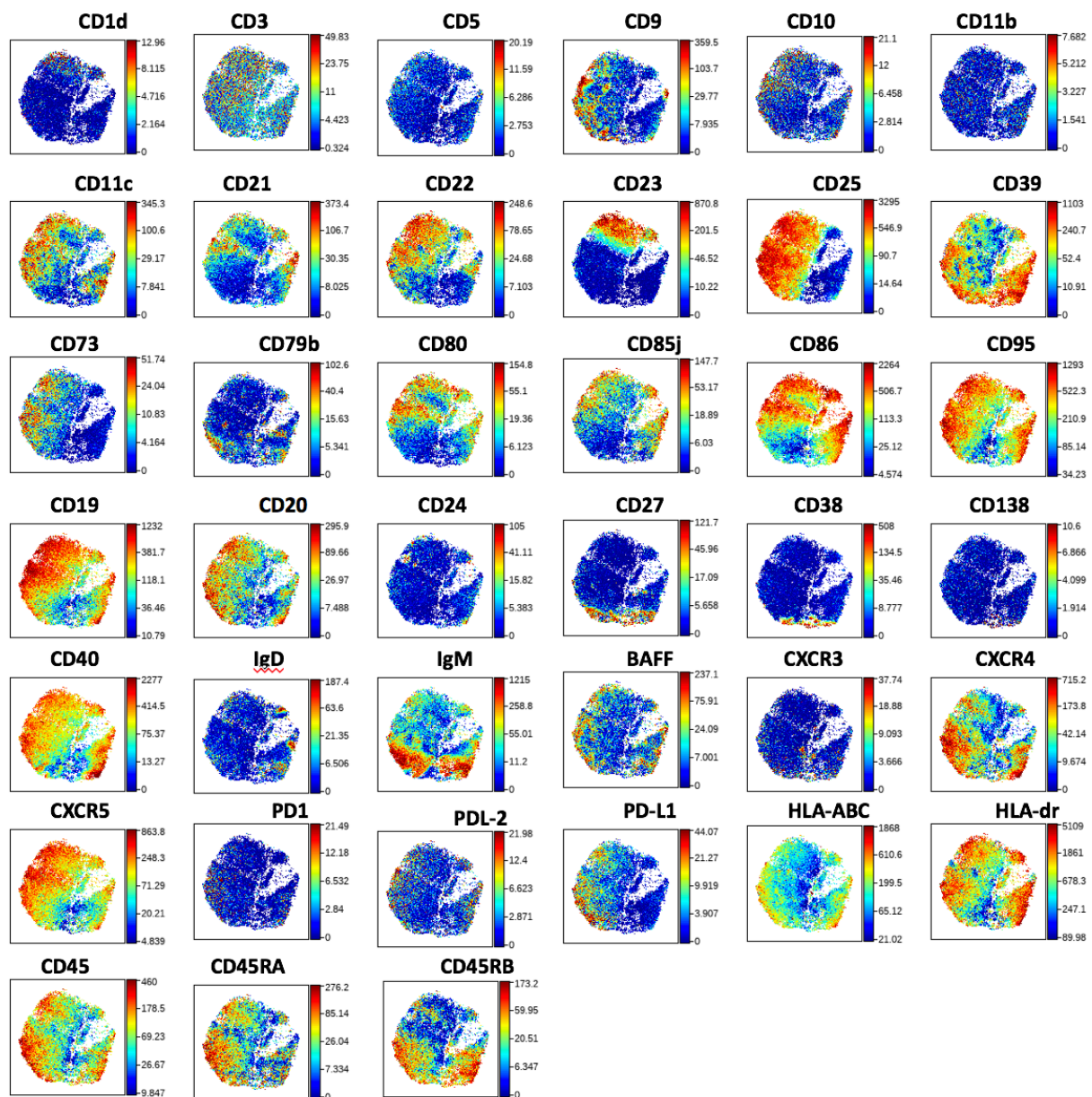


Figure 12. CD40L-IL21 transduced B cells expanded in conditions supplemented with IL-2 and IL-4 lose CD25 and PD-L1 expression after 3 weeks of culture. B cells were activated with CpG and Anti-BCR for two days, followed by transduction with CD40L-IL21. Following transduction, the B cells were cultured in complete SCGM media and supplemented with IL-2 and IL-4. **A** t-SNE map displaying B cell populations in culture for 7 days (orange) or 21 days (blue). **B**. t-SNE maps displaying the expression intensity of 39 surface markers. Low expression levels are represented by the color blue while high expression levels are represented by the color red.

4. Discussion

The immunomodulatory functions of B cells make them attractive candidates for cell therapy of inflammatory immune diseases. However, a reliable method for the large-scale expansion of B cells with suppressive capabilities has not yet been developed. We hypothesized that using genetic engineering tools we can generate a large number of B cells with an immunosuppressive phenotype and with the potential to be used for future cell therapy. Here, we demonstrate that B cells can acquire immunoregulatory function following activation transduction with CD40L, or CD40L and IL-21. Moreover, we provided evidence of T cell suppression by B cells through functional assays assessing T cell cytotoxicity and proliferation. We found that our B cells are capable of effectively suppressing T cell cytotoxicity and proliferation but that this effect was transient. In order to gain a deeper understanding of why expanded B cells only transiently suppress T-cell activity, we performed CytoF to interrogate the phenotype of the cells at the single cell level. We found that upon activation, B cells gain CD25 and PDL-1 expression. However, after 3 weeks of culture, the expression of CD73, CD25 and PDL-1 is lost. These data

suggest that these molecules play an important role in the underlying mechanism through which regulatory B cells gain their immunosuppressive function.

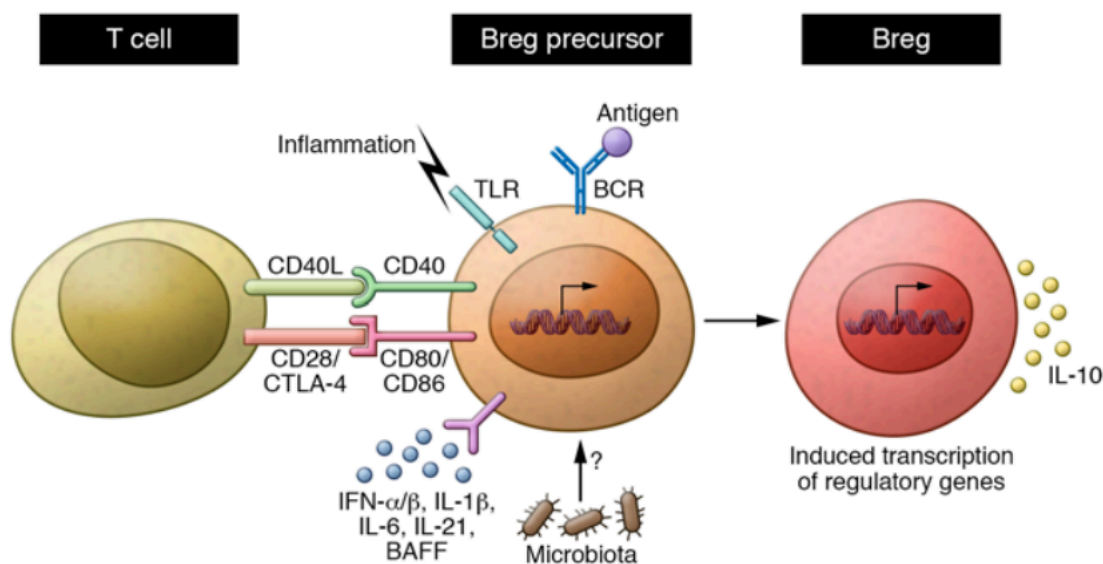


Figure 13. Signals shown to play a role in Breg formation. Multiple signals can contribute to Breg formation, including T-cell derived signals, cytokines, and microbial derived antigens. Reproduced with permission from C. Mauri, "Human regulatory B cells in health and disease: therapeutic potential" (2017). License number: 1081976-1

Since our goal was to create a protocol for large scale B cell expansion, we were interested in understanding the signals required for B cell proliferation. We chose to first activate B cells by stimulating them through their BCR and TLR, which has been previously shown to induce Breg formation (**Figure 13**). Philippe Musette and colleagues (Bouaziz 2010) demonstrated that stimulating B cells with CpG and anti-BCR will promote Breg formation. However, stimulation of B cells through these signals alone failed to induce large scale expansion of B cells. In an attempt to increase the proliferation of these

B cells, we genetically engineered them with retroviral constructs containing CD40L, a marker normally expressed by T cells that has also been shown to induce Breg formation (**Figure 13**). Thomas Tedder and colleagues used adoptive B cell transfers in a mouse model of experimental autoimmune encephalitis (EAE), commonly used to study multiple sclerosis, to show that CD40 stimulation can aid in Breg formation. Their studies revealed that B cells that do not express CD40 fail to diminish the severity of EAE when infused into B cell-deficient mice. However, infusion of B cells expressing CD40 that were cultured with CD40L and IL-21 expressing feeder cells were able to significantly diminish EAE disease severity. This study highlights the importance of CD40 signaling in Breg formation (Lykken 2015).

Our findings confirmed the importance of IL-21 in B cell proliferation, as B cells transduced with CD40L that were not supplemented with IL-21 in the media had significantly lower expansion rates (**Figure 6**). However, B cells transduced with CD40L-2A-IL21, which had the ability to secrete IL-21, had similar expansion rates of those transduced with CD40L alone that were supplemented with exogenous IL-21. These data support the notion that IL-21 drives B cell proliferation.

In order to promote the survival and proliferation of B cells, we tested the addition of different cytokines to the culture, including IL-2 and IL-4. IL-4 has been previously used to drive Breg formation (Lykken 2015). In addition, IL-2 has also been shown to promote B cell proliferation. Our studies showed that IL-2 and IL-4 cytokine supplementation significantly increased B cell proliferation rates.

We next asked if the expanded B cells could suppress T cell function in vitro. Previous research has shown that coculturing CD19⁺ CD24^{hi} CD38^{hi} B cells with T cells can limit T cell cytokine production and proliferation (Flores-Borja 2013). While our functional studies confirmed the immunosuppressive capacity of expanded B cells, this effect was lost when the B cells were kept in culture over time.

To understand why the expanded B cells lost this suppressive capability overtime, we used mass cytometry (CyTOF) to interrogate the phenotype of the B cells at the single cell level. We found that upon activation with CpG and anti-IgG/IgM, B cells express PD-L1 and CD25 (**Figure 11**). We next examined the surface expression of various markers on the surface of B cells cultured for 7 days (when they were immunosuppressive) versus 21 days (by which time they had lost their immunosuppressive function). We found that B cells lose expression of PD-L1, CD73 and CD25 by day 21 of culture (**Figure 12**), suggesting that these markers may be important the ability of B cells to suppress T cell cytotoxicity and proliferation.

In summary, we were able to achieve a 300-fold expansion of primary B cells after retroviral transduction with CD40L and supplementation with cytokines, including IL21 (+/- IL2 and/or IL4) (**Figure 6**). In addition, we were able to use functional studies to demonstrate the suppressive capacity of B cells on T cell function and proliferation. However, the ability of B cells to suppress T-cell function was transient and depended on the B:T cell ratio. To gain insight into the potential mechanisms that could explain why

these B cells lose their capacity to suppress T cell proliferation and cytokine secretion, we performed mass cytometry to phenotypically profile the suppressive B cells over time. The data generated from these experiments pointed to a number of signaling pathways that we could potentially manipulate in future studies in an attempt to prolong the B cell suppressive function.

5. Future directions

In this study we successfully developed the protocol for the genetic modification and expansion of primary B cells with immunosuppressive properties. However, these B cells lost their suppressive capacity over time. Understanding the mechanisms underlying this loss of suppression will provide us with valuable insight that could help us design future experiments to expand B cells with long term suppressive capacity.

There are multiple factors that could be contributing to this transient suppression. One possibility could be their inability to secrete suppressive cytokines over time. In order to test this, we would like to perform ELISA (enzyme-linked immunosorbent assay) to measure the levels immunosuppressive cytokines such as IL-10, IL-35, and TGF- β in supernatants collected from B cells cultured over time. We anticipate a decrease in IL-10, IL-35, and/or TGF- β secretion in B cells that lose their suppressive capacity over time.

Another possibility could be alterations in the metabolism of B cells as they are cultured over time. In the future, we would like to study the metabolic profile of expanded B cells over time and correlate our findings with their suppressive function. There is compelling evidence suggesting that activated Tregs have altered lipid metabolic pathways that contribute to their survival and immunosuppressive functions (Field 2019). Preliminary data from our lab also suggest that regulatory B cells may work through similar mechanisms. We plan to perform single cell RNA sequencing to define the metabolic transcriptomic profile of regulatory B cells as they are kept in culture. In addition, we plan

to use the Seahorse assay to determine the mitochondrial respiration and glycolytic capacity of B cells generated under different conditions. These experiments will allow us to determine how the B cell metabolic profile is changing over time.

We would also like to assess changes in telomere length of B cells as they continue to divide in culture. Telomere length has been previously identified as a way of protecting chromosome integrity, and that telomere length can be associated with B cell antibody responses. Najarro and colleagues demonstrated that B cells that produce a robust antibody response are associated with longer telomeres, while B cells that produce a weak antibody response are associated with shorter telomeres (Najarro 2015). However, elucidating the telomere length in suppressive B cells has not yet been shown. We are interested in seeing if the telomere length changes as B cells are kept in our culture conditions, as we may be able to associate shorter telomere length with a loss in suppressive capacity.

We plan to analyze the clonality of B cells expanded using these culture methods, to determine if the cells with suppressive function are oligo or polyclonal. This information will provide insight to the B cell repertoire of the expanded B cells and whether certain clones could have a greater suppressive capacity.

Lastly, we would like to test the *in vivo* suppressive activity of induced regulatory B cells in a xenogeneic NOD *SCID* gamma mouse model of graft versus host disease (GvHD). We will use the established xenogeneic mouse model of GVHD in our lab to test the *in*

vivo suppressive activity of Bregs. In addition, to determine the optimal cell dose, we will ascertain the optimal scheduling of Breg administration as well as the dose of B cells needed to mitigate signs of GVHD. These studies will determine if ex vivo engineered and activated B cells can suppress T cell inflammation in an *in vivo* setting.

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