The differential role of regulatory B cells in cancer and allo-immunity

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for the degree of Doctor of Philosophy

By

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Declaration of Originality

I herein, Anushruti Sarvaria, declare that the work in this thesis is my own and has not been submitted for the award of any degree at any other university. Where colleagues have made valuable contributions they have been acknowledged.

Copyright Declaration

Dedication

*This thesis is lovingly dedicated to my family; my father "**Naresh"** and my mother "**Manju"** to whom I owe my life.*

*I would also like to dedicate this work to my sister "**Pulkita"** and my brother "**Abhaas"**; your love, friendship, encouragement and faith in me have enabled the hours of research, contemplation and writing necessary to complete my study. Thank you for listening to my problems and providing perspective. I would not be who I am today without you all.*

*This thesis is also in debt to **Prof. Katy Rezvani**, the finest mentor and role model, without whom none of my study would be possible. My heartfelt gratitude for teaching and guiding me through my study and for being my family in Houston.*

*To **Houston**, thank you for giving me the most memorable 2 years of my life. I never thought I loved you so much!*

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" I can no other answer make, but, thanks, and thanks."
- Shakespeare, cited in Craig (1914).

Abstract

A new wave of research recognizes a distinct subset of B regulatory cells (Breg) that maintain immune tolerance. Breg cells have been shown to exert immunoregulatory functions through the production of interleukin (IL)-10 and appear to play important roles in autoimmunity and in cancer. Despite the extensive body of evidence reinforcing the notion of B cells as potential regulatory cells, some controversy over the paucity of markers that can unequivocally identify Bregs still exists. To study the role of Breg in immune surveillance, I designed a comprehensive multi-parameter panel of surface antibodies to define B-cell subsets in peripheral blood (PB) and cord blood (CB). The intracellular detection of IL-10 combined with flow cytometric phenotyping presented in my thesis demonstrate the presence of IL-10–producing Bregs with Treg-independent immunosuppressive functions in both the IgM memory (CD19+IgM+CD27+) and transitional (CD19+CD24hiCD38hi) PB B-cell subsets in healthy donors. The regulatory function PB Bregs against CD4+T cells and CD56+NK cells required both cell-cell contact and IL-10 production. Moreover, I demonstrate that Breg populations are expanded in the PB of AML patients and exert potent suppression of NK function mediated through 2B4-CD48 signaling. I further demonstrated the presence of IL-10-producing B cells with Treg-independent immunosuppressive properties in CB with the ability to suppress allogeneic-CD4+T cells through IL-10, as well as cell-cell contact mediated mechanisms involving CTLA-4 and CD80/CD86. I found an early and robust recovery of IL-10+B cells post-CBT. High Breg frequencies in CB may attenuate T-cell responses and contribute to the lower rates of cGVHD.

My findings have important clinical implications and suggest that Bregs may be exploited to treat immune-mediated diseases. Whereas, strategies to deplete Bregs for optimal anti-cancer immunotherapy may benefit antitumor activity in AML and other cancers, adoptive transfer of donor-derived Bregs post transplant may offer a potentially effective immunomodulatory therapy for the treatment of GVHD.

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List of Abbreviations

ABCB1	ATP-binding cassette B1
ADCC	antibody-dependent cell-mediated cytotoxicity
Ag	antigen
AHSCT	Allogeneic Hematopoietic Stem Cell Transplantation
AML	acute myeloid leukaemia
APC	antigen presenting cells or allophycocyanin
B10	IL-10 producing regulatory B cells
BAFF	B cell activating factor
BCR	B cell receptor
BD	Becton Dickinson
BFA	Brefeldin A
BM	Bone marrow
Breg	Regulatory B cells
CB	Cord blood
CBMC	cord blood mononuclear cells
CBT	Cord blood transplantation
CD	cluster of differentiation
CFSE	carboxyfluorescein diacetate succinimidyl ester
CIA	collagen induced arthritis
CLL	chronic lymphocytic leukaemia
Cntl	control cells
CpG	cytosine-p-guanine dinucleotide
CR	complement receptor
CTLA-4	cytotoxic T-lymphocyte associated protein 4
DC	dendritic cells

DLI	donor lymphocyte infusion
DMSO	dimethylsufoxide
DUCBT	Double unit cord blood transplantation
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
FACS	fluorescence-activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanat
FMO	fluorescence minus one
FSC	forward side scatters
Foxp3	forkhead box P3 transcription factor
G-CSF	granulocyte-colony stimulating factor
GC	germinal centre
GVHD	Graft versus host disease
GVL	Graft versus leukaemia
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigens
HSC	hematopoietic stem cells
HSCT	hematopoietic stem cell transplantation
IBD	inflammatory bowel disease
IFNγ	Interferon-gamma
Ig	immunoglobulin

IL	interleukin
ILT	inhibitory receptors Ig-like transcript
iNKT	invariant natural killer T cells
Iono	Ionomycin
ITP	idiopathic thrombocytopaenia purpura
ITSM	immuno-receptor tyrosine-based switch motifs
JAK	Janus Kinase
KIR	killer-cell immunoglobulin-like receptor
KO	knockout
LN	Lymph node
LPS	Lipopolysaccharides
mAb	monoclonal antibodies
MFI	median florescence intensity
MHC	major histocompatibility antigens
mHA	minor histocompatibility antigens
µl	microliter
MLN	mesenteric lymph node
MS	multiple sclerosis
MSC	mesenchymal stem cells
mTOR	mammalian target of rapamycin
MZ	marginal zone
MZB	marginal zone B cells
MZP	marginal zone progenitor cells
NF-κB	nuclear factor kappa- B
NK	natural killer
NKG2	natural killer group 2

NKT	natural killer T cells
PB	Peripheral blood
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
Pg	pictogram
PMA	phornol 12-myristate 13-acetate
pSHP-1	phosphorylated Src homology region 2 domain-containing phosphatase-1
pSTAT	phosphorylated signal transducer and activator of transcription
RIC	reduced intensity conditioning
RPMI	Rosewell Park Memorial Institute medium
RT	room temperature
SAP	SLAM associated protein
SH2	src-homology 2 domain
SHM	somatic Hypermutation
SIGLEC	sialic acid-binding immunoglobulin-type lectins
SLAM	Signaling lymphocytic activating molecule
siRNA	Small interfering RNA
SLE	systemic lupus erythematosus
SHP-1	Src homology region 2 domain-containing phosphatase-1
SSC	side scatter
SHM	somatic Hypermutation
STAT	signal transducer and activator of transcription
Tr	Transitional B cells

TD	T cell dependent
TGFβ	transforming growth factor beta
Th	T helper
TLR	Toll-like receptor
TNFα	tumour necrosis factor alpha
Treg	regulatory T cells
XLP	X-limked lymphoproliferative disease

Chapter I. Introduction

I.1 Allogeneic Haematopoietic Stem cell Transplantation (AHSCT)

Allogeneic haematopoietic stem cell transplantation (AHSCT) is considered an established curative option of effective adoptive cellular immunotherapy for the treatment of many haematological disorders (Barrett and Battiwala., 2010, Antin, 2002). In the ensuing years since the first successful allogeneic bone marrow transplantation in humans was performed (Gatti et al., 1968, Bach et al., 1968), an extensive body of evidence has highlighted AHSCT as an optional therapy for autoimmune conditions, metabolic disorders, severe immunodeficiencies and malignant and non-malignant diseases (Svenberg et al., 2009, Gyurkocza et al., 2011). AHSCT aims to improve heamatopoietic function through sustained engraftment, which is commonly monitored by chimerism, a valuable detection method for the most fatal causes of treatment failure in transplanted patients; GVHD outcome and graft failure or rejection (Svenberg et al., 2009). In contrast to autologous HSCT, allogeneic HSCT aims to implement induced tolerance through the transfer of a genetically different healthy immune complex (Svenberg et al., 2009). The increased use of modulated reduced-intensity conditioning (RIC) regimens to reduce acute toxicity and improve supportive care have widened the application of AHSCT to a broader spectrum of medical disorders and older patients who tend to have more aggressive disease (Barrett and Battiwala., 2010, Gyurkocza et al., 2011). The benefits of AHSCT rely heavily on the immunological consequences of the graft versus tumor (GVT) effect or graft-versus-leukemia (GVL) effect and immunosuppressive features of the conditioning regimen on the recipient's haematopoietic system (Pidala et al., 2011, Arnout et al.,

2014). Current clinical strategies are exploiting T-lymphocyte-mediated GVT effects to improve the prospect of survival after AHSCT (Barrett and Battiwala., 2010). However, despite a reduction in non-relapse mortality rates following AHSCT witnessed in the last decade, acute and chronic graft-versus-host disease (GVHD) and infections remain a major obstacle to success (Barrett and Battiwala., 2010, Pidala et al., 2011). These potentially fatal complications are a result of accompanying immunodeficiencies, toxicities of the preparative regimens and the organ damage induced by GVHD, leading to treatment-related mortality and morbidity associated with AHSCT (Barrett and Battiwala., 2010). Thus, the ultimate goal of AHSCT is to eradicate GVHD and augment GVL effect in transplant recipients.

I.2 Cord Blood Transplantation

Due to their less stringent requirement for human leukocyte antigen (HLA) matching, human cord blood (CB) is widely used as a source of hematopoietic stem cells (HSC) for many patients with haematological diseases who can be cured by AHSCT but lack a fully matched related or unrelated donor (Beaudette-Zlatanova et al., 2013, Komanduri et al., 2007, Stanevsky et al, 2009). Approximately, 30% of patients who require an allograft through AHSCT will have an HLA-identical sibling donor, and despite over 20 million adult volunteers registered on the National Marrow Donor Program and affiliated registries, many in this group, especially patients of ethnic minorities, will lack a suitably matched unrelated donor (Barker et al., 2010, Ballen et al., 2013). Hence, CBT has extended treatment options to patients that require AHSCT but are ineligible for this therapeutic approach.

In 1989, the first human related CBT was performed in France in a child with Fanconi Anemia (Gluckman et al., 1989). Almost 7 years later the first adult received an unrelated CBT (Laporte et al., 1996). Since this pivotal approach, over 30,000 CBT have been performed in children and adults with subsequent international cord blood banks that have been founded with over 600,000 units available for both related and unrelated CBT (Ballen et al., 2013). High rates of success have been reported with both related and unrelated CBT for several haematological diseases (malignant and non-malignant) in the pediatric setting as children require a lower cell dose than adults (Ballen et al., 2013). Successively, with improved supportive care practices, use of double cord blood units with a greater focus on units with sufficient cell dose, use of non-myeloblative conditioning, clinical strategies aimed at augmenting engraftment and improved donor selection by HLA matching, CBT outcome in adults has progressed (Ballen et al., 2013, Komanduri et al., 2007). Since cord blood has been reported to contain fewer nucleated cells/kg (by 1–2 logs) than mobilized peripheral blood or bone marrow, most adult patients are given two mismatched cord blood units (matched at 4/6 HLA alleles), with the purpose of overcoming the limited cell dose recovered in a single cord blood unit, to overcome delayed engraftment (Barker and Wagner., 2003, Komanduri et al, 2007, Stanevsky et al., 2009, Newell et al., 2013). Further, the use of umbilical cord blood (UCB) in comparison to AHSCT results in a more rapid availability of grafts (cryopreserved CBU that have been banked), a broader and more extensive donor pool due to less stringent requirements for HLA-matching (especially for ethnic minorities) due to superior immune plasticity of CB grafts, low risk of infection via latent viral transmission and

lack of risk to donor (Ballen et al., 2013, Gluckman and Rocha, 2009). However, despite these advantages, studies have reported delayed haematopoietic immune reconstitution after CBT (Komanduri et al, 2007). Limited data exist on immune reconstitution in adult CBT recipients in the course of immune recovery during which infection is a leading cause of mortality (Komanduri et al., 2007). Previous evidence has reported delayed T cell recovery and prolonged T cell lymphopenia predominantly in adult CBT recipients and a compensatory expansion in B cells and natural killer (NK) cells (Klein et al., 2001, Komanduri et al., 2007, Beaudette-Zlatanova et al., 2013). Additionally, late memory T cell skewing to the naïve compartment has been associated with thymopoietic failure in CBT patients (Komanduri et al., 2007). Thus, development of clinical strategies to augment engraftment and the recovery of thymopoiesis may improve outcomes after CBT. Additionally, although an increased incidence of acute GVHD (aGVHD) after double-unit CBT (DUCBT) compared with single CBT has been described (Cutler et al., 2011, Ballen et al, 2007) a lower incidence and severity, of chronic extensive GVHD has been reported after CBT with compared with other stem cell sources, despite broader HLA disparity (Beaudette-Zlatanova et al., 2013, Komanduri et al., 2007, Stanevsky et al., 2009). Although the exact cause instrumental for this reduced alloreactivity is not well understood it has been associated with decreased numbers of the naive T cell repertoire in CBT recipients (Cohen and Madrigal., 1998, Komanduri et al., 2007). Notably, due to high numbers and exclusive properties of NK cells in cord blood graft the GVL effect has been described to be preserved after CBT (Dalle et al., 2005, Stanevsky et al., 2009).

Thus, a major goal in CBT would be the use of immune modulatory cells such as T regulatory cells and mesenchymal stromal cells in therapeutic approaches that have shown potential to control GVHD and preserve GVL effect by limiting donor T cells that possess the capacity to cause GVHD by reacting to alloantigens of the recipient (Tolar et al., 2009, Brunstein et al., 2011, Parmar et al., 2014). Advances in immune suppressor cells offer high potential in safe and more efficacious treatment of hematological diseases treatable by CBT.

I.3 Graft versus Host Disease (GVHD)

I.3.1 The classification and manifestation of GVHD

The limited understanding of GVHD hinders the clinical classification of the disease and hampers the development of therapeutic strategies to target GVHD (Pidala et al., 2011, Ferrara et al., 2009).

The adoptive transfer of stem cells from the donor is governed by several factors that mediate donor selection including HLA-matching, ABO blood group, sex, age and CMV serostatus (Tay et al., 2012). Other factors such as killer immunoglobulin (KIR) immunogenetics and minor histocompatibility mismatch are being increasingly considered in donor selection and the outcome of AHSCT (Gratwohl, 2007, Dickinson and Charron, 2005). The success of stem cell transplantation is determined by engraftment and homing of donor stem cells to the bone marrow, combined with education of the donor immune cells to mediate tolerance (Blazer et al., 2012, Lynch et al., 2009). However, this effect can be counterbalanced by concurrent alloreactivity of

donor T lymphocytes against the host's healthy tissues or organs (Blazer et al., 2012, Ferrara et al., 2009), giving rise to complications of GVHD.

The development and severity of GVHD in transplant recipients is reliant on several factors including, age of recipient, heamatopoietic graft source, toxicity of the conditioning regimen and practices used for GVHD prophylaxis (Pidala et al., 2011). Traditionally, GVHD has been divided into 2 forms, acute and chronic, based on the time of its onset. Acute GVHD (aGVHD) has been described to occur within the first 100 days post transplant with strong inflammatory components, whereas chronic GVHD (cGVHD) occurs after the first 100 days post transplant and is associated with autoimmune and fibrotic mechanisms that are involved in its pathophysiology (Ferrara et al., 2009). However, discrepancies involved in an overlap syndrome of classification between the time of onset of acute and chronic GVHD have led to the National Institutes of Healthy consensus development project that has identified a classification criterion (**Table I-1**) for the diagnosis of GVHD (Filipovich et al., 2005, Shimabukuro-Vornhagen et al., 2009). The onset of aGVHD target regions including the liver (50% of recipients), skin (81% of recipients) and the gastrointestinal tract (54% of recipients) (Ferrara et al., 2009). On the other hand, cGVHD targets specific organs including the skin, mouth, eyes, liver, genitalia and GI tract, which are commonly associated with autoimmune heamolysis and thrombocytopenia (Ferrara et al., 2009). In addition to risk of mortality, GVHD can further lead to organ dysfunction, impaired quality of life, augmented risk of infectious complications through prolonged periods of immunosuppressive therapy and intensified risk of mortality in the immunocompromised allogeneic recipient (Pidala et al., 2011,

Blazer et al., 2012). Despite, the harmful effects of GVHD in transplant recipients, beneficial effects of the alloimmune GVL effect have also been described. Previous studies have indicated that both GVL and GVHD can stem from alloimmune responses induced by discrepancies in minor histocompatibility antigens between HLA-matched AHSCT donors and recipients (Mutis et al., 2010). This implication was first deployed through DLI following AHSCT in the setting of relapsed leukaemia during the 1990's (Kolb et al., 1995, Deol and Lum, 2010, Helg et al., 1998). Succeeding studies have thus demonstrated several leukaemia specific antigens considered to be targets for cytotoxic responses by T lymphocytes in GVL (Mutis and Goulmy, 2002).

Treatment and prophylaxis of GVHD require the use of immunosuppressive agents such as corticosteroids, tacrolimus, mTOR inhibitors (rampamycin), calcineurin inhibitors and monoclonal antibodies targeting CD25 and TNFα, as a result of non-specificity (Blazer et al., 2012, Vogelsang, 2001, Abouelnasr et al., 2013, Rodriguez et al., 2007), all of which carry significant side-effects. Furthermore, studies using a number of targeted agents such as anti-CD20 (Kim et al., 2010), bortezominb (Koreth et al., 2012) and cellular therapy approaches such as mesenchymal stem cells (MSC) are underway. However, as these strategies have not yet been approved by the FDA, the development of novel selective agents and immunosuppressive therapeutic strategies is required.

Table I-1: NIH consensus criteria for diagnosis of acute and chronic GVHD

Category	Time of symptoms after SCT or DLI (days)	Presence of aGVHD features	Presence of cGVHD features
Acute GVHD			
Classic GVHD	<100	Yes	No
Persistent, recurrent, late onset aGVHD	>100	Yes	No
Chronic GVHD			
Classic cGVHD	No time limit	No	Yes
Overlap syndrome	No time limit	Yes	Yes

Adopted from Filipovich et al., 2005

I.3.2 The pathophysiology of GVHD

Billingham described the triad of contributors to the development of GVHD: (i) the presence of functional immune cells in an allograft; (ii) disparity in the expression of tissue antigens between the recipient and donor; (iii) the incapability of the recipient to reject the donor cells (Billingham et al., 1966). Thus, immunocompromised transplant patients are at a high risk of developing GVHD.

Alloreactive reactions between donor-derived CD4+ and CD8+ T lymphocytes have typically been considered to be the chief effector cells arbitrating GVHD pathogenesis (Shimabukuro-Vornhagen et al., 2009, Rezvani et al., 2006). Clinical practices exemplify this paradigm by use of allografts depleted of T cells and T cell directed immunosuppression for the treatment of GVHD, which in some cases has failed to achieve success (Khaled et al., 2009). Host cells can also contribute to the development and pathogenesis of GVHD by

production of pro-inflammatory cytokines such as TNF-α and IL-1 (Blazer et al., 2012, Ferrara et al., 2009). Consequently, upon activation in the host, donor T cells can also secrete IL-2 and IFN-γ and induce direct tissue damage within the host through cell-mediated cytotoxicity by cytotoxic T lymphocytes (Weber et al., 2014).

Further, previous evidence has described an immunomodulatory role of IL-10 in controlling alloreactive T cell activation in HSCT and attenuating GVHD (Weber et al., 2014, Rowe et al., 2006, Holler et al., 2000, Zeller et al., 1999). However, murine studies have found that IL-10 can either suppress (Zeller et al., 1999) or enhance (Blazar t al., 1995) GVHD depending on the cell releasing it (Moore et al., 1993). There is considerable evidence that IL-10-producing donor T regulatory cells, bone marrow cells, dendritic cells and host B cells (Fillatreau et al., 2002, Lampropoulou et al., 2008, Rowe et al., 2006) are possible candidates for immune modulation that may aid to improve GVHD post transplantation. Although T cells have been known as the chief effector cells involved in GVHD, recent data have provided evidence for the suppressive role of B cells in the pathogenesis of GVHD (Shimabukuro-Vornhagen et al., 2009). However a more profound insight into their role is required to tailor strategies to suppress the development of GVHD and improve patient outcome following transplantation.

Acute GVHD is thought to be predominantly driven by alloreactive donor T cells that mediate cytotoxicity against the host's healthy tissue through secreted factors that perpetuate the process (Pidala et al, 2011). Profound genetic disparity between HLA antigens in the donor and host has been described as a major contributor to the severity of aGVHD (Pidala et al., 2011,

Goulmy et al., 1996). The resultant tissue damage from radiation, conditioning regimens or chemotherapy subsequently leads to the recruitment of other immune effector cells, pathogen associated molecular patterns and release of chemokines, which direct effector cells to migrate to target organ and amplify tissue injury as illustrated in **figure I-1** (Ferrara et al., 2009). Activated donor and host antigen presenting cells (APCs) also orchestrate a strong proinflammatory cytokine response by enhancing alloantigen presentation to T cells (Blazer et al, 2012, Pidala et al., 2011). Ultimately, the increased recruitment of proinflammatory cytokines (e.g. IFNγ, TNF, IL-2), macrophages, NK cells and effector T cells cause increased tissue destruction and severe organ damage (e.g. skin, gut, lungs and liver), which is clinically recognized as aGVHD (Blazer et al., 2012). Previous evidence has shown that T cell depletion from the allograft has almost completely abrogated the development of GVHD (Ferrara et al., 2009). Hence, further studies have focused on inhibiting T cell function to develop prevention and therapeutic strategies for aGVHD. However, if untreated, the severity of GVHD pathology could amplify.

Chronic GVHD (cGVHD) is the leading cause of transplants related morbidity and mortality that transpires in 30-65% of AHSCT recipients and signifies 30-50% of 5-year death rates resultant from opportunistic infections and immune dysregulation (Blazer et al., 2012). Clinical manifestations of cGVHD resemble features of autoimmune diseases such as scleroderma, cirrhosis and can lead to debilitating consequences such as blindness, lung disease and joint contractures (Ferrara et al., 2009). Whilst the pathophysiology of aGVHD is propagated through a cascade of inflammatory events, cGVHD is

dependent on the recruitment of polarised CD4+ T cells (Blazer et al. 2012). Resultant damage to the thymus epithelium caused by conditioning regimens, or possibly the prior manifestation of aGVHD consequently impairs negative selection of alloreactive CD4+ T cells initiating a cytokine response that propagates the release of pro-fibrotic cytokines such as IL-10, IL-2 and transforming growth factor β1 that augment the activation and proliferation of tissue fibroblasts as illustrated in **figure I-2** (Blazer et al., 2012). Clinical studies have provided support for an inverse relationship of TGF- β signalling in CD4+ and CD8+ T cell and risk of cGVHD (McCormick et al., 1999, Pidala et al., 2011). Recent evidence has underscored the significance of regulatory dysfunction mainly attributing to T regulatory cells during cGVHD development (Rezvani et al., 2006). However, a new wave of research has postulated a role for B cells in the regulation of cGVHD. (Shimabukuro-Vornhagen et al., 2009, Sarantopoulos et al., 2015). The potential role B cells in cGVHD is supported by reports of successful treatment of GVHD following B cell depletion (Cutler et al., 2006, Cutler et al., 2013). However, further insights into the mechanistic role of B cells in the pathogenesis of this debilitating disease is required to harness the therapeutic benefits of targeting these cells in acute and chronic GVHD.

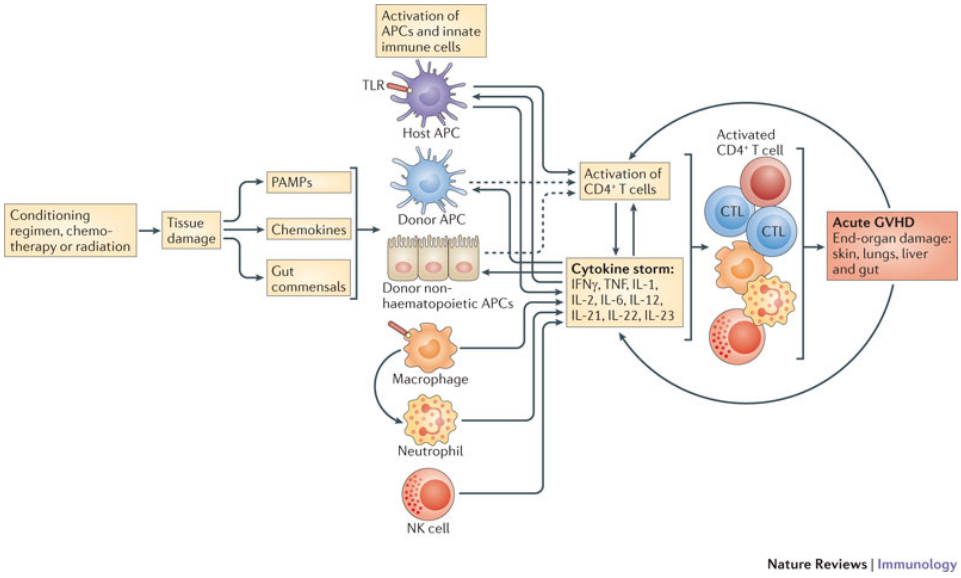

Figure I-1: The sequential series of inflammatory mechanisms simplifying distinct events that occur in the biology of aGVHD.
Resultant underlying damage from the HCT conditioning regimen provokes the release of proinflammatory cytokines and chemokines, which augment expression of antigens on APCs and subsequently lead to increased recruitment, differentiation and migration of effector immune cells leading to further tissue destruction. Figure adopted from Blazer et al., 2012.

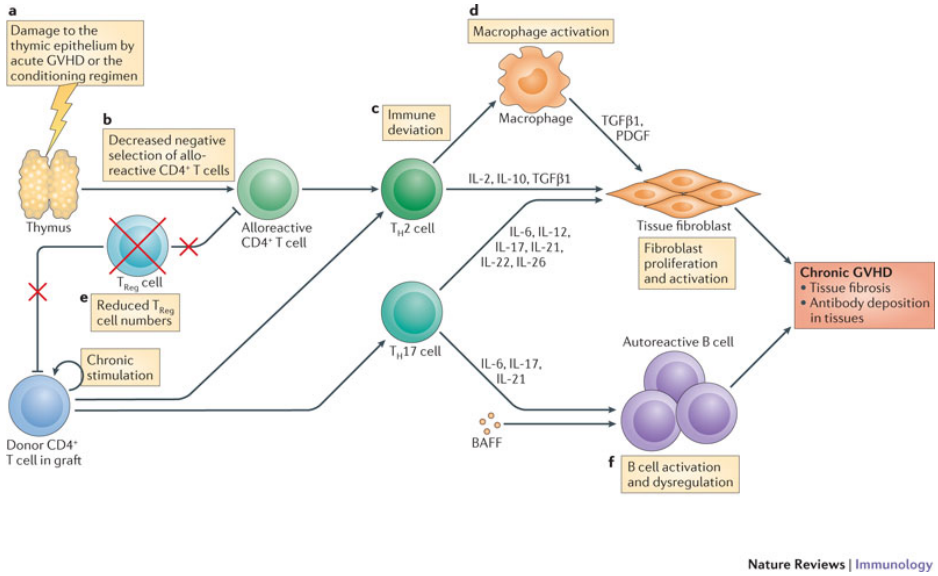

Figure I-2: The sequential series of inflammatory mechanisms simplifying distinct events that occur in the biology of cGVHD.
Resultant damage to the thymus caused by conditioning regimens or prior manifestation of aGVHD, leads to decreased negative selection of alloreactive CD4+ T cells, which become polarized and produce pro-fibrotic cytokines such as IL-2, IL-10 and TGF-β1. Macrophages that produce TGFβ1 and platelet-derived growth factor (PDGF) are also activated. Together the release of these molecules propagates fibroblast activation and proliferation causing tissue fibrosis. Low numbers of T regulatory cells and B cell dysregulation through production of autoreactive antibodies suggested to be the result of high B-cell activating factor (BAFF) levels, all contribute to fibroproliferative changes and antibody deposition in the tissue leading to cGVHD. Figure adopted from Blazer et al., 2012.

I.3.3 The role of B cells in GVHD

I.3.3.1 The role of B cells in human aGVHD

Donor effector T cell mediated destruction of host tissues and organs remains a common contributor to acute GVHD pathology (Pidala et al., 2011). However, the exact role of B cells in this disease remains controversial. Evidence from previous studies has suggested that B cell depletion is associated with prevention of aGVHD and improved outcome after AHSCT (Shimoni et al., 2003, Ratanatharathorn et al., 2009). Previous murine studies have reported a lower incidence of aGVHD with B cell depletion (Schultz et al., 1995). In accord with murine data, clinical studies have also associated the effectiveness of rituximab administered shortly before or after transplantation or as part of conditioning regimens with low rates of GVHD (Shimoni et al., 2003, Ratanatharathorn et al., 2009, Christopeit et al., 2009). In humans, a higher content of B cell progenitors in the donor graft was shown to be associated with less aGVHD (Michonneau et al., 2009). Kamble et al also highlighted successful response in 3 patients with refractory aGVHD treated with rituximab (Kamble et al., 2006). However, other studies failed to show a lower incidence of GVHD in patients with lymphoma after AHSCT after administration of rituximab post transplantation (Glass et al., 2008). These results suggest that early B cell depletion may play a role in the prevention GVHD. Further, high numbers of activated donor B cells in apheresis products was reported to increase the risk of aGVHD (Lori et al., 2008). In contrast, although recipient APCs are essential contributors to aGVHD pathology, Rowe et al (2006) demonstrated that recipient mice that were unable to produce IL-10+ B cells developed a greater severity of aGVHD

than wild-type mice. Further, the induction of IL-10 production by host B cells was found to attenuate experimental aGVHD. This study underlined a protective role for host B cells in GVHD. In agreement with these findings, Weber et al (2014) has also shown a regulatory function of IL-10 produced by host and donor B cells to suppress aGVHD after AHSCT. The results designate a unique regulatory function of B cells, which could be used to improve outcome after AHSCT. Overall, the role of B cells in aGVHD remains controversial, as they have been reported to induce both a protective and pathogenic consequence in aGVHD. Thus, an in-depth imperative understanding on B cells in aGVHD may aid the development of targeted therapies to exploit regulatory B cells to improve patient outcome.

I.3.3.2 The role of B cells in human chronic GVHD (cGVHD)

Classically, donor T cells have been considered as the chief effector cells contributing to the biology of GVHD however, accumulating evidence has clearly demonstrated the fundamental role of B cells in this disease (Kharfan-Dabaja and Cutler., 2011, Shimabukuro-Vornhagen., 2009). Delayed B-cell reconstitution and aberrant B-cell homeostasis after AHSCT has indicated a potential role for B cells through the presence of alloantibodies and high levels of B-cell activating factor (BAFF) in cGVHD development (Sarantopoulos et al., 2015).

B cells have generally been considered to positively regulate inflammation in cGVHD (Socie., 2011, Shimabukuro-Vornhagen., 2009) and B-cell depletion therapy with rituximab has been found to prevent steroid-refractory cGVHD in humans (Cutler et al., 2006, Cutler et al., 2013). Other studies have shown

the presence of B cells in an activated state in the cGVHD environment (Allen et al., 2012). Indeed, evidence of typically activated B cells by increased signaling networks through ERK and AKT pathways have been discovered in B cells isolated from patients with cGVHD (Allen et al., 2012). Furthermore, the presence of autoantibodies has been associated with the onset and severity of cGVHD, postulating a role for donor B cells in this disease (Patriarca et al., 2006, Kier et al., 1990, Svegliati et al., 2007). After AHSCT in humans with gender disparity, presence of alloantibodies to H-Y minor histocompatibility antigens has correlated with cGVHD development (Miklos et al., 2005). Whether this phenomenon is a result of antigenic disparity causing tissue injury or merely a marker for the presence of B cells during recovery post-transplant is not fully known (Svegliati et al., 2007). Moreover, high levels of autoantibodies found in mice with cGVHD and sclerodermatous skin damage has suggested that donor B cells amplify CD4+ T cell expansion and thus induce autoimmune manifestations in cGVHD (Zhang et al., 2006, Young et al., 2012). To date, at least 35 autoantibodies have been related with cGVHD (Kapur et al., 2008).

In addition to these findings, Rozendaal et al described the presence of autoreactive autoantibodies, usually expressed by circulating class-switched IgG memory B cells, during cGVHD. The development of these antibodies, reactive to recipient cells, was suggested to be a product of hyperstimulated B cells induced by alloreactive CD4+ T cells (Rozendaal et al., 1990). The emergence of autoreactive antibodies in cGVHD patients suggests that a critical breakdown in B cell tolerance occurs during cGVHD development after AHSCT. In contrast, other studies have found no association between

autoantibodies and onset or severity of cGVHD (Martin et al., 1997, Rouquette-Gally et al., 1988, Chan et al., 1997). Indeed, no evidence of autoimmune pathology was detected in healthy mice that were injected autoantibodies from mice with GVHD, suggesting that such antibodies are not themselves pathogenic per se (Rolink et al., 1988). Hence, the direct involvement of B cells or autoantibodies in pathogenesis of cGVHD still remains elusive.

Further, a dysregulated B cell homeostatic environment has also been associated with active cGVHD (Greinix et al., 2008). BAFF, also known as THANK, BlyS, TALL-1 and zTNF4, belongs to the TNF family and is a critical survival factor in B cell proliferation and differentiation during B cell development and is secreted by monocytes, some T cells and dendritic cells at increased levels in response to reduced B cell numbers (e.g. following B cell depletion therapy by rituximab) (Saito et al., 2008). Elevated BAFF/B cell ratios have related to the onset and severity of cGVHD compared to patients without cGVHD (Sarantopoulos et al., 2007., Allen et al., 2012., Jacobson et al.,2014). The results support the role of excess BAFF and altered B cell homeostasis in cGVHD. Murine models have demonstrated that B cell survival and differentiation is dependent on a balance of BAFF and BCR signalling during B cell development (Sasaki et al., 2004). Increased BCR responsiveness has been noted in cGVHD patients (Allen et al., 2014). Lesley et al indicated that autoreactive B cells were capable of evading negative selection in the lymphoid germinal center as a result of elevated BAFF levels leading to development of autoimmunity (Lesley et al., 2004). These results

suggest that minimal levels of BAFF prevent B-cell autoreactivity. In accord, increased counts of circulating pre-germinal center B cells and post-GC plasmablasts were also associated with high BAFF levels in cGVHD patients (Sarantopoulos et al., 2009).

This dysregulation in B cell tolerance involving BAFF may play an important role in the pathogenesis of cGVHD and it has been suggested that BCR pathways are candidates for the development of targeted therapeutic strategies (Sarantopoulos et a., 2015).

Moreover, in addition to these studies, altered frequencies of B cell compartments have also been implicated in cGVHD. Higher frequencies of CD25+ B cells in GCSF mobilized graft has been related with higher risk of cGVHD development post-transplant (Tavebi et al., 2001). Additionally, variable impact on the class-switched and nonswitched CD27+ memory B cells has been observed. D'Orsogna et al reported a reduced population of IgM+ CD27+ memory B cells in patients with a history of cGVHD (D'Orsogna et al., 2009). Further, subsequent evidence has reported a reduced population of both class-switched and IgM+ memory B cells in patients with cGVHD when compared to their non-GVHD counterparts (Hilgendorf et al., 2012, Greinix et al., 2008). On the contrary, Sarantopoulos et al observed patients with cGVHD displayed delayed reconstitution of naive B cells despite elevated BAFF levels and a sustained population of CD27+ memory B cells (Sarantopoulos et al., 2009). Further, decreased transitional B cell population (Hilgendorf et al., 2012, Storek et al.,1993), reduced CD5+ B1 like cells (Moins-Terssercne et al., 2013) and an abnormally expanded $CD21^{lo}$ B-cell

population (Kuzmina et al., 2011) have also been described in cGVHD. This observation is in line with studies of autoimmune and immune conditions that have also described an expanded population of CD21- B cells, which suggests a role for CD21- B cells in cGVHD biology as an auto-allo-condition (Sarantopoulos and Ritz., 2015). Furthermore, relatively reduced numbers of immature B cell subsets described in cGVHD suggests that a disturbed B cell homeostasis is a key component of this disease (Sarantopoulos et al., 2009). Although accumulating studies have provided compelling evidence that B cells play a fundamental role in human cGVHD pathology, the mechanisms that initiate and maintain B cell promote and sustain B-cell participation have not been fully elucidated.

Recent research has provided compelling evidence that B cells can suppress or amplify immune responses through cytokine production, designating discrete regulatory and effector B cell sub populations (Sanz et al., 2007). Rowe et al., highlighted the presence of IL-10 producing B cells, which could inhibit alloreactive T-cell expansion, and subsequent induction of GVHD (Rowe et al., 2006). IL-10 producing B regulatory cells have been associated with prolonged allograft survival suggesting a protective role for this population post-transplant (Lee et al., 2012). In accord with this evidence, elevated GC-derived CD24hiCD27+ plasmablast-like IL-10 producing B cells have been described in the regulation of human cGVHD (de Masson et al., 2015). A recent study has highlighted the importance of adoptively transferred donor derived regulatory B cells in attenuating the augmented manifestations of murine sclerodermatous cGVHD (Huu et al., 2013). Moreover Khoder et al.,

has recently described that IL-10 producing regulatory B cells are deficient in recipients of HLA-matched sibling or matched unrelated donor HSCT with cGVHD than healthy donors and patients without cGVHD (Khoder et al., 2014). Collectively, these findings provide compelling evidence for a fundamental role of IL-10 producing B cells in the pathogenesis of cGVHD.

I.4. B cell development

B cell development progresses through several distinctive phases, which occur in the bone marrow and peripheral lymphoid tissues as depicted in **figure I-3** (Cambier et al., 2007). Several micro-environmental constituents and cellular interfaces in the bone marrow niche direct the differentiation of irreversibly committed B cell precursors, which express PAX5 (Nagasawa et al., 2006). Following the rearrangement of the D_H (diversity) and J_H (junction) segments of the immunoglobulin (Ig) heavy chain gene, pro B cells (CD34+CD19+) undertake further rearrangement of the V_H segment yielding a pre-B cell. Subsequently, upon coupling of the VDJ segments (also known as VDJ recombination), at a later phase of their differentiation, pre B-cells express pre B-cell receptor (pre-BCR), which is expressed within a cell and not on the surface (Cambier et al., 2007, Pieper et al., 2013). Pre-BCR signalling averts further recombination and propagates positive selection and proliferation of B cells (Pieper et al., 2013). Moreover, cells that fail to function undergo developmental arrest, apoptosis or receptor editing (Edry et al., 2004). This stage of development is antigen independent and takes place in the bone marrow stromal environment (Maddalay et al., 2010). Hardy et al, highlighted that the sequential transition of B cells through different development stages is delineated by differential expression of surface markers (Hardy et al., 1991). Consequently, surface co-expression of IgM and IgD, namely the mature BCR which is capable of antigen binding is presented on pre-B cells as a result of preferential k light chain rearrangement during receptor editing (Marie-Cardline et al., 2008). The expression of BCR marks the first checkpoint in B-cell development (Cambier et al., 2007). Following

this stage, the vast majority of immature autoreactive B cells are removed from the B cell repertoire through negative selection to avoid any additional development of self-reactive B cells (Sims et al., 2005, Hartley et al., 1991, Duty et al., 2009). In this process, the immature B-cells that survive proceed to the peripheral blood as transitional B cells (Forster et al., 1990, Warderman et al., 2003). Depending on the strength of BCR signalling and antigen specificity, transitional 'immature' B cells populate niches and mature within the splenic microenvironment into follicular or marginal zone (MZ) B cells (Allman et al., 2008). Following an immune response, antigen-activated naïve B cells can obtain T-cell help to form germinal centers and undergo somatic hypermutation and isotype switching to produce memory B cells and plasma cells (antibody-secreting cells), forming the basis for adaptive humoral immunity (Carsetti et al., 2004). In contrast, MZ B cells produce T-cell independent-Ag responses and endure reduced levels of somatic hypermutation (Weill et al., 2009).

The knowledge of B cell development is essential for better understanding and treatment of a number of non-malignant medical conditions.

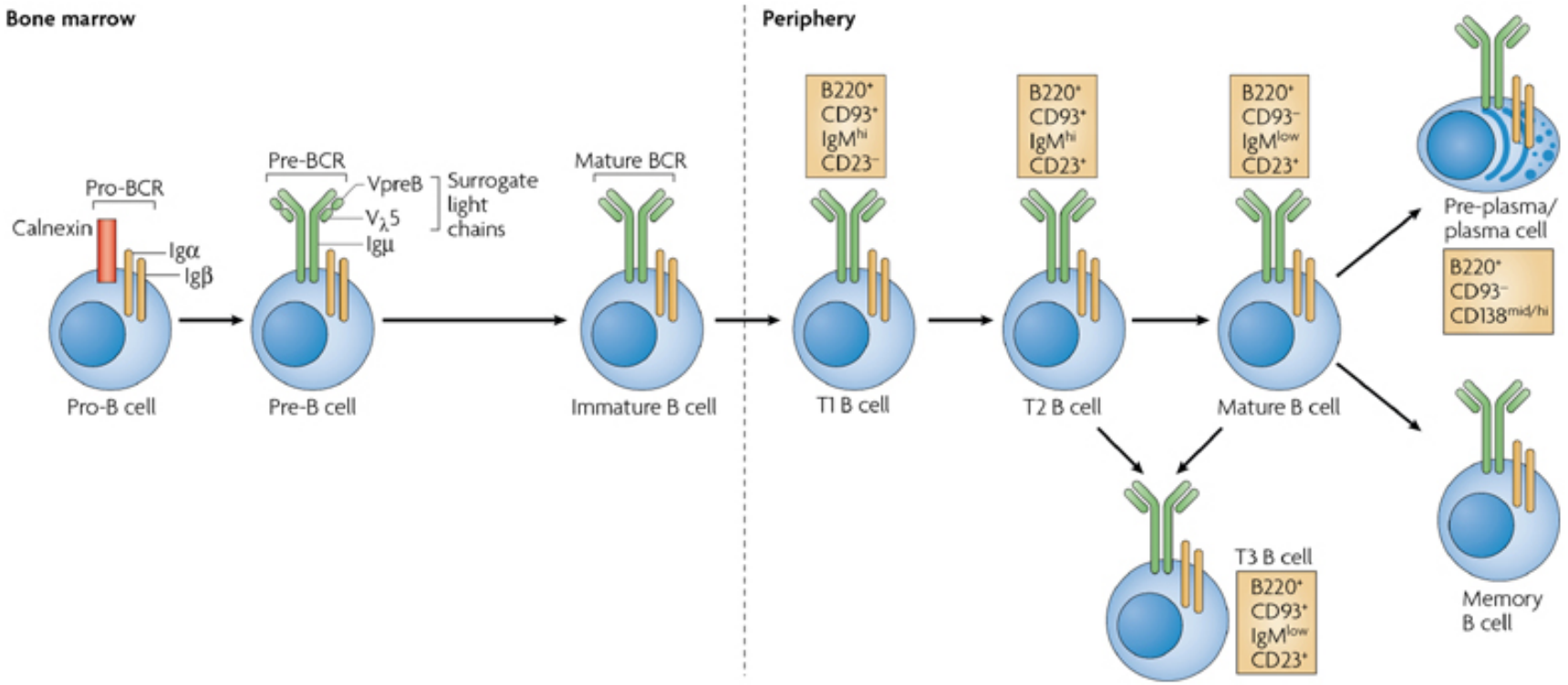

Figure I-3 An illustrative figure showing the distinct phases of B cell development through the bone marrow and periphery. B cell generation evolves through the pro-B-cell, pre-B-cell, immature-B-cell phases and eventually maturing into mature B cells. Adopted from Cambier et al.,2007

I.5 B cell subsets

Immature B cells migrate from the bone marrow and into transitional B cells; the early emigrants from the bone marrow defined by their short lives that must generate a functional BCR in order to overcome negative selection pressures (Hartley et al., 1991, Sims et al., 2005). Transitional B cells preserve the capacity to reconstitute the B cell pool following bone marrow transplantation (Marie-Cardine et al., 2008) and B cell depletion therapy (Anolik et al., 2007). Transitional B cells represent 2.2-7% of B cells in healthy adult peripheral blood and approximately 50% of B cells in cord blood and are commonly distinguished as $CD19^{+}$ $CD24^{high}$ $CD38^{high}$ (Sims et al., 2005, Carsetti et al., 2004, Marie-Cardine et al., 2008). Transitional B cells have been extensively studied in murine spleen based on the expression of heat stable (HAS, CD24) and IgM receptors, transitional B cell population could be distinguished from immature to mature splenic B cells (Loder et al., 1999). Additional phenotypic profiling has further subdivided transitional B cells into T1 ($CD19^{+}$ $CD21^{low}$, $CD23^{low}$, $CD24^{high}$, IgM^{high}, IgD^{low}), T2 ($CD19^{+}CD21^{low}$, $CD23^{high}$, $CD24^{high}$, IgM^{high}, IgD^{high}) and a T3 subset ($CD19^{+}$ $CD21^{low}$, $CD23^{high}$, $CD24^{high}$, IgM^{low} IgD^{high}) (Sims et al., 2005, Allman et al., 2001, Marie-Cardine et al., 2008). T1 cells are known to be susceptible to apoptosis whereas T2 subsets undergo proliferation and differentiation into follicular mature B cells upon BCR ligation mainly through the B-cell activation factor receptor (BAFF-R) pathway (Sims et al., 2005, Mackay et al., 2010, Marie-Cardine et al., 2008). Furthermore, Allman et al identified the expression of CD93 (the B cell lineage precursor marker) in all 3 transitional subsets, which distinguishes their classification from MZ B cells and mature splenic follicular

B cells (Allman et al., 2001). Additionally, transitional B cell are CD27- and express decreasing levels of CD10 and IgM with increasing levels of CD21, CD23 and CD44 in accord with their maturation as their percentage progressively decreases (Palanichamy et al., 2009).

In human peripheral blood, transitional B cell subsets phenotypically resemble murine T1 and T2 subsets (Sims et al., 2005). Additionally, ATP-binding cassette transporter (ABCB1) expression has previously been found to define T3 transitional B cell subset following rituximab therapy in adult peripheral blood (Palanichamy et al., 2009). Transitional B cells are present in the bone marrow, predominantly (T1), spleen (T2), and peripheral blood and cord blood mostly (T3) and in small numbers in lymph nodes (Carsetti et al., 2004, Palanichamy et al., 2009). They mature in the spleen to follicular naïve B cells or MZ B cells depending on the strength of BCR signaling (Carsetti et al., 2004, Weill et al., 2009). However, weak BCR signalling and reduced antigen affinity direct the differentiation of T2 B cells to marginal zone B cells (CD27+IgM+IgD+), which mediate T-cell independent responses and differentiate into short-lived plasma cells (Allman and Pillai., 2008, Carsetti et al., 2004, Weil et al., 2009, Srivastava et al., 2005). Moreover, previous reports have proposed that transitional B cells could mature into IgM producing memory cells upon TLR-9 triggering *in vitro* (Capolunghi et al., 2008). Hence, transitional B cells are often termed as developmental intermediates for maturing B cells.

Human naïve B cells constitute approximately 40-70% of circulating B cells in both peripheral blood and cord blood and are often characterized as

$CD19^{+}CD27^{-}IgM^{low}IgD^{high}$ $CD24^{int}CD38^{int}$ bearing a functional BCR (Carsetti et al., 2004, Sims et al., 2005). Additionally, they fail to express CD21, CD10 and CD5 (Carsetti et al., 2004, Palanichamy et al., 2009). Their functional maturity is reflected by acquisition of surface IgD expression, longer life-span and proliferation upon BCR engagement and signalling (Allman et al., 2001). Having successfully passed the elimination checkpoints, naive B cells circulate through peripheral blood and have high affinity to specific non-self antigens. Additionally, naïve B cells possess the ability to home to and survive in the follicular niches of secondary lymphoid tissues (Allman and Pillai, 2008). Any naïve B cells that fail to encounter an antigen exit the lymph nodes through the lymphatic vessels and die within a few days (Perez-Andres et al., 2010). However, a fraction of auto-reactive anergic naïve B cells expressing IgD are detected in the periphery (Duty et al., 2009). Activation of the follicular naïve B cells is T-cell dependent and requires BCR engagement and CD40 signaling (Allman and Pillai, 2008). Mature naïve B cells are able to participate in T-dependent immune responses by presenting processed peptides on MHC complexes on their surface, which is recognized by cognate CD4+ T cells (Perez-Andres, 2008). Accumulating evidence has further suggested that cytokine production by B cells (i.e. IL-10) can also modulate T-dependent immune responses (Mauri and Bosma., 2012). Following activation, antigen specific B cells migrate to the germinal center and undergo numerous rounds of division, somatic hyper-mutation, class-switch recombination, and affinity selection (Palanichamy et al., 2009). Naïve B cells are distinguished from germinal center B cells by increased expression of CD44 and bcl2 and decreased expression of CD10, CD95, CD38 and HLA-

DR (Allen et al., 2007, Perez-Andres, 2010). Activating signals from the T cell propagate B cell activation resulting in proliferation, germinal center formation and eventual differentiation of naïve B cells into memory or antibody secreting plasma cells (Perez-Andres, 2008). However, the factors governing the differentiation of activated naïve B cells into memory or plasma cells remain elusive (Good-Jacobson and Shlomchik, 2010).

Memory B cells form the basis for humoral adaptive immunity and are large antigen experienced cells, which persist in the absence of immunizing agents and possess increased responsiveness during a secondary response by secreting Ig upon stimulation (Weill et al., 2009, Good-Jacobson and Shlomchik, 2010). Memory B cells constitute approximately 20-30% of B cells in peripheral blood and are almost absent in cord blood but gradually increase in proportion during the first year of life (Kruetzmann et al., 2003, Weller et al., 2004). They are characterized by acquired somatic hypermutations in their rearranged Ig variable regions. Klein and Kuppers, classified memory B cells based on their CD27 surface expression, which is classed as a universal marker for memory B cells (Klein and Kuppers, 1998). Memory B cells are also noted to express CD24hi and CD38int, however the level of expression of these markers on the CD24/CD38 axis varies relative to the expression of CD27 (Sanz et al., 2008, Khoder et al., 2014). Interestingly, about a third of the circulating memory B cells with mutated BCRs are double negative CD27 and IgD and account for 5% of total B cells in peripheral blood, which contradicts the classification of memory B cells on the basis of a single marker (CD27) as too simplistic (Sanz et al., 2008, Fecteau et al., 2006, Weill et al.,

2009). Additionally, this subgroup of B cells has a lower mutation rate than CD27+ memory B cells, which may be a result of incomplete germinal center reactions; however, the function and origin of this subgroup remains to be elucidated (Fecteau et al., 2006, Sanz et al., 2008).

Furthermore, whilst the majority of naïve B cells recirculate between lymphoid tissues, memory B cells reside in areas of antigen drainage including the mucosal epithelium of the tonsils and the splenic marginal zone (Perez-Andres., 2010). As naïve B cells differentiate into memory B cells, they attain a higher affinity to bind to antigens and exhibit an augmented *in vitro* response when exposed to different stimuli that mimic antigen-recognition (i.e. anti-BCR antibodies) or upon follicular T-helper cell interaction via CD40L (Perez-Andres., 2010). Upon such T-cell dependent activation that takes place in GCs within the spleen and lymph nodes, germinal center-derived memory B cells rapidly undergo further rounds of cell-cycle division and a proportion differentiates into antibody secreting cells (plasmablasts), which represent about 1–3% of all circulating healthy adult PB B-cells and are the main providers for Ig (Perez-Andres et al., 2008, Odendahl et al., 2005). Once generated, plasmablast cells migrate from secondary lymphoid tissue and mature within the bone marrow niche, which equips them with factors required for their survival and differentiation before entering the peripheral blood compartment (Odendahl et al., 2005, Perez-Andres., 2010).

Peripheral blood CD27+ve memory B cells can be divided into two major groups, isotype switched (IgM-ve IgD-ve) and IgM memory (IgM+ve IgD+ve) (Perez-Andres., 2010). IgM only memory B cells have been suggested to be germinal center derived and the direct precursor of isotype switched cells

(Weill et al., 2009). Memory B cells that have undergone somatic hyper-mutation are recognized as isotype switched memory B cells, which account for approximately 20% of all memory B cells in PB and are the final product of a successful T-cell dependent germinal center reaction (Good-Jacobson and Shlomchik., 2010, Klein et al., 1998). The mutations affecting components of class-switched B cells prevent the formation of plasma cells and memory B cells expressing IgA, IgG, and IgE (Perez-Andres et al., 2010).

On the other hand, IgM memory B cells account for approximately 50% of memory B cells in PB (Perez-Andres et al., 2010). IgM memory B cells are large cells with abundant cytoplasm and have been known to express IgMhi, IgD+, CD1c+, CD21hi, CD5-, CD23- and CD27+ as well as chemokine receptors such as CXCR4, CXCR5 and CCR7 (Weller et al., 2004, Weill et al., 2009, Tangye and Tarlinton, 2009). The high level of expression of CD21 on this subset is essential for the recognition of bacterial polysaccharides (Zandvoort et al., 2001). IgM memory B cells predominantly produce IgM and have a higher level of CD72 expression but lower CD80, CD86 and CD95 expression than switched memory B cells (Shi et al., 2003). Further, in contrast to the pentamer structure of secreted IgM, membrane bound IgM (BCR receptor) on the surface of IgM memory B cells has a dimer or monomer configuration (Ehrenstein and Notley, 2010). IgM memory B cells develop in the MZ of the spleen with lower numbers of somatic mutations and have not undergone class switching and retain higher clonal diversity than switched memory B cells (Weller et al., 2004). Thus, the spleen and possibly other lymphoid organs have been recognized as a source for IgM memory B cell maintenance (Kruetzmann et al., 2003). Upon antigen activation, this pre-

diversified subset of memory cells produce T cell independent IgM, IgG and IgA (Weill et al., 2009, Weller et al., 2004). IgM memory B cells are involved in providing rapid protection against bacterial infections such as Streptococcus pneumonia through their interaction with MZ macrophages (Kruetzmann et al., 2003). Previous evidence has suggested that upon a primary response to bacterial antigens, IgM memory B cells are able to mature into CD20-IgM producing plasmablasts within 3 days that express high levels of CD38 and CD27 and higher rate of CD40 and BCR engagement (Shi et la,. 2003). Although previous studies have detected their presence in neonatal cord blood, bone marrow, lymph nodes and foetal liver at 14-16 weeks of gestation, others have found them completely absent in cord blood (Kruetzmann et al., 2003). However, previous studies have found that early post-rearrangement Ig diversification of IgM memory B cells through induction of somatic hyper-mutations may occur during foetal life (Scheeren et al., 2008). In accord, evidence has highlighted that the maturation of cord blood-derived transitional B cells into IgM memory B cells *in vitro* after CpG stimulation (Capolunghi et al., 2008). Thus the ontogeny of IgM memory B cells remain unclear; whether this subset is derived as a product of germinal center differentiation or as a derivative from precursor transitional B cells (Weller et al., 2005).

Further, IgM memory B cells essentially serve as a bridge between innate and adaptive immunity and by virtue of their high avidity and poly-reactivity they are known to promote self-tolerance (Ehrenstein and Notley, 2010). It has been suggested that autoreactive MZ B cells or IgM B cells may be potential

regulators of immune function by secreting the immunomodulatory cytokine IL-10 (Zhou et al., 2011, Gray et al., 2007).

Beyond the commonly recognized role of peripheral B cells in mediating humoral immune responses through antibody secretion and in antigen presentation, more recently they have been highlighted to secrete cytokines important in B cell homeostasis and pathogenesis of multiple diseases (Mauri and Bosma., 2012, Lund and Randall., 2010). Accumulating evidence has highlighted a potential role for some B cell populations with regulatory capacity in immune modulation. This revolutionary discovery has led to the subdivision of B cells into effector and regulatory subsets.

I.6 Regulatory B cells

1.6.1 Origin of Regulatory B cells

The hallmark of effective immune modulation in autoimmunity, infection, inflammation and during cancer immune surveillance is regulation by the release of anti-inflammatory mediators and cytokines, such as interleukin-10 (IL-10), produced by regulatory immune cells (Rosser and Mauri., 2015). On one hand, B-cells arbitrate an effector immune response by producing pro-inflammatory cytokines (i.e. IFN-γ and TNF-α), whereas on the other hand, B-cells can contribute to the maintenance of immune tolerance through the expression of immune-regulatory cytokines (i.e. IL-10 and TGF-β) (Harris et al., 2000, Mauri and Bosma., 2012).

The concept that suppressor B cells could orchestrate immune modulation was first proposed nearly 40 years ago, where unlike total splenocytes, B-cell depleted-splenocytes in guinea pigs were associated with an increase in the severity and duration of contact hypersensitivity suggesting that B-cells possessed the ability to inhibit T-cell activation (Katz et al., 1974, Neta and Salvin., 1974). In accord with these findings, B-cells were noted to suppress anti-tumor T-cell responses highlighting their role in modulating immune homeostasis (Gorczynski et al., 1974). These initial findings were later supported by a series of *in vivo* studies showing that adoptive transfer of activated splenic B-cells induced tolerance and differentiation of suppressor T-cells in recipient naïve mice (Shimamura et al., 1982, Shimamura et al., 1984). Further, an enriched pool of splenic B-cells induced tolerance to MHC-alloantigens *in vivo*, which are required for T-cell recognition and activation (Ryan et al., 1984). Moreover, antigen presentation by resting B-cells to

resting T-cells was similarly found to cause loss of T-cell function associated with induction of tolerance (Evans et al., 2000). Well-founded support for the tolerogenic role for B cells was further proposed by two independent studies indicating that B-cell deficient mice cultivate aggravated, chronic forms of colitis or autoimmune encephalitis, indicating that B cells possess regulatory properties (Mizoguchi et al., 1997 and Wolf et al., 1996). Although these seminal observations designated a role for suppressor B-cells in immune modulation and homeostatic balance, the term 'regulatory B-cells' (Breg) was first introduced by Mizoguchi and Bhan in 2002, nearly 30 years later after primary observations indicated their presence (Mizoguchi et al., 2002, Mizoguchi et al., 2006). Mizoguchi et al, described a gut-associated Il-10 producing B-cell subset with upregulated CD1d expression that suppressed the progression of colitis-related intestinal inflammation by downregulating inflammatory cascades in a chronic inflammatory setting (Mizoguchi et al., 2002). Shortly thereafter, the recovery in mice with experimental autoimmune encephalomyelitis was dependent on the presence of IL-10 producing splenic B cells (Fillatreau et al., 2002). Further support for Bregs comes from reports in transplant recipients that withdrawal of immunosuppressive drugs results in higher levels of B cell activation compared to patients who continued immunosuppressant therapy, suggesting a protective role for suppressor B-cells in the transplant setting (Newell et al., 2010). Similar to T regulatory cells, the suppressive capacity of Bregs have been found to be mediated via the production of regulatory cytokines; namely, IL-10 and TGF-β and the expression of inhibitory molecules through cell-to-cell contact-dependent mechanisms that suppress pathogenic T cells (Lundy et al., 2009).

Furthermore, previous studies have indicated that Bregs exist as a heterogeneous population that can be derived from total B cells under appropriate stimulatory conditions (Gray and Gray., 2010). Despite the extensive body of evidence accumulating in the ensuing years since these studies were published, some controversy over the paucity of markers that can unequivocally identify Bregs, particularly in humans, still exists (Mauri and Bosma., 2012). Hence, most current strategies to definitively identify Bregs in a reproducible manner rely on the detection of IL-10, which inhibits proinflammatory cytokine production and differentiation of effector T cells (Mauri and Bosma., 2012). However, it is questionable whether all mechanisms of suppression rely exclusively on the suppressive effect of IL-10. Accumulating evidence has conclusively designated a pivotal role of Bregs that exert their suppressive functions though different mechanisms in divergent models of disease, including autoimmunity, infection, cancer and inflammation (DiLillo et al., 2010). Here, I review the recent advances made in our understanding of both the phenotypic and functional characterization of Breg cells in murine models and humans.

1.6.2 Characterization and Identification of Regulatory B cells

1.6.2.1 Identification and development of regulatory B cells (B10 cells in mice

In recent years, a diverse population of regulatory B cell (Breg) subsets has been identified in murine models of collagen induced arthritis (CIA), experimental autoimmune encephalomyelitis (EAE) and inflammatory bowel disease (IBD) where adoptive transfer of Breg attenuated disease pathology (Yanaba et al., 2008, DiLillo et al., 2010, Mauri and Bosma., 2012). B10 cells were first highlighted in a contact hypersensitivity murine model in which CD19-/- mice exhibited augmented T-cell mediated inflammation whereas mice with hyperactive B cells overexpressing human CD19 transgene (hCD19Tg) presented substantially reduced inflammation regulated by an IL-10 producing $CD1d^{hi}$CD5+ B cell subset (Yanaba et al., 2008). This unique regulatory subset was absent in CD19 deficient mice but represented 1-2% of spleen B cells in wild-type mice, which expanded to approximately to 10% in hCD19Tg mice. CD19-/- mice presented increased levels of ear swelling 96h after immunization with oxazolone. The resulting inflammation normalized upon adoptive transfer of $CD1d^{hi}$CD5+ B cells in CD19-/- mice and IL-10 secretion was found to be a prerequisite for the suppressive capacity of this subset. Thus, the term B10 was introduced to represent this subset of potent regulatory B cells to functionally distinguish this defined B cell subset from other B cell types. Although this study provided evidence for BCR signaling in B10 cell induction, the data remains contradictory (Yanaba et al., 2009, Mauri and Bosma et al., 2012). Further, B cell activation in response to various stimuli including protein kinase C activator phorbol 12-myristate 13-acetate (PMA) and ionomycin allowing measurable levels of IL-10+B cells to be

detected by immunofluorescence staining has been reported (Yanaba et al., 2009). Further, the addition of lipopolysaccharide (LPS) to these cultures was shown to augment the frequency of B10 cells and is commonly employed to identify IL-10+ murine B cells ex vivo. In addition, B cell activation with apoptotic cells or CpG (TLR ligand) has also been found to produce high levels of B10 cells suggesting that B10 cells are antigen-experienced cells (Yanaba et al., 2009, Gray et al., 2007). In accord with these findings, Fillatreau et al proposed that Bregs were recruited in close proximity to an antigen during inflammation in an antigen specific model and expanded in the lymph nodes via T-cell help (Fillatreau et al., 2002). In contrast, non-antigen specific models have shown that Bregs are induced through activated CD154-expressing T-cell interaction regardless of antigen specificity and are abundant in the periphery (Wei et al., 2005). Additionally, Poe et al further noted that CD22-/- mice expressing CD40L presented increased numbers of $CD1d^{hi}CD5+$ B and B10 cells (Poe et al., 2011). Further support for CD40 engagement in inducing Breg function has been highlighted in models of CIA and EAE through IL-10 dependent mechanisms (Mizoguchi et al., 2006, Mauri et al., 2003). Furthermore, the role Toll-like receptors (TLR) in Breg function have also been suggested. Murine B cells lacking MyD88, TLR2, or TLR4 was associated with development of chronic EAE in mice (Lampropoulou et al., 2008). Additional evidence has also shown an expanded pool of B10 cells following CD40 signaling in-vivo and an increase in IL-10+B cells after stimulation by agonistic-CD40 monoclonal antibody (Yanaba et al., 2009, Poe et al., 2011). The expanded B10 population has been termed B10 progenitor cells (B10pro) and CD40 signals have been proposed to mature B10

progenitor cells to B10 cells, whereas BCR cross-linking has been proposed to inhibit this progression (Yanaba et al., 2009). Thus, murine studies have demonstrated that Bregs are able to respond in both an antigen specific and polyclonal manner.

The identification of B10pro cells following *in vitro* stimulation has suggested that selected B cells possess the capacity to produce IL-10 but require additional signals to actively secrete IL-10 (Nouel et al., 2014). Collective evidence has highlighted signals involved in B10 cell development (**figure I-4**). B10pro cells are suggested to have already received appropriate BCR signals for molecular events required for IL-10 secretion but require additional signals such as CD40 stimulation, which induces IL-10 expression in B10pro cells and LPS stimulation with PMA and ionomycin, which acts as a potent stimulus to promote IL-10 secretion (Kalampokis et al., 2013, Candando et al., 2014). Following IL-10 production, some B cells differentiate into memory B10 cells or into plasma cells that are capable of secreting poly-, auto- or self-reactive antibodies depending on their BCR specificity (Kalampokis et al., 2013, Candando et al., 2014). Alternatively, following these events T-cell derived signaling is required for B10 cell expansion, active IL-10 secretion and immune regulation *in vivo*. Yoshizaki et al., supported this theory by showing that Bregs require cognate interactions with IL-21 producing T cells to exert IL-10 dependent suppressive function in autoimmunity (Yoshizaki et al., 2012). IL-21R signaling with CD40 and MHC-II interactions was shown to augment Breg frequency and IL-10 secretion (Yoshizaki et al., 2012). Thus B10pro cells have been suggested to mature into B10 cells that require T-cell derived signals to secrete IL-10 and exert immuno suppression. These data

suggest that cellular cross-talk may play pivotal role in immune regulation than cytokine-dependent immune modulation.

1.6.2.2 Immunophenotype of regulatory B cells identified in mouse models

Mouse B10 cells have been identified in the spleen, lymph nodes, peripheral blood (PB) and gut associated lymphoid tissues including peritoneal cavity and mesenteric lymph nodes (Mizoguchi et al., 2002, Yanaba et al., 2009). B10 cells described in the peritoneal cavity have been identified within the $CD5^{+}CD11b^{+}$ B1a B cell subset (38%), $CD5^{-}$ $CD11b^{+}$ B1b (18%) subset and the $CD5^{-}CD11b^{-}$ B2 (4%) subset (Candando et al., 2014). B10 cells within other mucosal tissues signify approximately 4% of *lamina propia*, 3% of Peyer's patch B cells and 1% of mesenteric lymph nodes (Candando et al., 2014). Additionally, a small proportion of B10 cells have been identified in the lymph nodes and PB (Yanaba et al., 2009). Although a number of B10 surface markers have been identified in murine models (**Table I-2**), a lack of consensual phenotype to define the B10 subset limits the study of IL-10+B cells (Mauri and Bosma., 2012). Hence, most current strategies to definitively identify Bregs in a reproducible manner rely on the detection of IL-10.

Matsushita and Tedder highlighted that only 1-3% of total B cells from wildtype C57BL/6 mice produce IL-10 upon stimulation with PMA and ionomycin (Matsushita and Tedder., 2011). Subsequently, the intracellular detection of IL-10 combined with flow cytometric phenotyping has highlighted that murine splenic IL-10+B cells were predominantly enriched in $CD1d^{hi}CD5^{+}$ B cell subset, where they represent 15-20% of the cells in C57BL/6 mice

(Kalampokis et al., 2013). Further, this unique subset shares phenotypic features with IL-10 producing $CD1d^{hi}CD23^{-}IgM^{hi}CD1d^{hi}$ marginal zone (MZ) B cells, $CD1d^{hi}CD23^{+}IgM^{hi}CD1d^{hi}$ T2-MZ precursor, and $CD1d^{hi}CD5^{+}$B-1a B cells, but does not exclusively belong to these B cell sub-populations (**figure I-5**) (Evans et al., 2007, Yanaba et al., 2008, Mizoguchi and Bhan., 2006, Matsushita et al., 2008). Contrastingly, Ding et al highlighted that although Tim-1 expressing IL-10+ B cells are also enriched in the $CD1d^{hi}CD5^{+}$ subset, $Tim\text{-}1^{-}IL\text{-}10^{+}$B cells are found within the non-$CD1d^{hi}CD5^{+}$ subset (Ding et al., 2011). Further, IL-10 producing plasma cells ($CD19^{+}CD138^{+}$) and plasmablasts have also been reported (Shen et al., 2014, Matsumoto et al., 2014). Similarly, Maseda et al highlighted that B10 cells expressing $IgM^{hi}CD1d^{hi}CD5^{+}CD19^{hi}CD23^{low}CD38^{hi}B220^{hi}$ were capable of differentiating into IgM and IgG secreting CD138+ plasma cells (Maseda et al., 2012). Despite discrepancies found in characterization of B10 cell phenotype, the capacity of B cells to produce IL-10 remains the gold standard to identify pure B10-cell populations for study (Rosser and Mauri., 2015). Nonetheless, the best current strategy for isolating an enriched population of B10-cells for use in adoptive transfer experiments in murine models is the isolation of $CD1d^{hi}CD5^{+}$ B cells or other well-defined B cell subsets enriched for IL-10+B cells (Kalampokis et al., 2013).

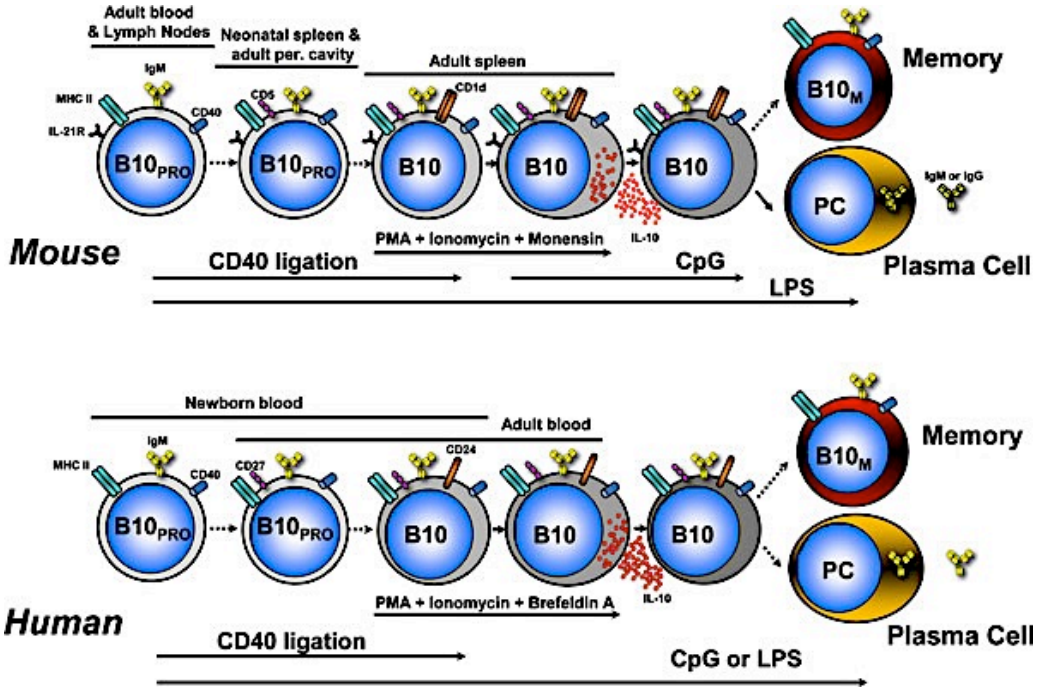

Figure I-4: B10 cell development *in vivo* in mice and human
BCR signalling following antigen encounter induces a small proportion of B cells to produce IL-10. In mice B10 cells mature from a progenitor population (B10pro). Following CD40 stimulation, B10pro cells become competent for IL-10 expression. The resulting CD1dhiCD5-IL-10 competent B10 cells are induced to produce IL-10 in response to LPS stimulation with PMA and ionomycin, which acts as a potent stimulus to promote IL-10 secretion. Following transient B-cell IL-10 production, a small proportion of B-cells differentiate into antibody secreting plasma cells or memory B10 cells. Development of human IL-10+B cells is thought to follow a similar pathway as observed in mice as both B10 and B10pro cells have been recognized in neonatal and adult blood. Whether human B10 cells differentiate into memory cells first or plasma cells remains unknown. Solid arrows depict known associations and dashed arrows represent contemplated links. Adopted from Kalampokis et al., 2013.

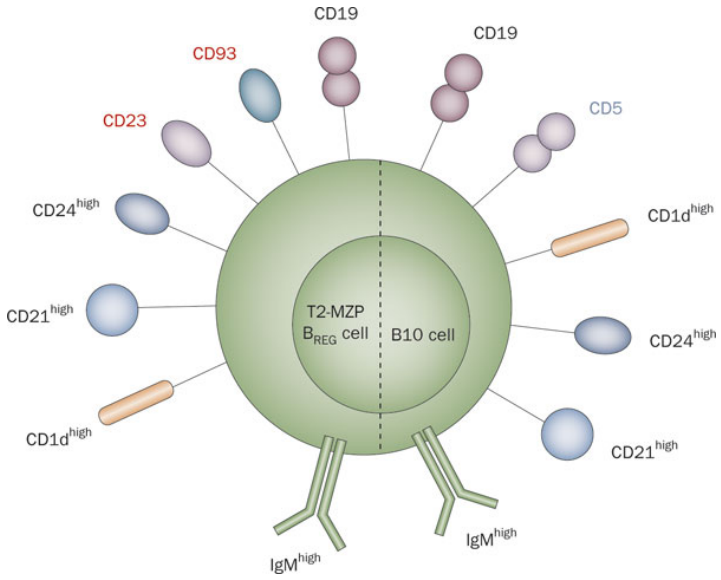

Figure I-5: Phenotypic markers for distinct Breg subsets in mice
A summary of the phenotypic profiles of IL-10 producing B cells: B10 cells and T2-MZP. The colour code illustrates shared and unshared markers by both subsets. Adopted from Mauri and Blair, 2010.

Table I-2: Regulatory B cell subsets identified in mice and human

Breg Cell Type	Mouse	Human	Key Features	Reference
B10	$CD5^{+}CD1d^{hi}$	$CD24^{hi}CD27^{+}$	Found in mice spleen and human blood, produce IL-10, and suppress effector CD4+ T cells, monocytes, DCs	Horikawa et al., 2013, Iwata et al., 2011, Matsushita et al., 2010 and Yanaba et al., 2008
MZ	$CD19^{+}CD21^{hi}CD23^{-}$	-	Found in spleen, produce IL-10, induce Treg cells and suppress effector CD8+ and CD4+ T cells	Bankoti et al., 2012, Gray et al., 2007 and Miles et al., 2012
T2-MZP cells	$CD19^{+}CD21^{hi}CD23^{hi}$ $CD24^{hi}$	-	Produce IL-10, found in spleen, induce Treg cells and suppress effector CD8+ and CD4+ T cells	Blair et al., 2009, Carter et al., 2011, Evans et al., 2007 and Schioppa et al., 2011
Plasma cells	$CD138^{+}$MHC-11^{lo} $B220^{+}$	-	Produce IL-10 and IL-35, found in spleen suppress NK cells, neutrophils and effector CD4+ T cells	Neves et al., 2010 and Shen et al., 2014
Tim-1+ B cells	$Tim\text{-}1^{+}CD19^{+}$	-	Produce IL-10, found in mice spleen and suppress effector CD4+ T cells	Ding et al., 2011 and Xiao et al., 2012
Plasmablasts	$CD138^{+}CD44^{hi}$	$CD19^{+}CD24^{hi}CD27^{int}$	Produce IL-10, found in dLNs (mice) and human blood and suppress DCs and effector CD4+ T cells	Matsumoto et al., 2014
Immature cells	-	$CD19^{+}CD24^{hi}CD38^{hi}$	Produce IL-10, found in blood and at site of inflammation, induce Treg cells, suppress Th1 and Th17 cells, suppress virus specific CD8+ T cell responses, are defective in patients with SLE and RA	Blair et al., 2010, Das et al., 2012, Flores-Borja et al., 2013, Khoder et al., 2014
Memory B cells	-	$CD19^{+}CD27^{+}IgM^{+}$	Produce IL-10 and found in Human blood	Khoder et al., 2014
Br1 cells	-	$CD19^{+}CD25^{hi}CD71^{hi}$	Found in blood and produce IL-10 and IgG4	Van de Veen et al., 2013

Br1, B regulatory 1; DC, dendritic cell; dLN, draining lymph node; IgG4, immunoglobulin G4; MHC, major histocompatibility complex; MZ, marginal zone; NK, natural killer; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; T2-MZP, transitional 2 marginal-zone precursor. Table adopted from Rosser and Mauri (2015)

1.6.2.3 Identification of regulatory B cells in humans

IL-10 producing B cells subsets with regulatory capacity have been recently identified in humans (Iwata et al., 2011). Low but detectable numbers of IL-10+B cells have been found in human blood, tonsils, spleen and neonatal cord blood (Candando et al., 2014). The general scheme of human IL-10+B cell development appears to follow mouse B10pro cell maturation where their response to LPS and CpG stimulation and CD40 ligation induces the maturation of B10pro cells into B10 cells that are capable of expressing IL-10 (Iwata et al., 2011). Some individuals have demonstrated increased IL-10+B cell frequencies in response to TLR4 (LPS) and TLR9 (CpG) stimulation, suggesting that in humans B10pro cells may respond preferentially to TLR stimuli (Candando et al., 2014). Additionally, other studies have observed that whereas CD40 signaling promotes B10pro cell maturation, BCR cross-linking inhibits this progress in human B cell cultures (Iwata et al., 2011, Duddy et al., 2007). In contrast, others have reported that human B10pro development is most optimally induced by BCR and CpG stimulation and independent of CD40 signaling (Bouaziz et al., 2010).

1.6.2.4 Immunophenotype of regulatory B cells identified in humans

Over the last decade, accumulating evidence has defined a subset of IL-10+B cells in humans, which represents <1% of PB-derived B cells with varying phenotypes (Iwata et al., 2011, Blair et al., 2010). Previous evidence highlighted that IL-10+B cells are contained within CD27- naïve B cell compartment, while memory B cells were found to produce pro-inflammatory cytokines in response to different stimulus (Duddy et al., 2004, Correale et al.,

2008). Consistent with these findings, Blair et al., elegantly described an enriched population of IL-10+B cells in the CD24hiCD38hi B cell subset, a phenotype that typically delineates human transitional B cells, which exerted IL-10 dependent suppressive function on CD25-CD4+ effector T cell cytokine production (Blair et al., 2010, Flores-Borja et al., 2013). Conversely, a subset of Breg, analogous to murine B10 cells, was found to be enriched within the CD24hiCD27+ B cell subset, of which 60% expressed CD38 (Iwata et al., 2011). These CD24hiCD27+ B cells exerted suppressive function on CD14+ monocyte proliferation and cytokine production. Extensive phenotyping of IL-10+B cells has also highlighted that they highly express CD48 (B-cell activation marker) and CD148 (marker for human memory B cells)(Iwata et al., 2011). Thus, the CD24hiCD148+ phenotype of IL-10+B cells may suggest that they are enriched in the memory B cell pool or share overlapping markers with memory B cells (Iwata et al., 2011, Candando et al., 2014). Further, Bouaziz et al, provided evidence of IL-10+B cells that fall within both the CD27+ and the CD27-CD38hi transitional compartment (Bouaziz et al., 2010). In accord with this finding, 60% of IL-10+B cells were found within the CD27- naïve population in relapsing remitting MS patients and within the CD27+ subset during relapse (Knippenberg et al., 2011). However, a recent report identified that human IgM+CD27+ memory B cells produced greater levels of IL-10 than CD27- naïve B cells (Miles et al., 2012). A recent report by Khoder et al supported previous studies and in addition showed that IL-10-secreting CD19+IgM+CD27+ memory B cells coexist with IL-10+CD24^{hi}CD38^{hi} transitional B cells in healthy human donors and significantly suppress the proliferation and cytokine production of autologous CD4+ T cells through both

IL-10-dependant and cell-to-cell contact mediated mechanisms (Khoder et al., 2014). Other phenotypes for IL-10 producing CD19+ B cells have also been described. Matsumoto et al identified a subset of $CD19^{+}CD24^{hi}CD27^{int}$ IL-10+ plasmablast regulatory B cells (Matsumoto et al., 2014). Further, a recent study characterized IgG4 expressing human inducible IL-10-secreting B regulatory 1 (BR1) cells that had a high expression of surface CD71 and CD25 but low CD73 and potently suppressed antigen-specific CD4+ T cell proliferation, highlighting a role for Breg in allergen tolerance (van de Veen et al., 2013). Thus, although IL-10+B cells can be modestly enriched in selected B cell compartments, their ability to produce IL-10 remains the gold standard method to unequivocally define Bregs.

1.6.2.5 IL-10 independent mechanisms of regulatory B cell suppression

In addition to IL-10 production, Breg have been reported to exert immune-suppression through other mechanisms including the release of immunomodulatory cytokines such as transforming growth factor β (TGF-β) and IL-35 (Rosser and Mauri., 2014). LPS activated B cells have been shown to induce both apoptosis of CD4+ T cells and anergy in CD8+ effector cells (Tian et al., 2001, Parekh et al., 2003). Additionally, the recent identification of IL-35 producing Breg in negative regulation of immunity demonstrated increased protection against experimental autoimmune encephalitis but markedly improved resistance to *Salmonella*-induced sepsis in murine models as lack of IL-35+ B cell expression augmented Th1 cell responses and the number of splenic macrophages (Shen et al., 2014). Further evidence has also supported the regulatory function of IL-35+Breg, in which the adoptive

transfer of IL-35 producing B cells inhibited experimental uveitis in mice (Wang et al., 2014). In addition to cytokine production, granzyme production by B cells has been found to initiate apoptosis of infected cells following the cleavage of caspases (Trapani and Sutton., 2003). The expression of Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has further identified in a distinct subset of suppressive B cells designated 'killer B cells' (Lundy, 2009). Further, expression of programed death ligand (PD-L) 1 and 2 has also been reported to participate in B cell immune-suppression (Lundy, 2009). Hence in addition to soluble factors, direct cell-to-cell contact may play a vital role in B cell mediated immune-suppression.

1.6.3 Regulatory B cells in immune regulation

Bregs have been reported to modulate immune responses through diverse mechanisms that target different immune cell types (**figure I-6**), including dendritic cells and Th1 and Th2 cells (Matsushita et al., 2010, Tian et al., 2001). Previous studies have highlighted the ability of Bregs to suppress T cell proliferation and production of IL-17 and IFN-γ by Th17 and Th1 cells, respectively (Carter et al., 2012). Consistent with these findings, Yang et al further demonstrated a role for Bregs in T-cell plasticity by observing that IL-10+B cells suppressed Th17 differentiation through attenuating levels of pStat3 in an IL-10-dependent fashion (Yang et al., 2012). Additionally, IL-10+Bregs have been shown to suppress CD4+ T cell proliferation and pro-inflammatory cytokine production through IL-10 (Khoder et al., 2014, Blair et al., 2010) and induce CD4+T cell death by expression of FasL (Tian et al., 2001). Further, adoptive transfer of GIFT-15 induced Breg has been observed to suppress CD4+IFN-γ production and symptoms of EAE (Rafei et al., 2009).

Hence, the dynamic and diverse interaction between Breg-CD4+T cells plays a pivotal role in immune regulation.

Moreover, studies have demonstrated that Bregs can not only suppress Th1-mediated immune responses but also generate conventional Treg cells and IL-10 producing T cells from effector T cells through the production of IL-10 (Carter et al., 2012, Flores-Borja et al., 2013). CD4+ Tregs sustain immunological self-tolerance and homeostasis and within the human CD4+ Treg compartment exist distinct subsets (Abbas et al., 2013). Firstly, naturally occurring FOXP3+ Treg cells that either originate from the thymus or peripheral sites (referred to as thymus- or peripherally- derived Tregs) can further be divided into three subpopulations by the expression of FOXP3, CD45RA and CD25 (Miyara et al., 2014). Additionally, functional activated human FOXP3+ Treg cells express a unique pattern of cell surface markers (high expression of CD25 and low or negative expression of CD127) that can facilitate their isolation (Miaya et al., 2014, Ohkura et al., 2013). Secondly, induced FOXP3+ Treg populations are generated *in vivo* or *in vitro* through the use of TGF- β (Abbas et al. 2013, Ohkura et al., 2013). Although the transcription factor FOXP3 plays a pivotal role in Treg function it is not sufficient for conferring and maintaining Treg function and phenotype (Ohkura et al., 2013). Hence, a third subset of Tregs are described as $CD4^{-}Foxp3^{-}$ Treg cells that mediate their suppressive functions primarily through IL-10 and TGF- β (Ohkura et al., 2013).

Carter et al indicated that IL-10-producing B cells partake in a longer period of contact with CD4+CD25- T cells than IL-10-deficient B cells, which allows IL-10+B cells to convert effector T cells into Il-10 producing suppressive T cells

(Carter et al., 2011). Further, Carter et al highlighted that lack of Bregs coincided with a decrease of peripheral Foxp3+ Treg (Carter et al., 2011). In accord with these findings, CD40-stimulated murine B cells were also found to generate IL-10+Treg cells from effector T cells (Blair et al., 2009). Subsequently, Ray et al reported that the adoptive transfer of wild-type B cells restored both the recovery of EAE and Treg numbers in mice (Ray et al., 2012). Bregs have also been observed to suppress immune responses by CD8+T cells (Bankoti et al.,2012). Human transitional B cells have been implicated in suppressing HBV-specific CD8+ T cell responses in an IL-10 dependent fashion (Das et al., 2012). Additionally, IL-10 producing MZ-B cells have also been shown to suppress CD8+ T cell responses (Bankoti et al., 2012). Further to these observations, the effect of Bregs to exert immune-modulation by cellular interactions has also been noted. Breg can also induce dendritic cells to produce IL-4 and downregulate IL-12, thereby affecting theTh1/Th2 balance (Moulin et al., 2000). Moreover, induction of IL-10+B cells via CD40L interaction on tumor cells can suppress IFN-γ production by NK cells (Inoue et al., 2006). Furthermore, CD1d-expressing MZ B cells, which share phenotypic characteristics with IL-10+B cells has been observed to modulate peripheral tolerance by inducing IL-10 producing T cells in the presence of DCs (Sonoda et al., 2002). Moreover, this process is mediated through glycolipid presentation via CD1d, which are recognized by NKT cells and CD1d−/−mice lacking NKT cells have demonstrated aggravated EAE (Croxford et al., 2006). The role of CD1d in Breg-NKT interaction was further highlighted in a study where human transitional B cells were shown to activate and expand the iNKT pool and thereby indirectly exert their regulatory function

in healthy individuals but not in SLE patients as they have defects in CD1d (Bosma et al., 2012). Overall, Bregs have been reported to exert their regulatory function during immune responses by both direct and indirect mechanisms.

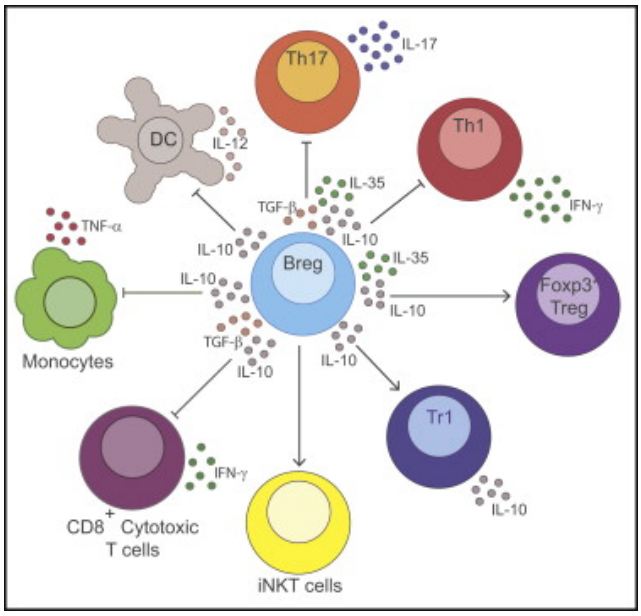

Figure I-6: Mechanisms for human Breg-mediated immune-suppression
The proposed mechanisms by which Breg cells may modulate immune responses through IL-10, IL-35 and TGF-β including: suppression of Th1 and Th17 differentiation and effector cytokine production, suppression of TNFα production by monocytes and IL-12-producing dendritic cells, inducing differentiation of Tr1 cells and Foxp3+T cells, maintenance of iNKT cells through CD1d expression and suppression of cytotoxic CD8+T cells. Adopted from Rosser and Mauri, 2015.

1.6.4 Human Regulatory B cells in disease

IL-10 producing Bregs have been identified in humans as critical regulators of immune tolerance in disease pathology such as autoimmunity, tolerance, infection and cancer.

1.6.4.1 Regulatory B cells in Autoimmunity

Extensive studies in mice have highlighted the crucial role of Breg in immunosuppression of autoimmune diseases, however the role of human Bregs in autoimmunity remains understudied. Blair et al, described a subset of IL-10+CD19^{+}CD24^{hi}CD38^{hi} human transitional B cells that were functionally impaired in SLE patients and lacked suppressive capacity associated with defective IL-10 production in response to CD40 ligation when compared to their healthy counterparts, suggesting that altered Breg function may impact the maintenance of immune tolerance in SLE and other autoimmune diseases (Blair et al., 2010). Incidentally, following rituximab treatment, SLE patients had an increased ratio of CD19^{+}CD24^{hi}CD38^{hi} B cells to memory B cells suggesting a role for B-cell depletion in generating tolerogenic B cells (Palanichamy et al., 2009, Anolik et al., 2007).

Moreover, a subset of human B10 cells (CD24^{hi}CD27^{+}) identified by Tedder et al that can suppress TNFα production by monocytes was found to be expanded in autoimmune disorders such as SLE, RA, autoimmune skin disease and MS, supporting their role as a biomarker for autoimmune disease activity (Iwata et al., 2011). Subsequently reduced frequency of immature B cells has been associated with exacerbation of disease in RA as immature B cells re-locate to the inflamed joint (Flores-Borja et al., 2013). Further immature B cells isolated from RA patients lack suppressive capacity on Th17

differentiation and are unable to generate $FOXP3^+$ T cells from naïve T cells when compared to their healthy counterparts suggesting that mechanisms other than IL-10 production may be involved in Breg-mediated suppression (Flores-Borja et al., 2013).

The role of B cells as both initiators and contributors to autoimmune pathology has been further reported by recent advances in B-cell depletion strategy used to treat patients with autoimmune disease (Yanaba et al., 2008). B-cell depletion therapy using rituximab in RA patients was found to considerably attenuate ongoing joint inflammation; however, recrudescence of disease activity was found to be associated with B-cell recovery (Edwards et al., 2004, Leandro et al., 2006). Additionally, B-cell depletion in MS patients demonstrated beneficial effects after the onset of disease and ameliorated disease progression (Hauser et al., 2008). The beneficial effect of rituximab is further supported by clinical reports that correlated B-cell depletion with improvements in clinical manifestations of SLE (Looney et al., 2004). These reports suggest the appreciable role of rituximab as a powerful tool for researchers to study Breg function in the treatment of human autoimmune disease. Contrastingly, B-cell depletion has been shown to augment Th1-mediated immunity and thereby exacerbate ulcerative colitis and trigger psoriasis, further supporting a role for Bregs in controlling Th1 responses *in vivo* (Goetz et al., 2007, Dass et al., 2007). Taken together, the role of B cells as negative regulators of effector immune responses further supports their role in the pathogenesis of autoimmune diseases.

1.6.4.2 Regulatory B cells in Cancer

A new wave of research provides evidence that Bregs may play a role in cancer progression. DiLillo *et al* (2013) highlighted an expanded pool of IL-10 competent $CD5^{+}$ $CD20^{int}$ B cells in patients with chronic lymphocytic leukemia (CLL) that expressed high levels of IL-10 production following activation with LPS when compared to healthy controls (DiLillo et al., 2013). Further, high plasma levels of IL-10 have been reported in CLL, suggesting that IL-10 competent CLL cells may regulate immunosuppression and thereby attenuate patient responses to immunotherapies including rituximab. In accord with this finding, Horikawa et al observed that although B-cell depletion therapy was effective in treating non-Hodgkin lymphomas and CLL, some patients present resistance to this therapy or eventually relapse as a result of IL-10+ B cells that inhibit lymphoma depletion (Horikawa et al., 2011). Hence, IL-10+B cells may inhibit the efficacy of therapy in patients with malignant lymphoma, and B cell depletion may prevent cancer progression and metastasis. In addition, an increased frequency of circulating B cells was identified in human hepatocellular carcinoma (HCC) patients with advanced tumor staging (Lin et al., 2010). More recently, Shao et al further highlighted that a significantly higher percentage of circulating Bregs in HCC patients correlated with advanced tumor staging, tumor multiplicity and venous infiltration (Shao et al., 2014). Bregs promoted HCC growth and invasiveness by directly interacting with liver cancer cells through the CD40/CD154 signaling pathway (Shao et al., 2014).

Collectively, the studies highlight that Bregs may sustain malignant expansion and support cancer progression in humans.

1.6.4.3 Regulatory B cells in Infection

Growing evidence has associated increased frequencies of PB-derived immature B cells with immune responses in infectious diseases such as HIV and hepatitis B (Malaspina et al., 2006, Das et al., 2012). Human transitional B cells have been implicated in suppressing HBV-specific CD8+ T cell responses in an IL-10 dependent fashion (Das et al., 2012). Further, in a study investigating immune regulation in multiple sclerosis patients, parasitic infection has been shown to induce functionally suppressive Bregs that attenuate proliferation and IFNγ production by myelin-specific T cells (Correale et al., 2008). Additionally, an increased $CD1d^{hi}$ IL-10 producing B cell pool was observed in Gabonese children infected with *Schistosoma haematobium.* After treatment children had fewer $CD1d^{hi}$ B cells that correlated with reduced inflammation (van der Vlugt et al., 2012). Collectively, recent evidence of B regulatory cells in infection indicates a potential role for Bregs as potential therapeutic target for infectious diseases in humans however, the mechanistics of Breg suppression is not yet fully understood.

1.6.4.4 Regulatory B cells in Transplant Tolerance

Accumulating studies support a role for Bregs as crucial mediators of immune tolerance in allograft recipients (Pallier et al., 2010, Newell et al., 2010, Silva et al., 2012). In one study, an expanded pool of transitional/naïve B cells which shared phenotypic similarities with previously identified Bregs was found in graft-tolerant renal transplant recipients that were off immunosuppressants (Newell et al., 2010 and Sagoo et al., 2010). In addition,

increased frequency and absolute counts of peripheral B cell that were phenotypically reminiscent of murine B10 cells were reported in a study by Pallier et al. These elevated B cells were enriched within the transitional B cell compartment and were suggested to contribute to the maintenance of long-term graft function (Pallier et al., 2010).

1.6.4.5 Regulatory B cells in Pregnancy and Allergy

Interestingly, during pregnancy, another state of tolerance to alloantigen, IL-10 producing $CD19^{+}CD24^{hi}CD27^{+}$ Bregs were reported to increase with pregnancy onset (Rolle et al., 2013). These IL-10 producing CD19+B cells suppressed effector cytokine production by CD4+T cells, suggesting a novel role for Bregs in suppression of maternal T cells and acquisition of tolerance during pregnancy (Rolle et al., 2013). Further, a population of IL-10+Bregs producing IgG4 isolated from Bee Keepers that are known to exhibit long-term tolerance to bee venom allergens were shown to suppress antigen-specific CD4+T cells proliferation. These Bregs were induced in allergic patients following specific immunotherapy and demonstrated that induction of long-term tolerance to allergic antigens ay be partially dependent on B suppressor cells (van de Veen et al., 2013). The regulatory capacity of B cells to maintain tolerance could therefore be exploited for therapeutic purposes by triggering expansion of the Breg population *in vivo*.

Aims and Hypothesis

In recent years, a distinct newly described subpopulation of IL-10 producing human B regulatory cells, that have been shown to exert significant immunoregulatory functions, has been the focus of intense immunological research. However, seemingly conflicting data exist as to the phenotypic and functional characteristics of these immunomodulatory B cells. Further, little is known about these B cell subsets in cord blood. There is therefore, a compelling need to characterize human B cell subsets more comprehensively to better understand their immune modulatory function, which may in turn aid the development of therapeutics to treat immune-mediated diseases.

I hypothesize that an imbalance of effector and regulatory subsets may lead to loss of tolerance and induction of allo-reactivity in GVHD. Further, I propose that regulatory B cells play a crucial role in modulating the function of immune effector cells and the induction of GVHD.

The results of these laboratory findings will aid our understanding of their role in allogeneic HCT and possible application for the prevention and treatment of GVHD and a host of other immune-mediated diseases.

The specific aims of this thesis are:

1. To perform a comprehensive characterization of IL-10 producing regulatory B cells in peripheral blood and cord blood
2. To define the mechanisms of B-cell mediated suppression, specifically whether suppression is dependent on IL-10 or requires cell-to-cell interaction.

3. To study the interaction of Bregs with T cell subsets as well as other cells of the immune system (NK cells) that might unravel additional undiscovered functions of the nature of B cells in cord blood grafts.
4. To study the kinetics of Bregs reconstitution post-cord blood transplant and their impact on the risk of graft-versus-host disease (GVHD).

Chapter II. Materials and Methods

II.1 Patients and Healthy Controls

All samples were collected after written informed consent according to local policy guidelines at the MD Anderson Cancer Centre, and in accord with the Declaration of Helsinki. The study was approved by the institutional review board (IRB). Peripheral blood (PB) samples were collected from 13 consecutive AML patients [median age was 68 years (range 20–80 years)] at presentation. Patient samples were also studied in parallel with PB samples from healthy controls (6 female and 8 male, n=14) aged 24-50 years. All samples underwent Ficoll density separation (Lymphoprep), freezing and storage in liquid nitrogen, and were then used for the isolation of B regulatory (Breg) and natural killer (NK) cells. Cord blood transplant recipient samples were collected from 16 patients [median age was 42 years (range 21–64 years)] before and after CBT between 2007 and 2011 and at intervals of 90 days for up to 1 year post CBT and at 2 years. cGVHD status was classified according to the National Institutes of Health (NIH) criteria at the time the sample was collected and the modified Seattle criteria for limited versus extensive disease.

II.2 Sample processing

II.2.1 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated by use of density gradient separation technique (Lymphoprep). In summary, EDTA anti-coagulated whole blood was diluted 1:1 with RPMI 1640 media (GIBCO / Invitrogen) and 30 ml of the diluted blood was gently layered into 50 ml falcon

tubes containing 15 ml of Lymphoprep (Axis Shield). The tubes were centrifuged at 1800 rpm for 30 min and the interface layer containing mononuclear cells was collected. Collected PBMCs were washed with RPMI twice for 10 minutes at 1400 rpm. Cells were then re-suspended in RPMI containing 10% foetal calf serum (FCS) and counted using Trypan Blue for cell viability assessment.

II.2.2 Isolation of cord blood mononuclear cells

Cord blood units were kindly donated by the cord blood bank at MD Anderson Cancer Center, Houston, Texas, USA under an IRB-approved protocol. Cord blood mononuclear cells (CBMCs) were by use of density gradient separation technique (Lymphoprep). In summary, EDTA anti-coagulated whole blood was diluted 1:1 with RPMI 1640 media (GIBCO / Invitrogen) and 30ml of the diluted blood was gently layered into 50ml falcon tubes containing 15ml of Lymphoprep (Axis Shield). The tubes were centrifuged at 2000 rpm for 20 min and the interface layer containing mononuclear cells was collected. Collected PBMCs were washed with PBS for 10 minutes at 1400rpm. Cells were then re-suspended in RPMI containing 10% foetal calf serum (FCS) and counted using Trypan Blue for cell viability assessment.

II.2.3 Cell freezing

PBMC were suspended in freezing media on ice (RPMI 1640 media supplemented with 20% FCS) and 20% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and gently aliquoted into 1.5ml cryovials for storage in -80° C (max 1 week) before being cryopreserved in liquid nitrogen at a density of 5 x 10^6/ml cells.

II.2.4 Cell thawing

PBMCS stored in cryovials were transferred from liquid nitrogen on dry ice for thawing protocol. Cryovials were thawed at 37 ºC in the water bath and immediately transferred into falcon tubes containing 10 ml thawing media (RPMI supplemented with 20% FCS and 50,000 units of DNase). PBMC were centrifuged at 1100 rpm for 10 min, and washed again in RPMI. Pelleted cells were resuspended in media for cell count and viability assessment (PBMC viability >76% after thawing).

II.3 Cell lines

II.3.1 L cells and control cells

Frozen aliquots of CD154 (CD40L)-transfected fibroblasts (L cells) and CD154-negative control cells were stored in the -81° C freezer and thawed as per the protocol described in II-2.4. L cells were grown in complete media (CM) (complete RPMI 1640, supplemented with 5 µM HEPES, 12.5 µg/ml Gentamicin and 10% FCS). Once confluent in the T25 vented culture flasks, adherent L cells were incubated for 4 min at 37°C with 5 ml of 0.05% Trypsin-EDTA (GIBCO/ Invitrogen) after removing the culture media. Following gentle tapping of the flask, once the L cells were detached, an equal volume of CM was added and cells were centrifuged at 1200rpm for 10 min. To determine the expression of CD40L on transfected L cells, cells were stained with CD154-PE mAb and analyzed periodically by Fluorescence-activated cell sorting (FACS) analysis **figure (II-1),** to ensure that the CD40L expression was not affected by frequent passaging. L cells were sent to microbiology lab for testing of mycoplasma contamination every 6 months. After cells were trypsinized, the viability of L cells and expression of CD154 was assessed to

exclude any impact of trypsin. Following the wash, cells were frozen as described in II-2.3 and stored at – 81 ° C.

II.3.2 K562 cell line

Frozen aliquots of K562 tumour target cell line were stored in the -80° C freezer and thawed as required following the protocol outlined in II-2.4. K562 cells were grown in CM. Once confluent in the T25 vented culture flasks, non-adherent K562 cells were washed by centrifugation at 1200rpm for 10 minutes. After washing, the viability of K562 cells was assessed and cells were frozen as outlined in section II-2.3 and stored at – 80 ° C and then transferred to liquid nitrogen.

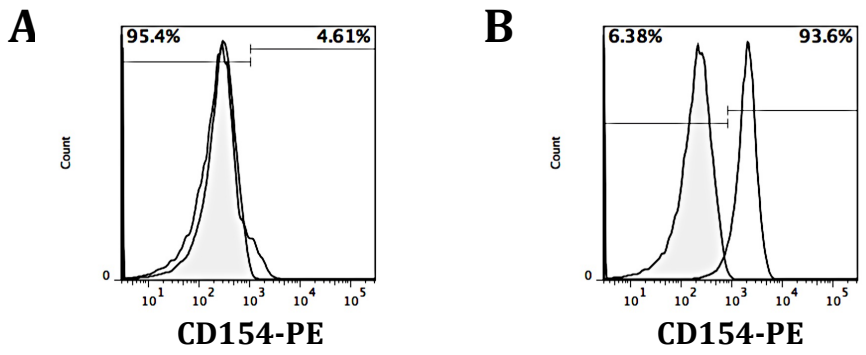

Figure II-1: CD154 (CD40L) expressing transfected L cells and their controls

A. Staining of CD154-control cells (white) and the isotype (tinted grey) and **B.** CD154+ L cells (white) with isotype control (tinted grey)

II.4 Cell selection

II.4.1 B cell selection

Following the manufacturer's protocol, untouched B cells were selected from either fresh or frozen PBMC using the human B cell isolation kit II (Miltenyi Biotec Ltd). In summary, PBMC were washed using MACS Buffer (MACS BSA and autoMACS^{TM} rinsing solution from Miltenyi Biotec Ltd). Upon determining the cell number, 10^7 cells were re-suspended in 40 µl of MACS buffer and 10 µl of Biotin-antibody cocktail against CD2, CD235a, CD36, CD16, CD43, and CD14 and incubated at 4°C for 10 minutes; 30µl/10^7 of MACS buffer and 20 µl/10^7 of anti-Biotin micro-beads were then added to the cells and further incubated for 15 minutes at 4°C. After incubation, cells were washed with MACS buffer and re-suspended in 500 µl of MACS buffer. The cells were applied to primed LD MACS columns (Miltenyi Biotec Ltd) followed by 3 washes of the column. The pass-through containing purified population of negatively isolated B cells was collected and centrifuged at 1200 rpm for 10 minutes. Cells were re-suspended in CM at the required cell concentration and stained with anti-CD19 PE (BD PharmingenTM Cat# 555413) to confirm B cell purity, which was consistently between 95.5%-99%, **figure (II-2)**

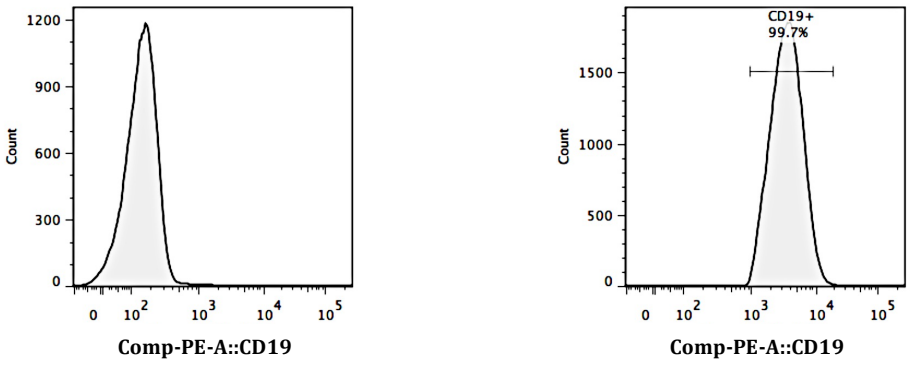

Figure II-2: B cell selection purity check
Purity of selected $CD19^+$ B cells from a healthy donor stained with CD19 PE

II.4.2 T cell selection

II.4.2.1 Total CD4+ T cell selection

Untouched CD4+ T cells were negatively isolated from fresh PBMC using human CD4 T cell isolation kit II (Miltenyi Biotec Ltd), following the manufacturer's protocol. Briefly, isolated PBMC were washed using MACS Buffer. Upon determining the cell number, the cell pellet was resuspended in 40µl per 10^7cells of MACS buffer. Subsequently, 10 µl per10^7 cells of Biotin-antibody cocktail against CD8, CD19, CD16, CD14, CD123, CD56, CD36, TCR γ/δ, and CD235a was added to the cells and incubated for 5 minutes at 4°C. Following incubation, 30 µl of MACS buffer and 20 µl of anti-Biotin micro-beads per 10^7 cells were added to the tube and incubated for a further 10 minutes at 4°C. The cell suspension was then applied onto the primed LD MACS columns and an additional 3 ml of MACS buffer was used to wash the columns 3 times. The pass-through containing a population of enriched CD4+ T cells was collected and centrifuged at 1400 rpm for 10 minutes. Cells were re-suspended in RPMI/10%FCS at the required cell concentration and stained with anti-CD4 APC (BD Cat# 340443) to assess the purity of selected CD4+ T cells. Purity was consistently between 92%-98%; **figure (II-3).**

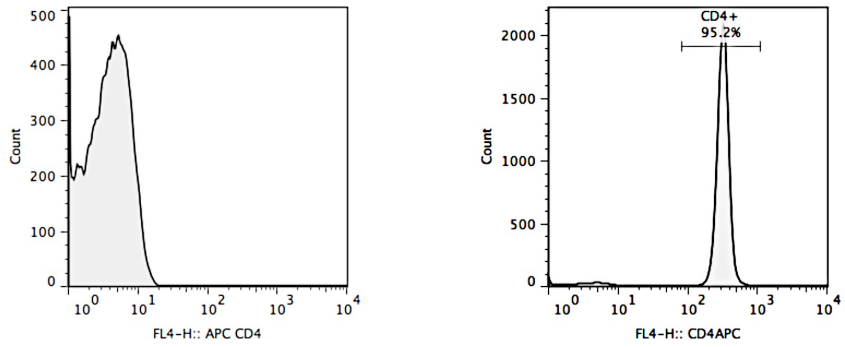

Figure II-3: T cell selection purity check
Purity of healthy donor derived selected $CD4^+$ T cells was determined by staining with CD4 APC

II.4.2.2 CD4+CD127loCD25- Treg magnetic selection/ "Treg depletion"

$CD4^{+}CD127^{lo}CD25^{-}$ cells were selected by a two step labelling procedure for the isolation of Treg cells from fresh PBMC using CD4+CD25+CD127dim/- regulatory T cell isolation kit II human (Miltenyi Biotec Ltd), as per the manufacturer's protocol. In summary, PBMC cell number was determined and cells were washed using MACS Buffer. The cell pellet was resuspended in 40µl/10^7 cells of MACS buffer and 10 µl/10^7 cells of Biotin-antibody cocktail was added to magnetically remove CD4- and CD127 hi cells. Cells were incubated for 7 minutes at 4°C, after which 30 µl of MACS buffer and 20 µl of anti-Biotin micro-beads per 10^7 cells was added to the cells and further incubated for 10 minutes at 4°C. Cells were washed and resuspended in 500 µl of MACS buffer and applied to LD MACS columns. Columns were washed three times with 3 ml of MACS buffer and the flow-through containing a population of enriched CD4+ T cells was collected and centrifuged at 1200 rpm for 10 minutes. The cell pellet was re-suspended in 90 µl of MACS buffer followed by 20 µl of CD25 MicroBeads per 10^7 cells and incubated for 15 min on ice for the second step of the labeling procedure. Cells were passed through an MS MACS magnetic column and the pass-through containing untouched CD4+ cells depleted of Treg cells ($CD4^{+}CD127^{lo}CD25^{hi}$) were collected. 1ml of MACS buffer was added to the column and labeled Treg cells were eluted by firmly pushing the plunger into the column. Cell number and viability were determined and cells were plated as required. Cells were stained with anti-CD25, anti-CD4 and anti-CD127 to confirm the purity of the

isolated subsets, which was consistently found to be between 88.6-97%; **figure (II-4).**

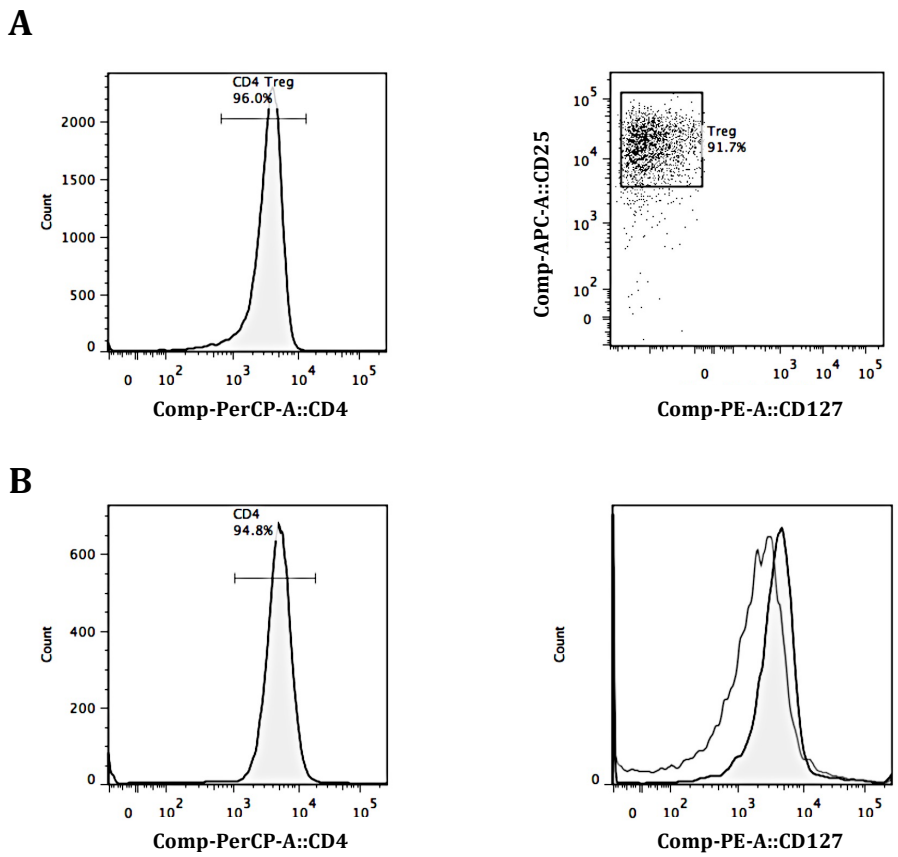

Figure II-4: Treg cell selection purity check

A. Purity of healthy donor derived selected Treg cells was determined through staining with CD4 PerCP, CD127 PE and CD25 APC **B**. Phenotype of flow through (enriched $CD4^+$ T cells depleted of Treg) shows CD4 purity of 94.8% and lower CD127 expression ($CD127^{lo}CD4^+$) (**grey**) compared to total $CD4^+$ T cells (**white**)

II.4.3 NK cell selection

Isolation of untouched NK cells from fresh or frozen PBMCs was performed using the human NK cell Isolation kit according to the manufacturers' protocol. In summary, PBMC were washed using MAC buffer and the cell number was determined. Cells (10^7) were suspended in 40 µl MACS buffer; 10 µl per10^7 cells of biotin-antibody cocktail against antigens not expressed by NK cells was added to the cells for 5 minutes at 4°C. Following incubation, 30 µl of MACS buffer and 20 µl of anti-Biotin micro-beads per 10^7 cells was then added and incubated for a further 10 minutes at 4°C. Cells were washed and the cell volume was adjusted to 500 µl and applied onto the primed LD MACS columns. An additional 3 ml of MACS buffer was used to wash the columns a total of three times. The flow-through containing an enriched population of unlabeled NK cells was collected and centrifuged at 1400 rpm for 10 minutes. Cells were re-suspended in CM to achieve the required cell concentration. Post-selection purity was confirmed using anti-CD56 Bv605 (BD Biosciences) and CD3 APC-Cy7 mAbs (Biolegend). Purity was consistently between 92%-98%; **figure (II-5).**

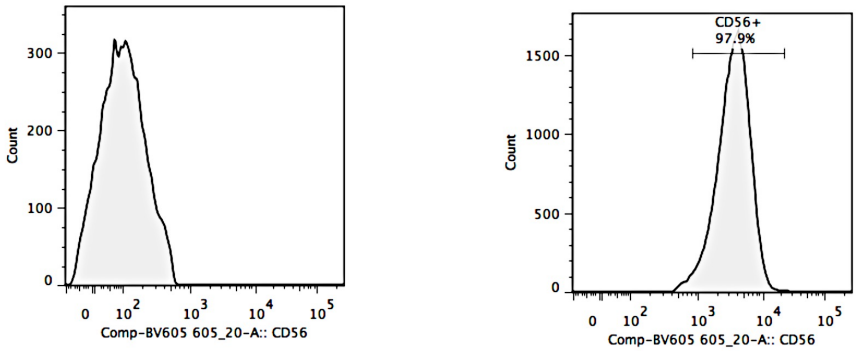

Figure II-5: NK cell selection purity check
Purity of healthy donor-derived selected $CD56^+$ NK cells was determined by staining with CD56 Bv605.

II.5 Cell sorting for B cell subsets

II.5.1 Cell sorting for peripheral blood-derived B cell subsets

B cell sorting was performed at the FACS core facility, Department of stem Cell Transplantation and Cellular therapy, MD Anderson Cancer Centre using BD FACS Aria IIIu instrument. In summary, 20 x10^7 freshly isolated PBMC were stained with 8 µL of anti-CD24-FITC (BD Biosciences), 10 µL of anti-CD19-APC (BD Biosciences), 7 µL of anti-CD38-Pe-cy7 (eBiosciences) and 7 µL of anti-IgM-PerCP cy5.5 (BD Biosciences) for 20 min. Positive controls stained for each parameter were included in each experiment in addition to unstained (negative) control. To prevent aggregates from clogging the 70 µm nozzle, all samples were filtered prior to sorting. A total of 10^7 cells/ml of PBS was used to achieve a sorting efficiency of > 80%. The gating strategy performed for B cell subset sorting is illustrated in **figure (II-6).** Sort-purified $CD19^+$ B cell populations including $CD19^+CD24^{hi}CD38h^iIgM^{hi}$ transitional (mean purity 92.1%), $CD19^+IgM^+CD24^{hi}CD38^{-/lo}$ IgM memory (mean purity 93.6%), $CD19^+CD24^{int}CD38^{int}IgM^+$ naïve (mean purity 91.4%) and $CD19^+IgM^-CD24^{hi}CD38^{-/lo}$ switched memory (mean purity 92.5%) B-cell subsets were collected in purity sorter mode; **figure (II-7)**. At the end of each sort, purity checks were undertaken to ensure high purity populations >90%.

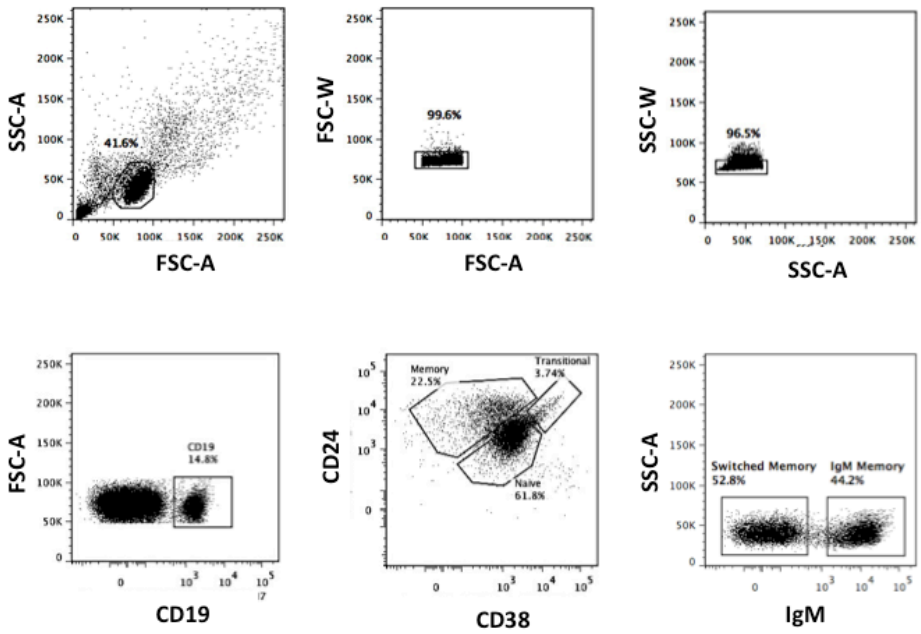

Figure II-6: Gating strategy of PB B cell sorting
Multi-parametric flow cytometric gating strategy for B cell subset sorting on BD FACS ARIA IIIu. Following lymphocyte gate and cell doublet discrimination, CD19+ B cells are then sort-purified based on CD24 and CD38 expression into 3 subsets, namely memory, transitional and naïve. Memory B cells are further divided into switched memory and IgM memory B cells based on IgM expression.

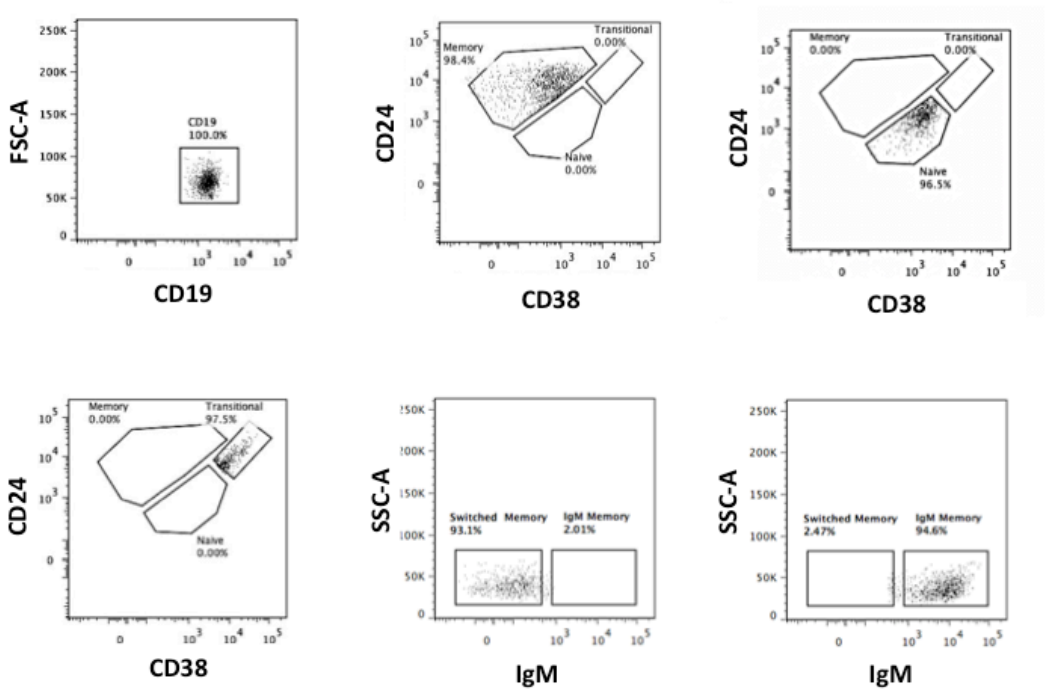

Figure II-7: Purity checks of PB B cell sorting
FACS plots illustrating the high purity of sorted B cell subsets are shown within the CD19+ gate including $CD19^{+}CD24^{hi}CD38h^{i}IgM^{hi}$ transitional (mean purity 92.1%), $CD19^{+}IgM^{+}CD24^{hi}CD38^{-/lo}$ IgM memory (mean purity 93.6%), $CD19^{+}CD24^{int}CD38^{int}IgM^{+}$ naïve (mean purity 91.4%) and $CD19^{+}IgM^{-}CD24^{hi}CD38^{-/lo}$ switched memory (mean purity 92.5%) B-cells (n=48)

II.5.2 Cell sorting for cord blood derived B cell subsets

Cord blood-derived B cell subsets were sort-purified on FACSAria IIIu instrument (Becton Dickinson) at the FACS core facility, Department of stem Cell Transplantation and Cellular therapy, MD Anderson Cancer Centre. Following staining with CD19 APC, CD24 FITC (all from Becton Dickinson, USA) and CD38 Pecy7 (eBiosciences, San Diego, USA).

Briefly, freshly prepared 10 x10^7 PBMC were stained with 8 µL of FITC-labelled anti-CD24 (BD Biosciences), 10 µL of anti-CD19-APC (BD Biosciences) and 7 µL of anti-CD38-Pe-cy7 (eBiosciences) for 30 min. Positive controls for each individual parameter were included in addition to unstained (negative) control.

To prevent aggregates from clogging the 70 µm nozzle, all samples were filtered prior to sorting. A concentration of 10^7 cells/ml of PBS was determined to achieve a sorting efficiency of > 80%. The gating strategy performed for CB B cell subset sorting is illustrated in **figure (II-8).**

Two distinct sort-purified CD19+ B cell populations, namely $CD19^+CD38^{hi}CD24^{hi}$ transitional B cells (a population that includes immature B cells) and $CD19^+CD38^{int}CD24^{int}$ naïve B cells (primarily mature B cells) were obtained in purity sorter mode; **figure (II-9)**. At the end of each sort, purity checks were undertaken to ensure high purity populations >90%.

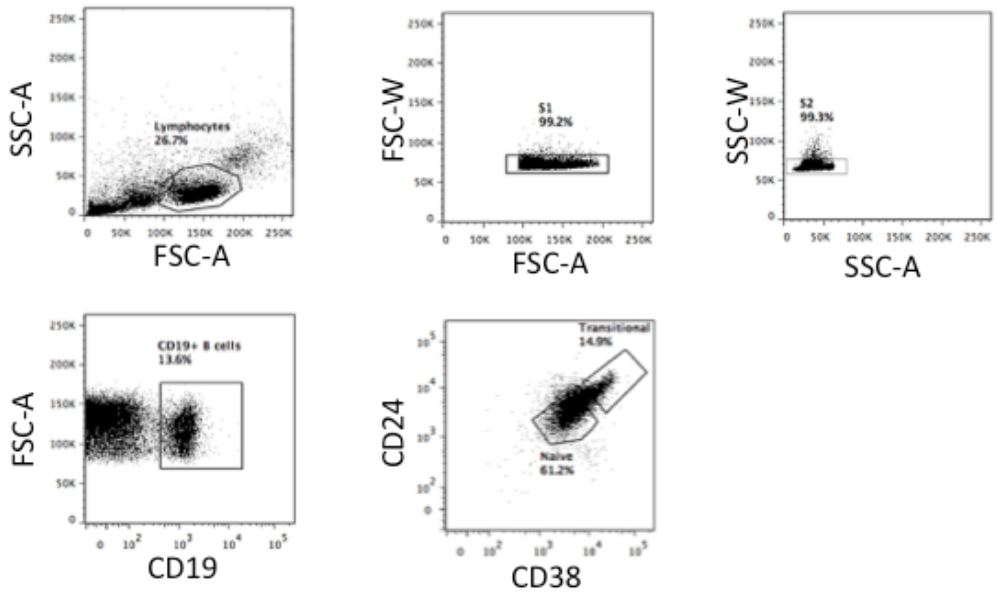

Figure II-8: Gating strategy of CB B cell sorting

Multi-parametric flow cytometric gating strategy for sorting B cell subsets on BD FACS ARIA IIIu. Following lymphocyte gate and cell doublet discrimination, CD19+ B cells are then sort purified based on CD24 and CD38 expression into 2 subsets, $CD19^{+}CD38^{hi}CD24^{hi}$ transitional B cells and $CD19^{+}CD38^{int}CD24^{int}$ naïve B cells

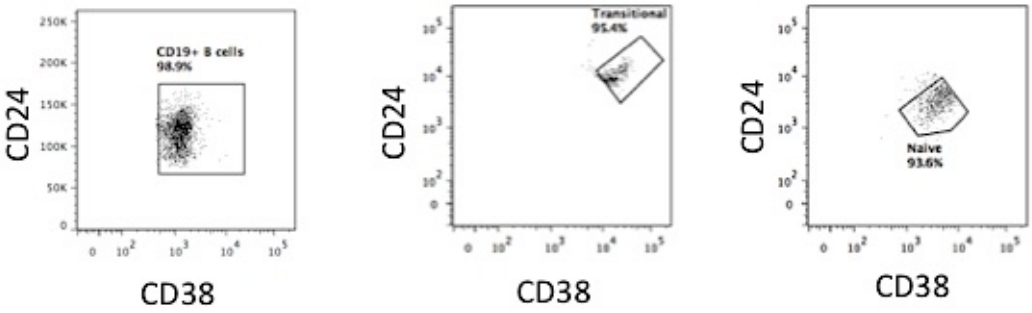

Figure II-9: Purity checks of PB B cell sorting

FACS plots illustrating the high purity of sorted B cell subsets are shown within the CD19+ gate, namely $CD19^{+}CD38^{hi}CD24^{hi}$ transitional B cells (mean purity 90.8%) and $CD19^{+}CD38^{int}CD24^{int}$ naïve B cells (mean purity 91.9%) (n=30)

II.6 Phenotypic and functional characterization of peripheral B cells using flow cytometry

II.6.1 B cell Phenotyping using an extended panel of surface antibodies

A laser-based, biophysical technology of flow cytometry enables simultaneous multiparametric analysis of particular cell populations of interest based on their properties through biomarkers with use of specific fluorochromes and different gating strategies. Fluorochromes are selected based on intended cell markers, commercially available options and instrument configuration. For B cell phenotyping, an antibody panel of 9-fluorochrome markers (as defined in **Table II-1**) was customized to determine the following B cell subsets:

Transitional B cells: $CD19^{+}$, $CD24^{hi}$, $CD27^{-}$, $CD38^{hi}$, $CD10^{+}$, $CD21^{-/lo}$, IgM^{hi} and IgD^{+}

Naïve B cells: $CD10^{-}$, $CD19^{+}$, $CD21^{+}$, $CD24^{-/lo}$, $CD27^{-}$, $CD38^{+}$, IgM^{-} and IgD^{+}

Pre Germinal Centre: $CD10^{+}$, $CD19^{+}$, $CD21^{hi}$, $CD24^{-}$, $CD27^{+}$, $CD38^{hi}$, IgM^{+} and IgD^{+}

Memory B cells; isotype switched: $CD10^{-}$, $CD19^{+}$, $CD21^{+}$, $CD24^{-/lo}$, $CD27^{+}$, $CD38^{+/-}$, IgM^{-} and IgD^{-}

IgM memory or marginal zone B cells (MZB cell): $CD10^{-}$, $CD19^{+}$, $CD21^{+/hi}$, $CD24^{-/lo}$, $CD27^{+}$ $CD38^{lo/-}$, $IgM^{+/lo}$ and $IgD^{+/lo}$

Tissue like memory cell, exhausted B cells: $CD10^{-}$, $CD19^{+}$, $CD21^{-}$, $CD24^{-}$, $CD27^{-}$, $CD38^{-}$, IgM^{-} and IgD^{-}

Plasmablasts: $CD10^{-}$, CD 21^{-}, CD24 $^{-}$, CD 38^{hi}, $CD27^{+/hi}$ IgM^{-} and IgD^{-}

Table II-1: Antibodies used for characterizing B cell subsets

Antibody	Volume µl	Manufacturer	Description	Clone
CD19 APC H7	4	BD Pharmingen™	B cell lineage differentiation marker	SJ25C1
CD38 PE Cy7	3	eBioscience	Surface antibody involved in calcium signalling	HIT2
CD24 FITC	4	BD Pharmingen™	B cell maturation	HB5
CD21 APC	4	eBioscience	complement receptor 2	HB5
CD27 PE	4	BD Pharmingen™	Tumor necrosis factor receptor	M-T271
IgD v450	4	BD Pharmingen™	Surface antibody	IA6-2
IgM PerCP cy5.5	3	BD Pharmingen™	Surface antibody	G20-127
CD10 Qdot® 605	1	Invitrogen™	membrane metallo-endopeptidase	MEM-78
Aqua florescent reactive dye	1	Invitrogen™	Live/Dead marker- binds to intracellular amines of dead cells	

II.6.2 Cell staining and FACS acquisition

Briefly, $1x10^6$ cells derived from fresh or thawed PBMC were stained with 1 µl of cell viability marker (invitrogen™ LIVE/DEAD Fixable Aqua Dead cell stain Kit) for 30 minutes at room temperature in the dark. Following incubation, cells were washed and incubated with an antibody cocktail for various cell markers (**Table II-1**) at titrated concentrations in x1 PBS for a further 30 min. Stained cells were washed with 2 ml of PBS and acquired on LSR Fortessa (BD Biosciences) at MD Anderson. The instrument was standardized by running Cytometer Settings & Tracking (CST) beads (Becton Dickinson) at designated PMT voltages daily to reduce variation between experiments or samples. Compensation controls (single stained controls) for each fluorochrome were made using pre mixed negative and positive antibody capture beads (BD™ Comp Beads), which were stained singly with each antibody. The

compensation controls were recorded at 5000 events per tube individually to ensure that each fluorochrome marker was the brightest in its channel. FACSDiva software was used to compute the automated compensation matrix of the controls. PMT voltages were also adjusted using unstained cells in order to exclude auto-fluorescence and control the background intensity i.e. by restraining the negative population below log 10^2. Manual compensation was used occasionally when the software did not allow optimal adjustment of the spectral overlap between the different fluorochromes. Acquired data was analysed using FlowJo (v.7.6.5) software (Tree Star).

II.6.3 B cell activation

Total CD19+ B cells selected from either fresh or frozen PBMC were plated at a concentration of $2x10^5$ cells/well in a total volume of 200 µl of CM in flat-96-well plates (nunc). B cells were stimulated for 24, 48 and 72 hours with either CpG OND200- 5G (InvivoGen, USA) at a dose of 0.2 µg/ml in a humidified incubator at 37 °C with 5% CO2, or L cells that were irradiated (60 Gy), rested for a minimum of 1 hour and plated with CD19+ selected B cells at a ratio of 1:10 (L cell to B cells), or with 0.6 µg/ml of F (ab') 2 Goat anti Human IgG+ IgM (H+L) (Jackson ImmunoResearch Laboratories, USA). At the end of culture, the plates were centrifuged at 1800rpm for 5 minutes and 150 µl of the supernatant was collected from each well for ELISA analysis. Cells were collected for detection of IL-10-producing B cells by intracellular staining and flow cytometric analysis.

II.6.4 Quantitative ELISA for IL-10

Detection of IL-10 by ELISA was performed using OptEIA ELISA kits (BD Pharmingen™, San Diego, CA), following the manufacturers' protocol. Briefly, following washing and blocking of the ELISA plate with 50 µl of ELISA diluent, 100 µl of sample collected from each culture condition was plated and incubated for 2 h at room temperature. Supernatants collected from un-stimulated B cells, CD154 positive fibroblasts and their CD154 negative control cells cultured alone were also used as controls to validate detection of IL-10 from CD19+ B cells. After 5 washings, 100 µl of detection antibodies were incubated with the samples for 1 h. Following a further 7 washings, 100 µl of the provided tetramethylbenzidine (TMB) was added to each well to develop colour for 30 minutes in the dark. 50 µl of stop solution was added to each well and the absorbance was read within 30 minutes at 450nm using microplate reader Gen5 2.0 data analysis software (BioTek). The linear ranges of detection were obtained using the dilution series of standard cytokine (1.95pg/ml – 500pg/ml) provided and were determined accordingly (**figure II-10**)

Un-stimulated selected B cells (mean purity 92%± 3.75) from 4 healthy donors produced either no detectable cytokine or only small amounts of IL-10 (mean 18±7.5 pg/ml, n=4). Of note, irradiated CD154 expressing fibroblasts (L cells) and their control counterpart also produced negligible amounts of IL-10 (mean 6.5± 2.4 and 21± 1.2 pg/ml respectively, n=3). Purified B cells stimulated with L cells at a ratio of 1:10 produced higher levels of IL-10 than B cells stimulated with BCR crosslinking or CpG in a time dependent manner.

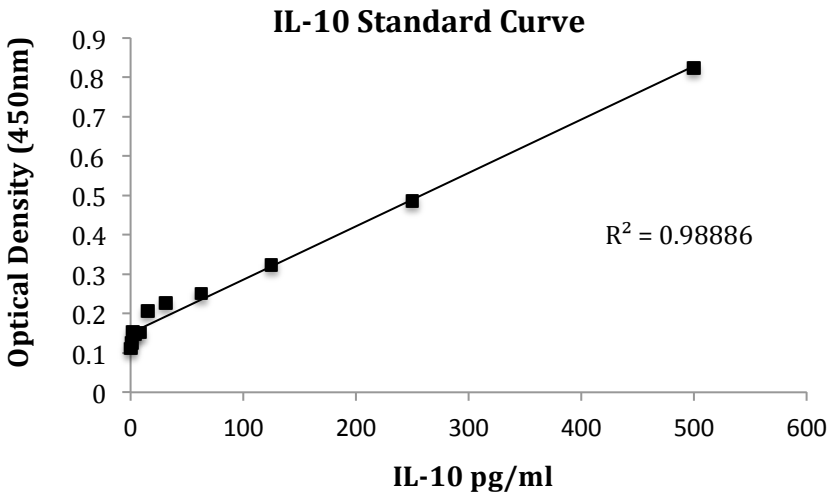

Figure II-10: Example of a standard linear curve analysis for IL-10 detection
using microplate reader Gen5 2.0 data analysis software (BioTek)

II.6.5 Intracellular cytokine assay for detection of IL-10+ B cells

Based on the ELISA experiments and a previously optimized protocol from our laboratory (Khoder et al Blood 2014), I used L cells for stimulation to optimise IL-10 intracellular cytokine (IC) staining of both selected and gated $CD19_+$ B cells derived from fresh or frozen PBMC. In order to determine the optimal time point at which maximal IL-10 production could be achieved without major activation-induced alterations of B cell phenotype, I performed kinetics of CD19+ IL-10 production using IC assay in which $CD19_+$ selected B cells or total PBMC were cultured with irradiated L cells at a ratio of (1:10) for 12, 18, 24, 48 and 72 hrs. PMA (50ng/ml), ionomycin (250 ng/ml) and brefeldin A (5 µg/ml) were added for the last six hours of the incubation. After incubation, cells were harvested and washed in staining buffer (1x PBS, 2% heat-inactivated FCS, 0.1% Sodium Azide) and incubated for 25 min in the dark at room temperature with a cocktail of CD19-PE, CD24 FITC, IgM PerCP Cy5.5, CD27 PE, all from BD Pharmingen and CD38 PE cy7 (eBiosciences).

Cells were then washed again and fixed/permeabilized for 45 min at 4°C with foxp3/transcription factor intracellular staining kit (eBioscience). Cells were then further incubated with 0.5 µl of either APC-conjugated IL-10 or IgG2aK isotype antibodies for 30 min at 4°C. As negative controls I used both the manufacturer's recommended isotype control, (IgG2aκ) (to control for non-specific binding effects of antibody and the fluorescence resulting from non-specific intracellular protein interactions), fluorescence minus one control (to control for autofluorescence) and unstimulated control incubated with BFA alone (unstimulated, BFA) and appropriately stimulated B cells without the addition of BFA (stimulated, no BFA).

Optimized volume of IL-10 monoclonal antibody was determined by MFI and maximal percentage of positively-stained cells with minimum background (negative control) experiments performed by previous member of the laboratory in dose titration experiments. Cells were washed 3 times with perm-wash and re-suspended in staining buffer prior to acquisition using BD LSRFortessa (Becton Dickinson). Data analysis was performed using FlowJo® software; **figure (II-11**). Stimulating gated or selected CD19+ B cells with L cells for 15h appeared to be optimal for sufficient numbers of IL-10 producing B cells to be recorded without significant changes in the overall B cell phenotype. I used combinations of 4-colour surface antibody panels, to determine the phenotype of IL-10+ B cells since combining these markers in one extended panel to confirm this phenotype with IC staining was unsuccessful due to the poor separation of IL-10- and IL-10+ populations (data not shown).

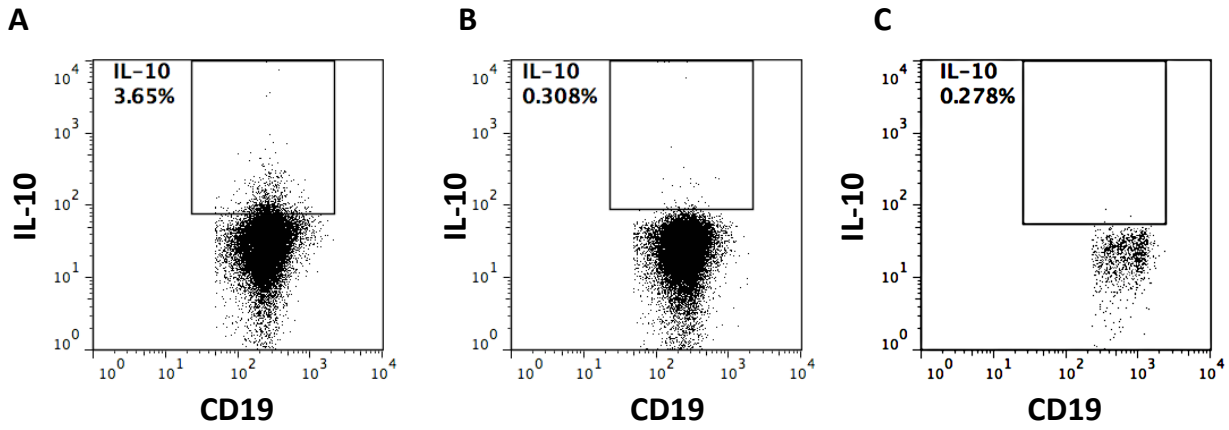

Figure II-11: Intracellular staining of IL-10 production by CD19+ B cells
A representative FACS plot depicting IL-10 production through intracellular staining when PBMC from a healthy control were stimulated with L cells for 48 hours (A). CD19+IL-10+ cells B IgG2ak isotype control (B) and fluorescence minus one control for IL-10 (C).

II.7 Phenotypic and functional characterization of cord blood derived B cells

II.7.1 Phenotypic characterization of cord blood derived B cells

A panel of 9 antibodies (as defined in **Table II-1**) was customized to determine the cord blood B cell subsets. Briefly, $1x10^6$ fresh or thawed CBMNC were stained with 1µl of the viability marker (invitrogen™ LIVE/DEAD Fixable Aqua Dead cell stain Kit) for 30 minutes at room temperature in the dark. Cells were then washed and incubated with the cocktail of mAb for various cell markers (**Table II-1**) at the titrated concentrations in x1 PBS for a further 30 min. Following incubation, stained cells were washed once more and then acquired on LSR Fortessa (BD Biosciences) at MD Anderson Cancer Centre.

II.7.2 Cord Blood B cell activation

Total CD19+ B cells or sort-purified B cell subsets selected from either fresh or frozen CBMNC were plated at a concentration of $2x10^5$ cells/well in a total volume of 200µl of CM in flat-96-well plates (nunc). To determine the optimal condition for CB B cell activation, B cells were stimulated for various time points: 24, 48 and 72 hours with either CpG OND200- 5G (InvivoGen, USA) at a dose of 0.2, 0.6 or 0.8 µg/ml in a humidified incubator at 37 °C with 5% CO2; or L cells that were irradiated (60 Gy), rested for a minimum of 1 hour and plated with CD19+ selected B cells at a ratio of 1:10 and 1:5 (L cell to B cells); or with 0.1, 0.6 or 1 µg/ml of F (ab') 2 goat anti Human IgG+ IgM (H+L) (Jackson ImmunoResearch Laboratories, USA). At the end of culture, the plates were centrifuged at 1800 rpm for 5 minutes and 150µl of the supernatant was collected from each well for ELISA analysis.

II.7.3 Detection of IL-10+ B cells through ELISA

Detection of IL-10 by ELISA was performed using OptEIA ELISA kits (BD PharningenTM, San Diego, CA), following the manufacturers' protocol. Briefly, following washing and blocking of the ELISA plate with 50 µl of ELISA diluent, 100 µl of sample collected for each condition was plated and incubated for 2h at room temperature. Supernatants collected from un-stimulated B cells, CD154 positive fibroblasts and their CD154 negative control cells cultured alone were also used as controls to validate detection of IL-10 from CD19+ B cells. After 5 washings, 100 µl of detection antibodies were incubated with the samples for 1h. Following a further 7 washings, 100 µl of the provided tetramethylbenzidine (TMB) was added to each well to develop colour for 30 minutes in the dark. 50 µl of stop solution was added to each well and the

absorbance was read within 30 minutes at 450nm using microplate reader Gen5 2.0 data analysis software (BioTek). The linear ranges and sensitivities were obtained using the standard cytokine and test sample dilutions provided and were determined accordingly (**figure II-10**).

Unstimulated selected B cells (mean purity 97%) from 4 healthy donors produced either no detectable cytokine or only small amounts of IL-10 (mean 1.2, n=6). Of note, irradiated CD154-expressing fibroblasts (L cells) also produced negligible amounts of IL-10 (mean 5.0± 3.5, n=6). Selected B cells stimulated with a combination of L cells at a ratio of 1:10, BCR crosslinking at 1µg/ml and 0.6µg/ml of CpG produced higher levels of IL-10 than B cells activated with these conditions alone in a time dependent manner.

II.8 *In vitro* suppression Assay of CD4+ T cells

II.8.1 Proliferation Suppression

To determine the suppressive ability of CB or PB derived sort-purified B cell subsets on proliferating CD4+ T cells, B cells were co-cultured with CFSE+ CD4+ T cells *in vitro*. Total CD4+ T cells were magnetically isolated as described in section II.4.2.1, and labeled with 2 µL per ml of prepared sample of aliquoted CFSE (65-0850; eBioscience, USA) from a stock solution (500µg/ml; stored at -20oC in DMSO) to achieve a final concentration of 2 µM CFSE. Cells were mixed well by vortexing and incubated for 8-10 minutes in the dark at room temperature after which 3 volumes of neat FCS was added to the cells for a further 5 minutes to stop CFE labeling. CFSE+CD4+ T cells were then plated into a flat bottom 96-well tissue culture plate at a concentration of 1x 10^5 per well in 100µl of CM with sort purified B cell subsets at a ratio of 1:1 to achieve a cell count of 2x10^5 per well.

Dynabeads® human T- activator CD3/CD28 (InvitroGen, Oslo, Norway) were added to each well for T cell activation at a concentration of 1 cell: 1 bead ratio (2µl/8x10^5) cells following the manufacturer protocol and incubated for 96h at 37ºC with 5% CO2. CFSE+CD4+ T cells were also cultured alone with CD3/CD28 coated beads (positive proliferation control) and without stimulation (negative control) for each experiment. Additionally, selected Treg cells were co-cultured with autologous CD4 T cells as quality control of suppression. Following 96 hours of culture, supernatants from each well was collected for analysis of secreted cytokine by ELISA (II.8.3) and cells were harvested and stained with APC anti-CD4 APC and anti-CD19 PE (both from BD Biosciences) for 25 min prior to acquisition on LSRFortessa. The percentage of dividing cells and proliferation index (to control for asymmetrical rate of cell distribution) were calculated using FlowJo's, proliferation tool; **figure (II-12).**

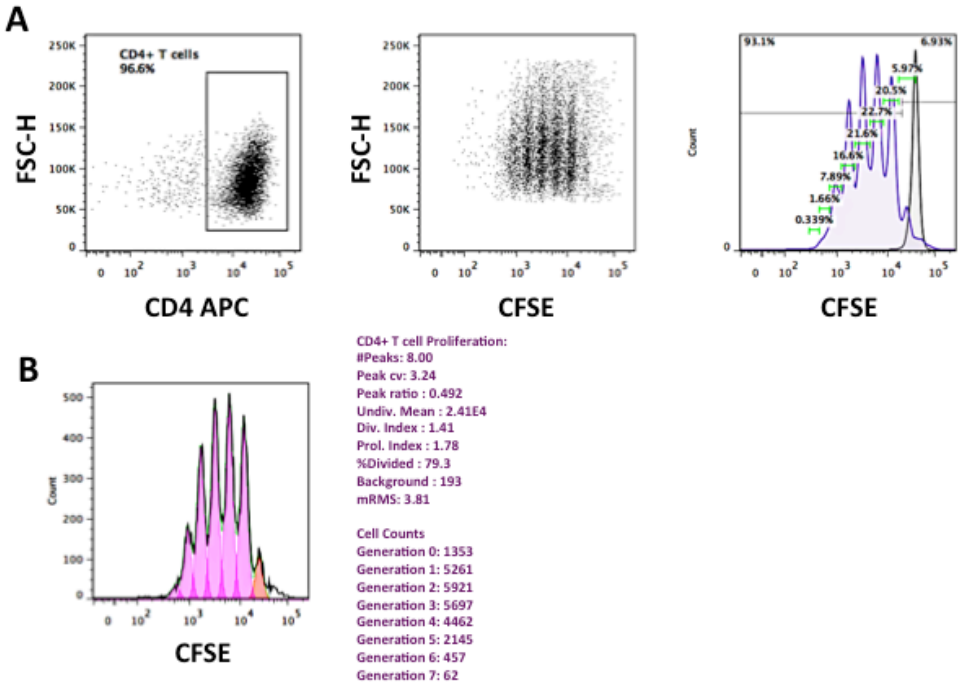

Figure II-12: Gating strategy for CD4+ T cell proliferation.
A. anti CD3/CD28 activated CD4+ T cells are gated on CFSE+CD4+ T cells. A representative

dot plot and histogram analysis of proliferating T cells (purple) gated on negative control (grey) illustrates the cell division with serial dilution of CFSE **B**. Generation of the proliferation index (1.78) through FlowJo

II.8.2 Suppression of cytokine production through flow cytometry

To assess the suppressive ability of CB or PB-derived sorted B cell subsets on CD4+ T cell cytokine production, magnetically-selected CD4+ T cells were co-cultured with B cell subsets at a B cell-to-effector T cell ratio of 1:1 into a 96-well flat-bottom tissue culture plate. Although in healthy PB the B to T cell ratio is approximately 1:4, the relevance of testing 1:1, 5:1 and 10:1 B cell to effector T cell ratio not only provides a model to understand Breg function but also contributes to gaining a better understanding of an altered ratio of regulatory-to-effector cells in diseases with expanded B cell frequencies or in transplant recipients in the context of immune reconstitution (associated with early B ell recovery and T cell lymphopenia) and cGVHD. Dynabeads® human T- activator CD3/CD28 (InvitroGen, Oslo, Norway) were added to each well for T cell activation at a concentration 1 bead: 1 cell (2µl/8x10^5 cells) following the manufacturer's protocol. Stimulated CD4+ T cells were also cultured alone (positive control) and un-stimulated T cells (negative control) for each experiment. The plate was incubated for 48 hours in the presence of 5% CO_2 at 37ºC. During the last 5 hours of incubation, 10µg/ml of Brefeldin-A (BFA)

(Sigma 5mg/ml DMSO cat# B-7651) was added. After 48 hours of culture, cells were harvested from each well and transferred into FACS tubes and washed with PBS. Cells were then stained with CD19 PE, CD4 PerCP (both from BD Biosciences) for 25 minutes and fixed and permeabilized using the eBioscience FoxP3 Staining Buffer Set (cat.72-5776) and eBioscience

Permeabilization Buffer (1x) (cat. 00-8333) for 45 minutes. Cells were washed again and stained with intracellular antibodies, IFNγ FITC and TNFα APC for 30 minutes, after which cells were washed once more and acquired on BD LSRFortessa and analyzed using FlowJo.

II.8.3 Detection of cytokine through IL-2, IFN−γ, TNF-α TGF-β and CD40L ELISA

Detection of secreted cytokines by ELISA was performed using ELISA kits obtained from R&D Systems for IFN-γ (cat# DIF50), TNF-α (cat# DTA00C), IL-2 (cat# D2050), TGF-β (cat# DB100B) and CD40L (cat# DCDL40) with aliquots taken from B cells/T cell co-cultures, following the manufacturers' protocol. Briefly, following adding of 100µl of assay diluent to each well, 100µl of samples or a dilution series of standard controls for appropriate cytokines were added to the appropriate wells and incubated at RT for 2 hours. The appropriate calibrator diluent served as the zero standard (0 pg/ml). Each well was then aspirated and washed a total of three times with wash buffer before addition of 200µl of conjugate to each well. The plate was sealed and incubated at RT for a further 2 hours. Following incubation, the wells were aspirated and washed again a total of three times and substrate solution was added to each well. The plate was further incubated at RT for 30 minutes -1 hour in the dark. 50 µl of stop solution was added to each well and the plate was read at 450 nm within 30 minutes. A standard curve was created by drawing a best-fit curve through the points plotted for the mean absorbance for each standard (y-axis) against the concentration (x-axis) using microplate reader Gen5 2.0 data analysis software (BioTek).

II.8.4 Luminex ProcartaPlex assay to measure T cell cytokine production following coculture with B cells

Detection of secreted cytokines by CD4+T cells in B cell/T cell co-cultures was assayed by ProCartaplex Human Th1/Th2/Th9/Th17/Th22/Treg Cytokine Panel (18 plex) assay (eBiosciences), as per the manufacturer's protocol. Briefly, antibody coated capture beads were prepared and added to each well for 2 min and then washed by 1X wash buffer. 25 µl of sample or standard controls were then added to the corresponding wells in duplicates and 50 µl of cell culture medium was added to the blank wells. The plate was then sealed and protected from light and incubated at RT for 2 hours on a plate shaker at 500 rpm. The wells were then washed again a total of 7 times and detection antibody was added to each well and incubated for 30 min. After a series of 7 washes, streptavidin phycoerythrin was also added to each well and incubated for a further 30 min after which wells were washed again and prepared for analysis on a luminex instrument by adding 120µl of reading buffer into each well.

II.8.5 Blocking experiments

Sort-purified B cells subsets and CFSE-stained CD4+ T cells were cocultured in 96-flat bottomed plates at a 1:1 ratio and were stimulated with anti-CD3/anti CD23 Dynabeads in the presence or absence of 5 µg/ml of anti-IL-10 (JEs#-9D7), anti–IL-10 receptor (3F9), anti-CD80 (10 mg/ml), anti-CD86 (10 mg/ml) (IT2.2), 10µg/ml anti-CTLA-4 (BNI3.1, Pharmingen) or anti–TGF-β (2 mg/ml) (TB21) for 96 hours. After co-culture, cells were harvested and stained with CD4 APC and CD19-PE (both from BD Biosciences) and washed with 2ml of

PBS before acquisition on the BD LSRFortessa. Gating on proliferating CD4+CFSE+ T cells assessed the impact of blocking cytokines.

For analysis of cytokine secretion, supernatants collected from cell cultures of sort purified B cells cultured with CD3/CD28-activated CD4+ T cells either directly or in separate transwell chambers after 96 hours was assayed by ELISA (R&D Systems) as described in II.8.3 or luminex ProCartaplex (eBiosciences) according to the manufacturer's instructions (II.8.4).

II.8.6 Transwell assays

A transwell system (Millicell, 1.0µm; Millipore) was used to block direct cell-to-cell contact between sort-purified B cell subsets (transitional, IgM memory, naïve and switched memory B cells) and selected $CFSE^+CD4^+$ T cells stimulated with anti-CD3/CD28 Dynabeads (Life Technologies). 1x 10^5/100µl $CFSE^+CD4^+$ T cells were either directly cultured with sorted B cell subsets at a ratio of 1:1 or placed in transwell chambers in the same well. After 96 hrs, cultured cells were harvested and analyzed for proliferating $CFSE^+CD4^+$ T cells. Supernatants from each well were also harvested for analysis of cytokine secretion by ELISA (R&D Systems) as described in II.8.3 or luminex ProCartaplex (eBiosciences) according to the manufacturer's instructions (II.8.4).

II.9 *In vitro* suppression Assay of CD56+CD3- NK cells

II.9.1 Proliferation suppression

NK cells were negatively bead-selected (Miltenyi Biotech) as described in II.4.3 and labelled with 5-carboxyfluorescein-diacetate-succinimidyl ester (CFSE) (eBioscience) as described previously (II.8.1). Briefly, labelled NK

cells were plated into 96-well flat-bottom tissue culture plates in Serum-free Stem Cell Growth Medium (SCGM) [CellGro® / CellGenix™] supplemented with 5% glutamine, 5 µM HEPES (both from GIBCO/ Invitrogen), and 10% FCS (Biosera) in the presence of 500 IU/ml of rhIL-2 (Proleukin, Chiron, CA, USA) for 8 days at 37°C in a 5% CO_2 humid atmosphere. Sort-purified B cell subsets (transitional, IgM memory, switched memory and naïve B cells) were then added to NK cells at a 1:1 ratio. CFSE-stained NK cells cultured alone with no stimulation (negative control) or with rhIL-2 (positive control) were included in each experiment. Magnetically selected Tregs (II.4.2.2) were co-cultured with autologous NK cells as a suppression control. The culture medium was replenished with fresh medium containing IL-2 every 2-3 days throughout the culture period. After the culture period, cells were stained with CD56 BV605, CD19 PE and PI (all from BD Biosciences) for 20 min and acquired on a BD LSRFortessa instrument (**figure II-13**).

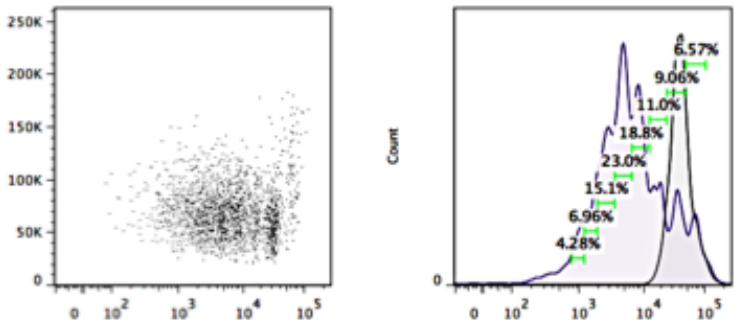

Figure II-13: A representative dot plot and histogram analysis of proliferating NK cells
Proliferating NK cells (purple) gated on negative control (grey) illustrates the cell division with serial dilution of CFSE

II.9.2 Suppression of cytokine production in NK cells by Bregs

For studies of NK cell suppression by Bregs, magnetically selected NK cells were cultured in Serum-free Stem Cell Growth Medium (SCGM) [CellGro® / CellGenix™] supplemented with 5% glutamine, 5 µM HEPES (both from GIBCO/ Invitrogen), and 10% FCS (Biosera) in 96-well flat-bottomed plates (Nunc) at 100,000/100µl in the presence of 5ng/ml of recombinant human IL-15 obtained from R&D Systems. NK cells were co-cultured either alone (positive control) or with autologous sort-purified B cell subsets at a 1:1 ratio for 48hrs at 37 °C. During the last 5 hours of incubation, K562 cell line target cells were added to the culture at an optimized E:T ratio of 10:1 together with CD107a PE-CF594 (BD Biosciences), monensin (BD GolgiStopTM) and BFA (Brefeldin A, Sigma). NK cells were incubated without targets as the negative control and stimulated with PMA (50 ng/mL) and ionomycin (2 mg/mL, Sigma Aldrich) as positive control. Furthermore, magnetically purified PB Treg cells isolated by negative selection (Miltenyi Biotec) were co-cultured with autologous NK cells as a suppression control.

Cells were collected, washed and surface stained with surface antibodies (table II-2) for 30 minutes at RT in the dark. Cells were then washed and fixed/permeabilized (BD Biosciences) and stained with intracellular antibodies IFN-γ v450 and TNF-α Alexafl700 (both from BDBiosciences). Cells were analyzed on a BD LSRFortessa flow cytometer equipped with FACS DIVA™ software. Secretion of TNF-a, IFN-y, IL-10, TGF-B (all from R&DSystems), granzyme B (eBiosciences) and perforin (MABTECH) was assessed in the supernatant collected after 48hours of B cell and NK co-culture by ELISA assays as per the manufacturers protocol (II.8.3).

II.9.3 Phenotypic characterization of inhibitory and activating markers on NK cells

The effect of B cell and NK cell co-culture on inhibitory and activating marker expressed on NK cells was assessed by using 10-colour multiparameter flow cytometry. Magnetically-selected NK cells were cocultured alone or with sort purified B cell subsets (transitional, IgM memory, switched memory and naïve B cells) at a 1:1 ratio at total concentration of $2x10^5$ cells/200µl in SCGM for 48 hours as described in II.9.2. Cells were then collected and washed with PBS and stained for surface expression of CD56 BV605, CD3 APC cy7, CD16 BV650, NGD2D PE, NTB-A PE, NKp30 Biotin in conjugation with Qdot800 Streptavidin (Invitrogen), DNAM-1 FITC (all Biolegend), NKp44 PerCP eflour710, NKp46-BV711 (eBioscience), NKG2A PEcy7, ILT2 PE (all Beckman Coulter), Pan KIR FITC (R&D Systems), Siglec-7 Pacific blue (Miltenyi Biotec) and 2B4 APC (Becton Dickenson) as shown in **Table II.2**, for 30 minutes in the dark at RT. Cells were washed and acquired on a BD LSR Fortessa flow cytometer equipped with FACS DIVA™ software and all data were analyzed using FlowJo software (Tree Star, San Carlos, CA, USA). Data are expressed as mean values of mean fluorescence intensity (MFI) ± standard error of mean (SEM).

Table II-2: Antibodies used for characterizing NK cells

Antibody	Volume (µl)	Manufacturer	Description	Clone
CD56 BV605	3	Biolegend	Surface antibody	HCD56
CD3 APC cy7	4	Biolegend	T cell co-receptor	HIT3a
CD16 BV650	3	Biolegend	Activates NK cells	3G8
NKG2D PE	4	Biolegend	Activating receptor	1D11
NTB-A PE	4	Biolegend	Co-receptor in NK Activation	NT-7
NKp30 Biotin	3	Biolegend	Natural cytotoxicity triggering receptor 3	P30-15
DNAM-1 FITC	4	Biolegend	Mediates cell adhesion	10E5
NKp44 PerCP eflur710	4	eBioscience	Natural cytotoxicity triggering receptor 2	44.189
NKp46 BV711	3	BD Biosciences	Natural cytotoxicity triggering receptor 1	9E2
NKG2A PE cy7	3	Beckman Coulter	Transmembrane protein	Z199
ILT-PE	4	Beckman Coulter	involved in inhibition of NK and T cell cytokine production	HP-F1
Pan KIR FITC	3	R&D systems	Inhibitory receptor	180704
Siglec-7 Pacific Blue	4	Miltenyi Biotec	Inhibits NK activation	REA214
2B4 APC	4	BD Biosciinces	Modulates NK cell activity	2-69
CD107a PE CF594	2	BD Biosciences	Marker of degranulation	H4A3

II.9.4 Suppression of NK cell mediated antibody dependent cell-mediated cytotoxicity (ADCC)

In brief, magnetically-selected NK cells were cocultured with sort-purified B cell subsets at a ratio of 1:1 at a concentration of $2x10^5$cells/200µl in SCGM in a 96-well flat-bottomed plate at 37C°. 96-well non-tissue culture plates were coated with 10 µg/ml of purified CD16 mAb (clone 3G8, BD Pharmingen) and incubated overnight at 4C°. The plate was then washed with 300µl of PBS per well a total of 3 times. During the last 5 hours of incubation, $1x10^5$ negatively selected NK cells were plated in the presence of CD107a, BFA and monensin either alone (positive control) as described in II.9.2 or with pre cultured NK and sort-purified B cell subsets, for 5 hours at 37C°. NK cells cultured without CD16 mAb were included in each experiment as a negative control. Cells were collected, washed and surface stained with CD3 APC cy7, CD56 BV605, fixed/permeabilized (BD Biosciences) and stained with IFN-γ v450 and TNF-α Alexafl700 (BDBiosciences) antibodies as described in II.9.2.

II.9.5 Blocking experiments

Purified B and NK cells were co-cultured and stimulated with K562 target cells as described in section II.9.2, in the presence or absence of 5 µg/ml of anti-IL-10 (JES#-9D7), anti–IL-10 receptor (3F9) (both from BD Pharmingen), anti-CD48 (5 µg/ml) (clone TU145 BD Biosciences), anti-2B4 (5 µg/ml) (clone C1.7 Beckman coulter, Fullerton, CA, USA), or anti–TGF-β (20 mg/ml) (clone TB21 Abcam). For analysis of cytokine secretion, sort-purified B cells were cultured with K562-activated $CD56^+CD3^-$ NK cells either directly or in separate transwell chambers for 48hrs as per the NK cytotoxicity assay described previously in II.9.2. Supernatants from cell cultures were collected after 48hrs,

and cytokine secretion was assayed by TNF-**α** and IFN-**γ** ELISA (R&D Systems) as per the manufacturer's instructions (II.8.3). For analysis of NK proliferation, sort-purifed B cells were cultured with $CFSE^{+}CD56^{+}CD3^{-}$ NK cells either directly or in separate transwell chambers (cultured with or without blocking mAbs) for 8 days, as described for the NK proliferation assay in II.9.1 Cells were collected and stained for $CFSE^{+}CD56^{+}CD3^{-}$ NK population.

II.9.6 Transwell assay

Magnetically selected NK cells ($1x\ 10^{5}$) were either directly co-cultured with sort-purified B cell subsets at a ratio of 1:1 or placed in transwell chambers (Millicell, 1.0 µm; Millipore) for 48 hours at 37°C. K562 target cells were added to NK cells for the last 5 hours of culture with CD107a, BFA and monensin for cytotoxicity assays (II.9.2). After 48 hours, cultured cells were harvested to measure NK cell cytotoxicity or proliferation and analyzed by flow cytometry.

II.10 Intracellular signalling

II.10.1 Phosflow analysis of JAK/STAT pathway in B cells

Sort-Purified B cell subsets were rested for 48 hours in medium alone (negative control) or with either L cells (1:10), magnetically selected NK cells at a 1:1 ratio or CD4+ T cells at a ratio of 1:1. Previous studies from our lab have shown that CD3/CD28 activated T cells are capable of inducing B cells to produce IL-10 via upregulation of CD154. After 48 hours of incubation, cells were surface stained with CD19 APC for 30 min in the dark at room temperature. Stained cells were washed with PBS and stimulated by incubation with 10 µg/ml of $F(ab')_2$ goat anti-human IgG+ IgM (H+L) (Jackson ImmunoResearch Laboratories) for 1 min at 37C°. 50 mM of hydrogen

peroxide solution (Sigma Aldrich) was added to positive control cells for 15 min at 37C°. Cells were then fixed for 10 min in the dark using the PerFix Kit for Phosflow assays (Beckman Coulter). After two washings, 1mL of X1 perm solution was added for 5 minutes at 37C°. Cells were washed twice and re-suspended in staining buffer (Beckman Coulter) for intracellular staining. Phosphorylated Stat3-PE (pSTAT3-PE) mAb Phosflow antibody (BD Biosciences) was used to stain the cells for 30 minutes in the dark at room temperature. Cells were then washed once more using staining buffer prior to acquisition, and gated on CD19+pSTAT3+ cells using both unstimulated B cells (negative control) and FMO (fluorescence minus one) control for pSTAT3 PE **figure (II-13).**

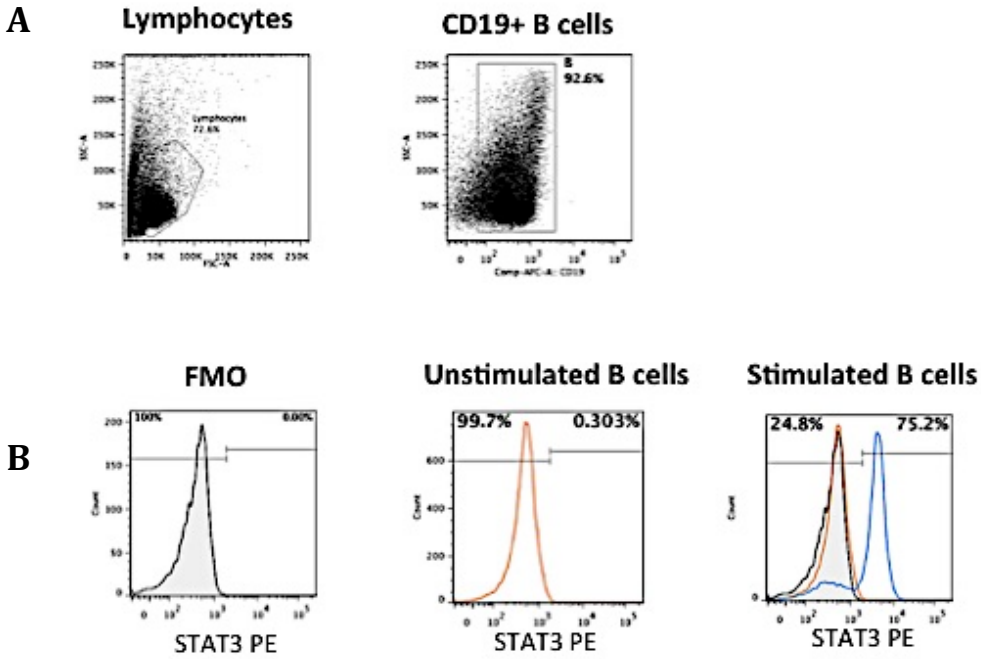

Figure II-14: Activated B cells upregulate JAK/STAT3 pathway.
A. A representative gating strategy-illustrating cells first gated on lymphocyte population and then sort purified CD19+ B cells. **B**. CD19+ B cell subsets were cultured with NK cells (1:1 ratio) for 48 hours and stimulated with 10µg/ml anti-BCR for 1 minute. CD19+STAT3+ cells (**blue)** were determined by PE FMO as indicated (**shaded grey**) and unstimulated B cells (**orange**).

II.10.2 Analysis of SAP expression on NK cells

Intracellular staining of the 2B4 signaling adaptor molecule SLAM-associated protein (SAP) was performed in negatively selected NK cells (Miltenyi Biotech) cultured either alone (positive control) or with sort-purified B cell subsets, namely, transitional, naïve, IgM memory and switched memory B cells at varying time points (**figure II-14**) to establish the optimum condition for B cell mediated NK+SAP+ suppression (in the presence or absence of 5 µg/ml of anti-IL-10 anti–IL-10 receptor, anti-CD48 (5 µg/ml) and anti-2B4 (5 µg/ml) for 48 hours at 37°C. Cells were collected and washed with 2 ml of PBS at 1400rpm for 10 min. Cells were then surface stained with CD56 BV605 (Biolegend) for 20 minutes at room temperature in the dark. Cells were then washed and fixed/ permeabilized for 45 minutes using the foxp3/transcription factor staining buffer set. Cells were washed using staining buffer and stained with anti-SAP PE (eBiosciences) for 30 minutes in the dark at RT. Cells were then washed again with 2 ml of staining buffer and analyzed on a BD LSRFortessa flow cytometer.

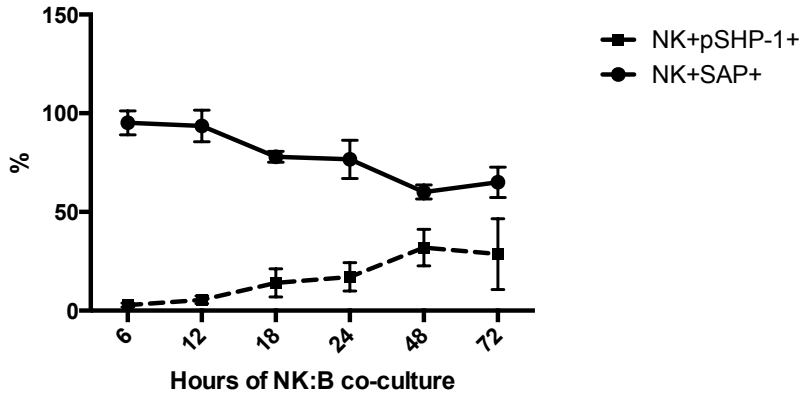

Figure II-15: A graph illustrating the relationship between B cell mediated suppression of NK+SAP+ cells and upregulation of inhibitory NK+pSHP-1 expression in a time dependent manner
(N=3)

II.10.3 Phosflow analysis of pSHP-1 expression on NK cells

The flow cytometry based measurement of pSHP1-Y536 in human PBMCs has been previously described. In brief, selected NK cells (1 x 10^6/ml) were cultured either alone or with sort-purified B cell subsets at a 1:1 ratio at varying time points to determine the optimized condition for upregulation of pSHP-1 expression in NK cells after B cell: NK cell co-culture (**figure II-14**). Having optimized the upregulation of pSHP-1 expression, cells were cultured for 48 hours at 37C°**.** Cells were harvested and suspended in PBS 1X at room temperature and stained with 2µg/ml of purified CD16 mAb (BD Pharmingen) for 20 min at 4C° for NK stimulation**.** After two washings with 2ml PBS 1X, 2µg/ml of goat anti-mouse IgG, F (ab')$_2$ fragment specific Ab (Jackson Immuno) was immediately added and cells were incubated at 37C° for 10 min. After stimulation, cells were fixed for 10 minutes at 37°C with fix solution 1 (Beckman Coulter, USA). After a further two washings, a 30 min surface staining was performed with anti-CD56 ECD (Beckman Coulter) and CD19 APC (BD Bioscience). Cells were washed and 1mL of perm solution2 was added for 5 min at 37°C. Cells were washed twice and stained with intracellular antibody pSHP-1 [p-SH-PTP1 Antibody (Tyr 536) Santa Cruz] self conjugated with Alexa Fluor® 488 Antibody Labeling Kit (Invitrogen) for 40 min in the dark at RT. After washing, cells were ready to be analysed on LSRFortessa (BD Biosciences) and gated on CD56+pSHP-1+ cells using both unstimulated B cells (negative control) and FMO (fluorescence minus one) control for pSHP-1 FITC **figure (II-15).**

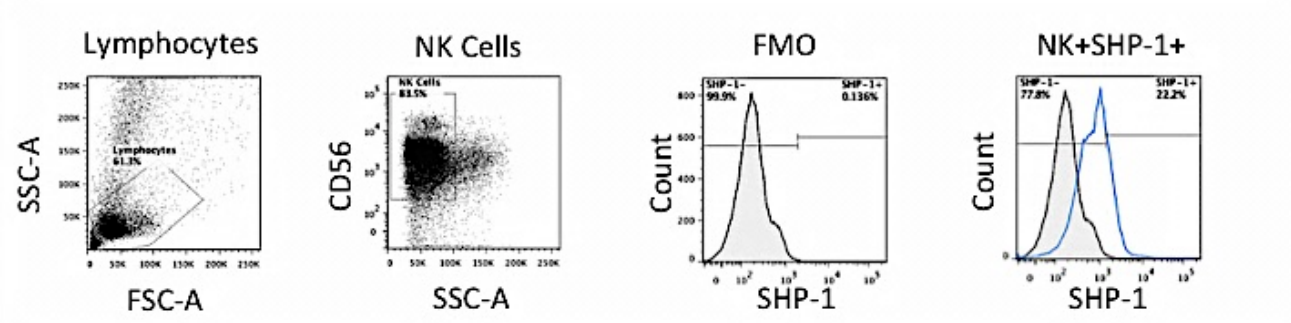

Figure II-16: Gating strategy of CD56+SHP-1+ cells
Cells were gated on lymphocyte population and then CD56+NK cells. CD56+SHP-1+ cells (blue) were determined by SHP-1 Alexa Fluor488 FMO (shaded grey).

II.11 Statistical Analysis

All values are expressed as mean, median and range as stated in the legends of each figure. We performed analysis of significance in Prism (GraphPad, La Jolla, USA) by unpaired or paired 2-tailed Student *t* test analysis and by nonparametric analysis of variance (ANOVA), as appropriate. Where p value was less than 0.05, the result was considered significant: *, $p < 0.05$; **, $p < 0.01$.

Chapter III. IL-10 producing B Cells With Immune Regulatory Capacity are Enriched Within Both the IgM Memory and Transitional B Cell Subset And Suppress CD4+ T cells

III.1 Introduction

In recent years, a distinct newly described subpopulation of interleukin-10 (IL-10) producing human B regulatory (Breg) cells, that have been shown to exert significant immunoregulatory functions both *in vitro* and *in vivo*, has been the focus of intense immunological research (Mauri and Bosma., 2012). In this chapter, I document work (introduction, findings and illustrations and discussion) from my recent published work (Khoder et al., Blood 2014) titled Regulatory B cells are enriched within the IgM memory and transitional subsets in healthy donors but are deficient in chronic GVHD. *Blood*, *124*(13), 2034.

The concept that suppressor B cells with regulatory properties could modulate the immune response, originated in the 1970's; however, the designated term 'B regulatory cells' (Breg) was first introduced nearly 30 years later (Katz et al., 1974, Neta and Salvin., 1974, Mizoguchi and Bhan., 2006). Despite the extensive body of evidence gathered, in the ensuing years since these studies were published, some controversy over the paucity of markers that can identify Bregs, particularly in humans, still exists. Tedder et al (Kalampokis et al., 2013) recognized IL-10-producing B cells as an emerging class of lymphocytes defined by an important set of regulatory functions that could be harnessed for therapeutic purposes. These Bregs have been shown to suppress inflammatory responses in murine models of experimental autoimmune encephalomyelitis (EAE) (Fillatreau et al., 2002), collagen-induced arthritis (CIA) (Mauri et al., 2003), colitis (Mizoguchi et al., 2002) and

the generation and maintenance of T-helper (Th) and T-regulatory (Treg) cells in the periphery (Wei et al., 2005). Thus far, a number of Breg phenotypic markers have been identified in murine models (Mauri and Bosma., 2012). Tedder et al demonstrated that IL-10-producing B cells (B10 cells) predominantly expressed $CD1d^{hi}CD5^{+}$ and shared phenotypic features common to marginal zone (MZ), T2-MZ precursor, and $CD1d^{hi}$ $CD5^{+}$ B-1a B cells, but did not exclusively belong to one of these B cell sub populations (Yanaba et al., 2008, Iwata et al., 2011). Hence, most current strategies to definitively identify Bregs in a reproducible manner rely on the detection of IL-10, which inhibits proinflammatory cytokine production and differentiation of effector T cells (Matsushita and Tedder., 2011). Thus, a more detailed investigation of the Breg 'signature' is needed to permit meaningful exploration of therapies based on B cells with regulatory potential.

The study of human Bregs, which share many functional characteristics with murine Bregs (Blair et al., 2010, Iwata et al., 2011) has been largely limited to IL-10-producing immature/transitional B cells in a small group of autoimmune diseases, including systemic lupus erythematous (SLE) (Blair et al.,2010), immune thrombocytopenia (ITP) (Li et al.,2012), multiple sclerosis (Correale., 2008) and more recently implicated in cGVHD (Khoder et al., 2014, Huu et al., 2013, de Masson et al, 2015). However, conflicting data exist as to the phenotypic characteristics of these cells. In the study by Blair et al (2010), IL-10^{+} Breg were shown to be enriched within the $CD24^{hi}$ $CD38^{hi}$ immature transitional B cell compartment. On the other hand, Tedder et al (2011) reported IL-10^{+} Breg to be enriched within the mature $CD24^{hi}$ $CD27^{+}$ memory

B cell subset. Although, the exact mechanism by which IL-10 producing regulatory B cells mediate immunosuppression is not clearly understood. Despite compelling evidence to support the role of Breg dysfunction in a number of autoimmune conditions, (Mauri and Bosma., 2012), very little is known about their role in immune regulation after allogeneic hematopoietic transplantation (HSCT) and their activities in chronic graft-versus-host disease (cGVHD), where CD4+CD25+ Treg cells have attracted the lions' share of attention (Rezvani et al.,2006, Mielke et al., 2007).

In this chapter my aim was to determine the presence of IL-10 producing regulatory B cells derived from peripheral blood mononuclear cells (PBMC) and to assess their suppressive potential on CD4+ T cell proliferation and effector function. My results demonstrate the presence of IL-10-secreting CD19+CD24hiCD38$^{-/lo}$IgM+ memory B cells that coexist with IL-10+CD24^{hi}CD38^{hi} transitional Breg cells in healthy human donors. Moreover, the regulatory capacity of these human Bregs on CD4+ T cell proliferation and cytokine production is depended on cell-cell contact through CD80/CD86 co-stimulatory signalling as well as IL-10 production.

III.2 Results

III.2.1 Phenotypic characterization of CD19+ B cell subsets

B cell characterization has been highlighted by a number of studies that recognize a number of classifying schemes, which are essential to the study of B cell biology in health and disease. Carsetti et al (2004) proposed to classify B cells through the CD24/CD38 expression profile, which characterizes B cell subsets as $CD24^{hi}CD38^{hi}$ transitional B cells, $CD24^{+}CD38^{+}$ naïve B cells, $CD24^{hi}CD38^{lo/-}$ memory B cells and $CD24^{-}CD38^{hi}$ plasmablasts. The use of additional markers such as IgM can further distinguish between switched memory and IgM+ memory B cells (Suryani et al 2010, Palanichamy et al, 2009). Additionally, Bohnhorst et al (2001), highlighted a classifying scheme referred to as Bm1-Bm5, which characterizes 5 B cell populations based on the phenotypic expression of IgD and CD38; Bm1 subset of naïve cells (IgD+CD38−); Bm2` subset of pre-GC cells (IgD+CD38++); GC cells (IgD−CD38++); and Bm5 memory cells (early IgD−CD38+ and late IgD-CD38-). Further, phenotypic expression of CD27 and IgD has also been proposed as a classification scheme to distinguish between CD27- antigen-naïve B cells and CD27+ memory B cells by Maurer et al (1992). However some memory B cells are also $CD27^{-}$, of which 20% are IgM-IgD-. Additionally, as transitional cells are also CD27-, further markers must be used to distinguish between the phenotypic differences of naïve and transitional subsets. With respect to the classification of B cells based on the CD24 and CD38 phenotype expression, I implemented this strategy to characterize B cell subsets and correlated the expression of transitional, naïve

and memory B cells with IgD/CD27, IgM/CD27 and IgD/CD38 axis (**figure III-1).**

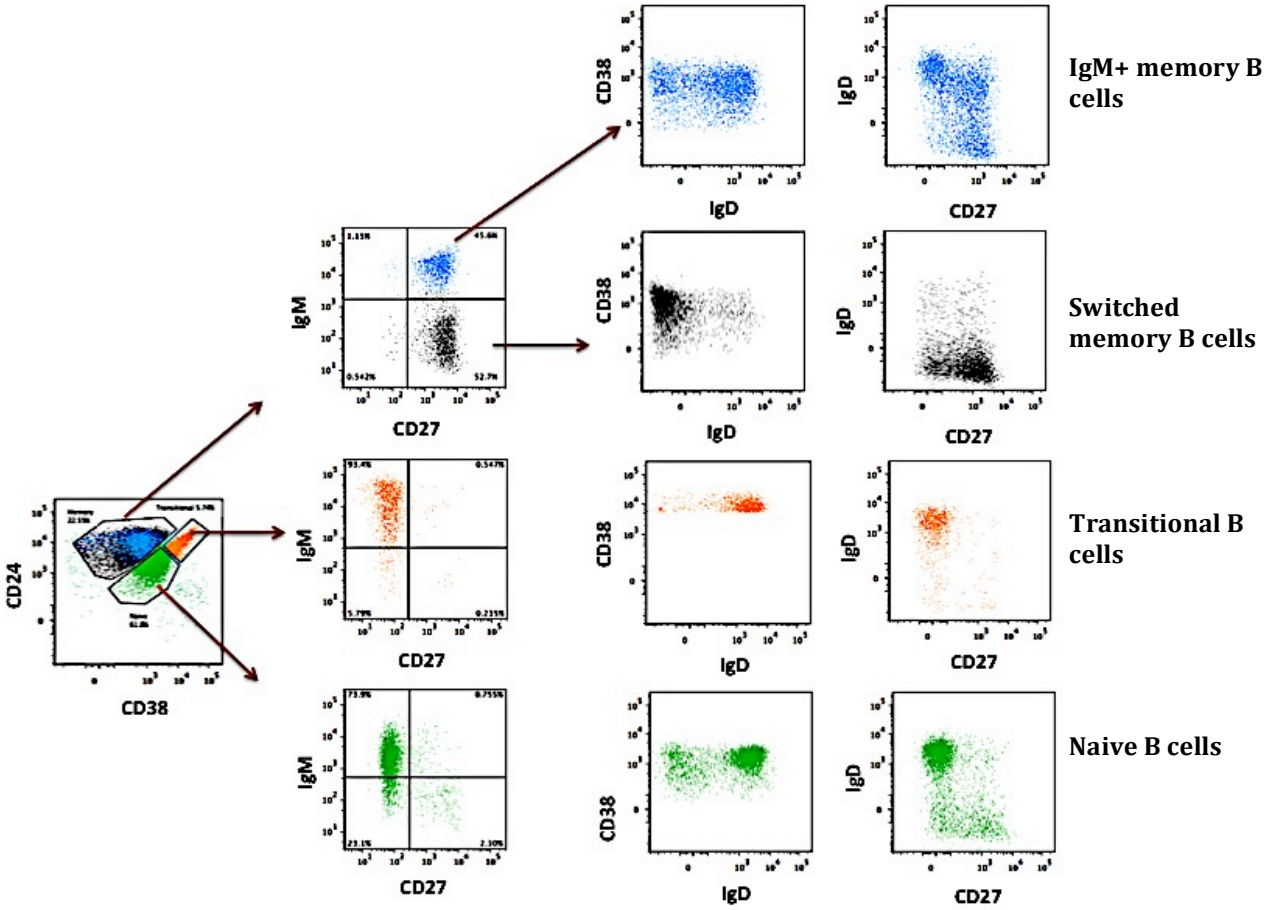

Figure III-1: CD19+ B cells subsets
Transitional (orange), naïve (green), IgM memory B cells (blue) switched B cells (black) were gated on CD38 and CD24 axis and correlated with different B cell classifying schemes IgM/CD27, CD38/IgD and CD27/IgD.

$CD19^{+}CD34^{hi}CD24^{hi}$ transitional B cells were further characterized as $CD27^{-}$ IgM^{hi} and IgD^{+}, $CD19^{+}CD24^{+}CD38^{+}$ naïve B cells were further characterized as $CD27^{-l}gM^{+}$ and IgD^{+}, $CD19^{+}CD24^{-/lo}CD38^{hi}$ memory B cells were further characterized as $IgM^{+}CD27^{+}IgD^{+/lo}$. However some IgM memory cells also contained $CD27^{-}IgD^{-}$ memory cells. $CD19^{+}CD24^{-/lo}CD38^{+}$ switched memory B cells were IgM^{-}, CD27+ and IgD-.

Further, in accord with previous studies (Suryani et al., 2010, Carsetti et al., 2004, Palanichamy et al., 2009), extended phenotypic characterization of B cells in 10 healthy donors using an extended panel outlined in table II-1, highlighted that PB-derived subpopulations of T1, T2 and T3 transitional subsets could be further characterized through the differential expression of CD10 and CD21 pattern where T1 cells illustrated highest expression of CD10, which is lost as the cells mature and acquire expression of CD21. T3 subsets had lower expression of CD10 and high expression of CD21, a marker of transition from immature to more mature and naïve B cells (**figure III-2**).

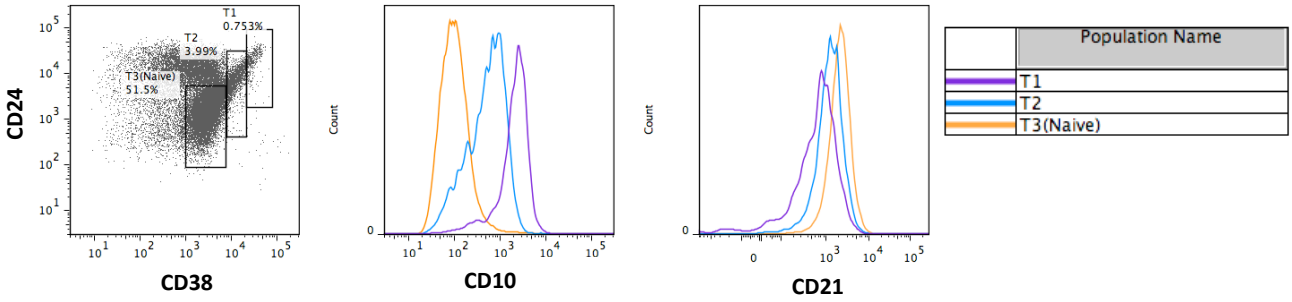

Figure III-2: Differential expression of CD10 and CD21
CD10 and CD21 expression distinguishes between T1 (most immature transitional cells) (purple), T2 (blue) and T3 (naïve) (orange) B cells based on CD24 and CD38 expression

Full phenotypic analysis of all four B cell subsets based on the expression of markers used in the extended B cell phenotype (section II.2.6.2) characterized transitional B cells as $CD19^{+}CD24^{hi}CD38^{hi}IgM^{hi}CD27^{-}CD10^{hi}CD21^{-}IgD^{hi}$, naïve B cells as $CD19^{+}CD24^{+}CD38^{+}IgM^{+}CD27^{-}IgD^{-}CD10^{-}CD21^{+}$, IgM+ memory B cells as $CD19^{+}CD24^{-}CD38^{hi}IgM^{+}CD27^{+}IgD^{+/lo}CD10^{-}CD21^{+}$ and switched memory B cells as $CD19^{+}CD24^{-}CD38^{hi}IgM^{-}CD27^{-}IgD^{-}CD10^{-}CD21^{+}$.

III.2.2 Peripheral blood B cell subset frequencies

B cell subsets in 10 healthy donors were characterized based on CD19+, CD38, CD24 and IgM expression as detailed in section II.6.2. The frequency of $CD19^+$ B cells in healthy PBMCs was 6.0% (3.21%-13.1%); 4.7%(2.55%-7.3%) of total $CD19^+$ B cells constituted of CD24hiCD38hi transitional B cells, 56.4%(38.9%-69.5%) were $CD24^+$ $CD38^+$ naïve B cells, 20.3%(12.9%-28.1%) were $CD24^{hi}$ $CD38^{lo}$ $CD27^+IgM^+$ memory B cells and 14.3%(10.6%-17.4%) $CD24^{hi}$ $CD38^{lo}$ $CD27^+IgM^-$ switched memory B cells (**figure III-3**). Further, I found no statistically significant difference on the impact of age on different subpopulations of CD19+ B cells. Additionally, there was also no significant difference in B cell subsets between gender groups, in females compared to males. This observation is in accord with previous reports that also did not find gender specific differences in CD19+ B cells (Jentsch-Ullrich et al., 2005).

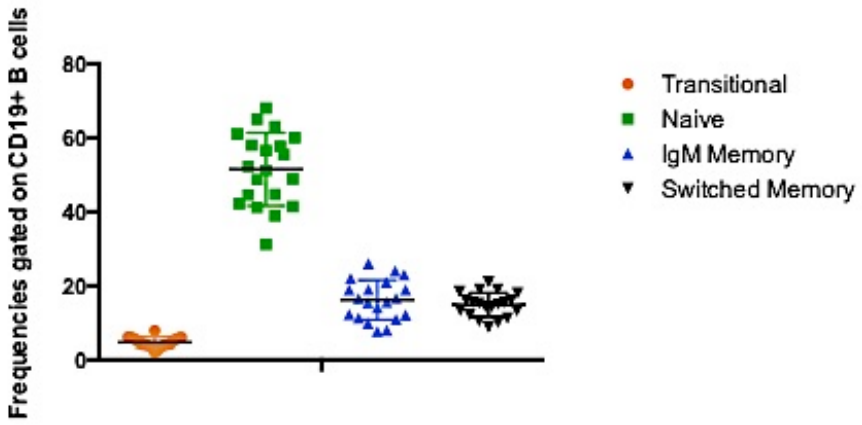

Figure III-3: Frequency of B cell subsets in healthy control PBMCs (n=20)
Frequency of B cell subsets out of total CD19+ B cells was determined through flow cytometric analysis of B cells subsets with respect to the CD24 and CD38 parameters. IgM+ and IgM- memory cells were subdivided as per expression of IgM. The line on the scatter dot plot represents median and range.

I next validated the effect of freezing on the signal intensity and the frequency of CD19+ B cell populations against the CD24 and CD38 axis and IgM

expression in the same 10 fresh healthy donor samples that were frozen at -80°c for a minimum of 8 days. No significant differences were found in the signal intensities or B cell subsets in paired fresh and frozen PBMC CD19+ B cell populations (**figure III-4**), which validates my panel to study frozen patient samples.

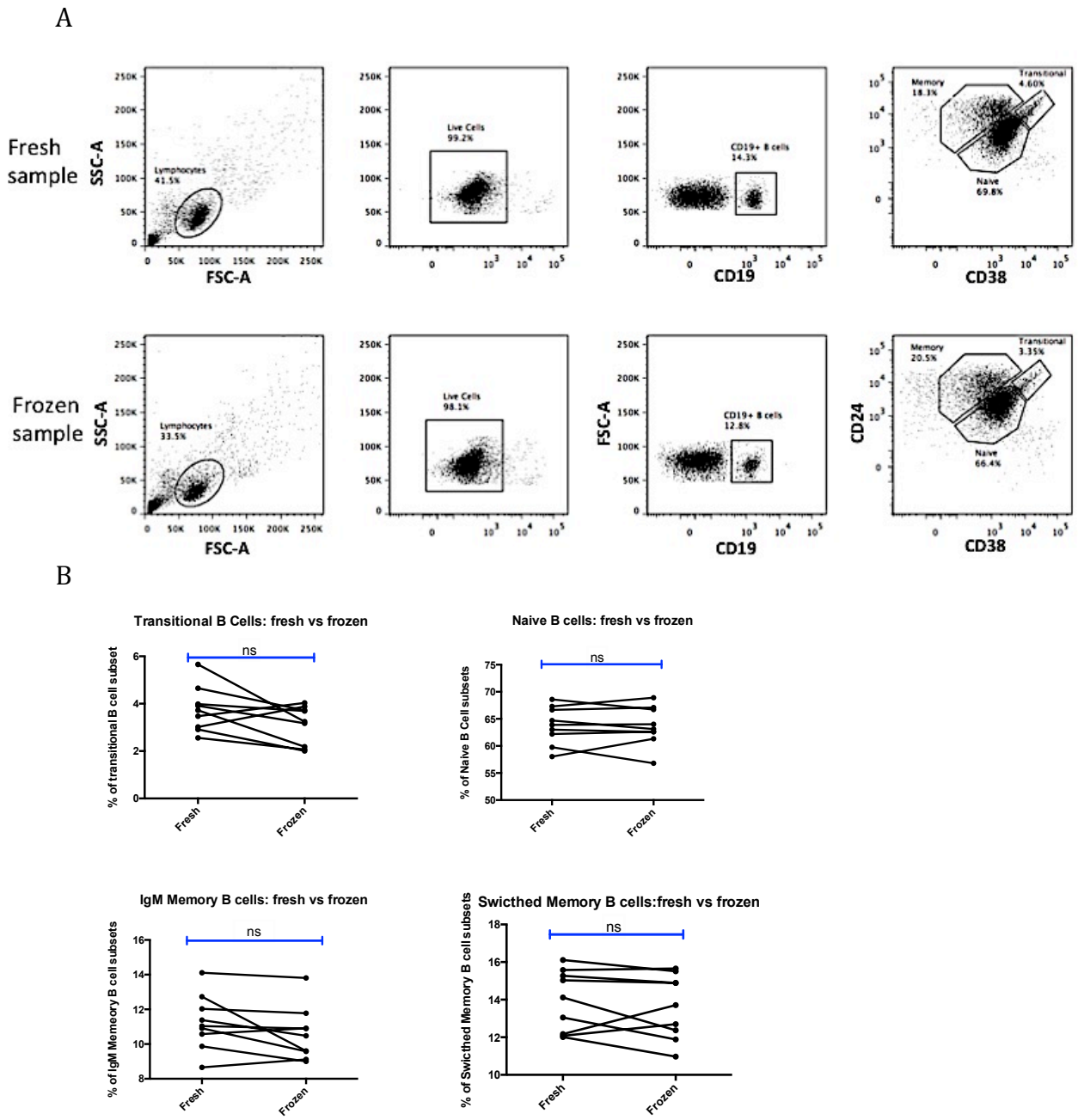

Figure III-4: Effect of cryopreservation on B cell phenotype
A. Representative FACS plots illustrating gating strategy of phenotyping CD19+ B cell subsets in both fresh and frozen samples. Cells gated on lymphocytes, live cells, CD19+ B cells and B cell subsets using CD24/CD38 axis. B. Each subset is

illustrated as bivariate plots representing data acquired from 10 paired fresh and frozen samples for transitional B cells (p= 0.0779), naïve B cells (p=0.8687), IgM memory (p=0.0969) and switched memory (p=0.3688) and analyzed using a paired t-test

III.2.3 Detection of IL-10 production by B cells isolated from PBMC

A number of studies have recognized the ability of PB-derived B cells to produce IL-10 in response to activation with CD40L, anti-BCR and CpG. Using the method described in II.6.3, I tested the kinetics of IL-10 production by freshly selected PB B cells from 14 healthy controls, stimulated with irradiated CD154 expressing fibroblasts (L cells), BCR crosslinking and CpG. Un-stimulated purified B cells, L cells and CD154- fibroblasts were also cultured alone as negative controls. I found that un-stimulated B cells produced negligible amounts of IL-10 (mean 14±4.5 pg/ml) and irradiated L cells and CD154- fibroblasts also produced small amounts of IL-10 (mean 5.5± 6.5 and 21± 12 pg/ml respectively). Activated CD19+ B cells produced IL-10 in a time dependent manner. In addition, B cells stimulated with L cells at a ratio of 1:10 produced higher levels of IL-10 than B cells stimulated with BCR crosslinking or CpG in a time dependent manner (**figure III-5**).

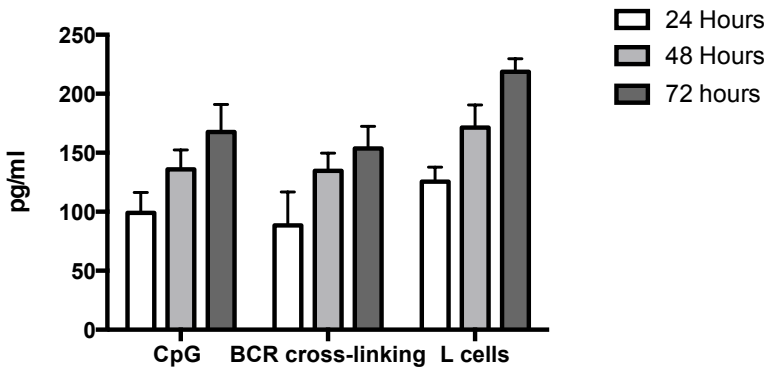

Figure III-5: Cumulative data illustrating levels of IL-10 production by L cell, CpG and BCR activated PB-derived total CD19+ B cells.

Based on these results I next determined the effect of cryopreservation on the ability of B cells to produce IL-10 in response to L-cells. PBMCs from the

same healthy donors were frozen at -80° C as described in II.2.3 for a minimum of 3 weeks. Cryovials were thawed as described in II.2.4 and rested for a minimum of 4 hours at 37° C before B cell selection was performed (II.4.1). Cells were activated as described previously and supernatant was assayed for IL-10 by ELISA. Minimal differences were observed in IL-10 levels between fresh and frozen paired samples and overall no significant differences were found (**figure III-6**). The results validate the use of frozen samples for the detection of IL-10 for future studies.

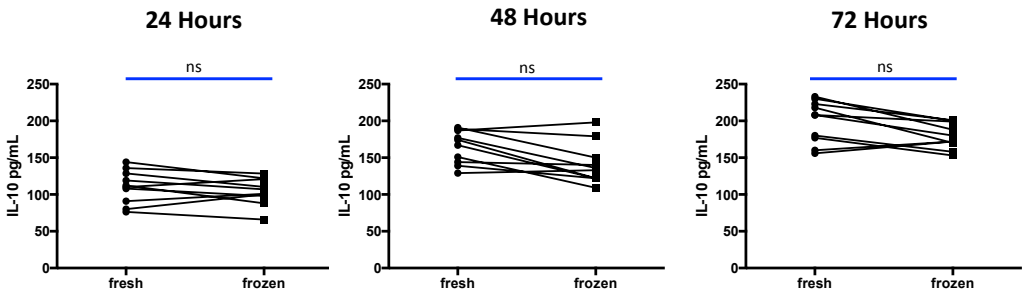

Figure III-6: Effect of cryopreservation on IL-10 production by B cells activated by L cells for 24, 48 and 72 hours.
Data was acquired from 10 paired fresh and frozen samples at 24 hours (p=0.4536), 48 hours (p=0.0717) and 72 hours (p=0.0934). Each sample is depicted on bivariate plot and analyzed using a paired t-test, $p<0.05$; ns,no significant difference

III.2.4 Detection of IL-10 production by B cells by intracellular staining

Based on ELISA results, I used L cells to study the kinetics of IL-10 production by B cells at different lengths of incubation (16hr, 24hr, 48hr and 72hr) by intracellular staining as described in II.6.5. PBMC or selected B cells were gated on CD19+IL-10+ population to determine the production of IL-10 in response to L cell activation. Un-stimulated cells, CD154- fibroblasts and an isotype control were used to validate the production of IL-10 by activated B cells. (**figure III-7A**).

In addition, the IL-10-producing B cell population was distinct from IFN-y

producing B cells, which suggests that B cells exist as 2 functionally distinct subsets: effector and regulatory **(figure III-7B**).

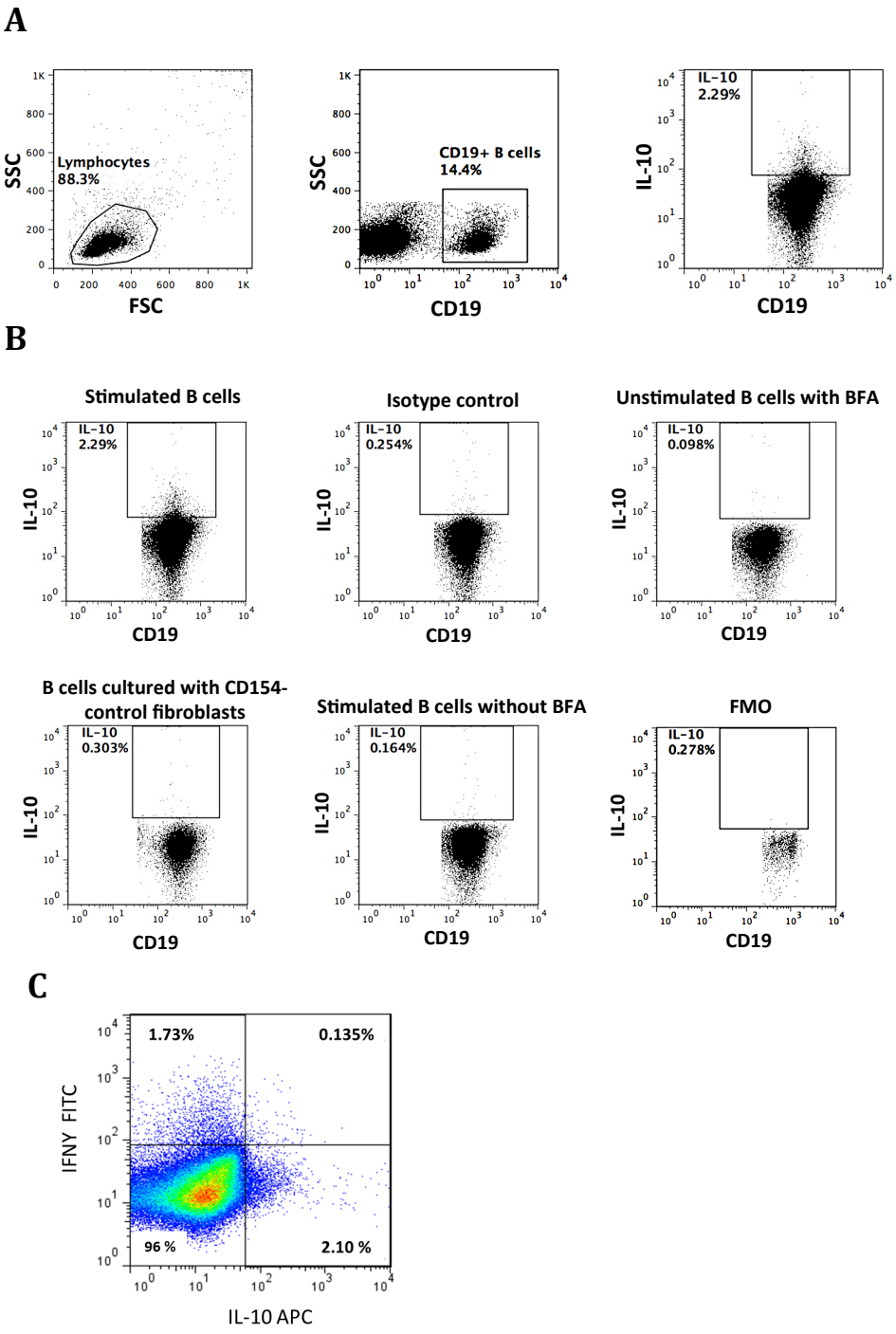

Figure III-7: Detection of CD19+ B cell IL-10 production by intracellular staining
A. Gating strategy for IL-10 producing CD19+ B cells. Gated CD19+ B cells from lymphocyte population were assessed for CD19+IL-10+ B cells **B** I gated on the CD19+IL-10+ population within PBMC or selected B cells to determine IL-10 production after 24 hours of coculture with L cell. The specificity of IL-10-producting B cells was validated by different negative controls as shown, including isotype control,

(IgG2aκ), unstimulated B cells incubated with BFA alone, PBMCs cultured with CD154- fibroblast (control cell line) and appropriately stimulated B cells without the addition of BFA. **C.** The IL-10-producing B cell population was distinct from IFN-y producing B cells

Further, gated CD19+ B cells produced IL-10 in a time dependent manner (**figure III-8A**). I next determined the effect of cryopreservation on B cell IL-10 production by intracellular staining. Paired PBMC from the same healthy donors (n=10) were frozen at -80° C as described in II.2.3 for a minimum of 3 weeks. Cryovials were thawed as described in II.2.4 and rested for a minimum of 4 hours at 37° C before the assay was performed (II.4.1). Minimal differences were observed in frequencies of IL-10 production between fresh and frozen paired samples after 24 hours of stimulation and overall no significant differences were found (1.98% vs. 1.65%; p=0.095) respectively (**figure III-8B**). The results validate the use of frozen samples for the detection of IL-10 for future studies.

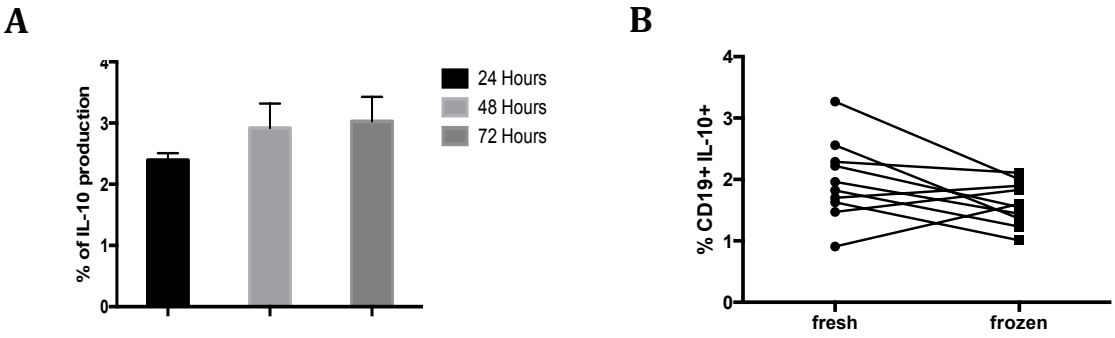

Figure III-8: Production of IL-10 from CD19+ B cells
A. Gated CD19+ B cells produce IL-10 in a time dependent manner (n=10) no significant was observed using an ANOVA test **B.** no significant difference was observed in the signal intensity of CD19+IL-10+ B cells between fresh and frozen paired healthy PBMC samples after 24 hours of stimulation (n=10)

III.2.5 Phenotypic characterization of IL-10 producing B cells

I next determined the phenotype of IL-10 producing B cells by using a panel of surface antibodies (CD19, CD24, CD38, CD27 and IgM) that have previously

been shown to identify a subset of IL-10 regulatory B cells (Khoder et al., 2014, Blair et al., 2010) in combination with intracellular IL-10 staining as detailed in section II.6.5. PBMC were stimulated with L cells at varying time points of 15h, 24h, 48 and 72hr. In accord with previous results from our laboratory (Khoder et al., et al 2014) I observed that the phenotype of B cells changes significantly within 24 hours of activation, characterized by downregulation of CD24 and the appearance of a population of plasmablasts ($CD24^{-}CD38^{hi}$). Therefore, in agreement with previous data from our lab, I found that 15 hours of B cell activation with L cells was optimal for IL-10 production without inducing significant changes in B cell phenotype. The majority of IL-10 producing B cells appeared to be enriched within the $CD19^{+}CD24^{hi}CD38^{hi}IgM^{hi}CD27^{-}$ transitional and $CD19^{+}CD38^{-/lo}$ $CD24^{hi}$ $CD27^{+}IgM^{+}$ memory B cell population and relatively fewer in the $CD19^{+}CD38^{+}CD24^{+}CD27^{-}$ naïve and $CD19^{+}CD24^{hi}CD38^{-/lo}CD27^{+}IgM^{-}$ switched memory B cell subsets. Median-fold increases in the frequency of IL-10 producing cells for IgM memory were 3.6 (range 2.5-4.6) and transitional were 4.42 (range 3.78-6.59) in comparison to naïve 0.48 (range 0.33-0.78) and switched 0.60 (range 0.43-0.80) B cell subsets (**figure III-9A**). In addition, CD19+IL-10+ B cells had a higher expression of CD24, CD27 and IgM. To validate if IL-10-producing B cells are enriched within transitional and IgM memory B cell subsets, I also measured IL-10 concentrations in the supernatants collected from sort-purified B cell subsets cultured with irradiated L cells. In keeping with my intracellular cytokine data, the highest level of IL-10 production was found in supernatants collected from transitional

and IgM memory B cell cultures, when compared to supernatants from naïve and switched memory B cell cultures (**figure III-9B**).

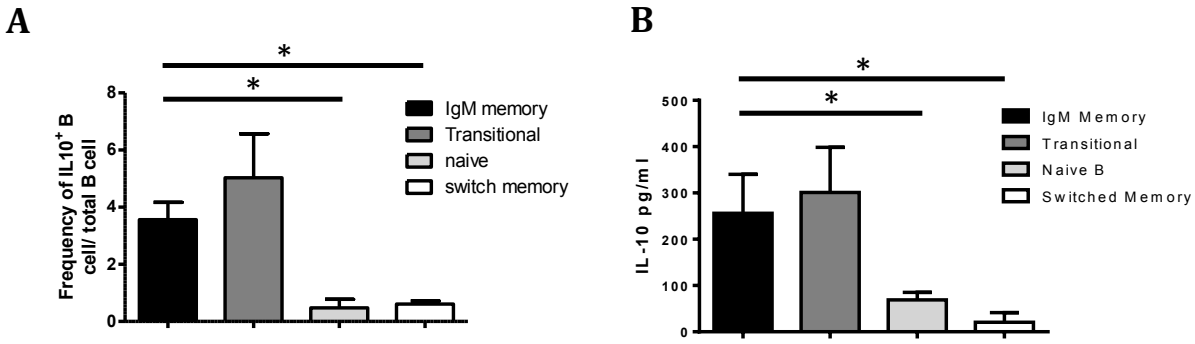

Figure III-9: IL-10 producing B cells are enriched within transitional and IgM memory B cell subsets
A. Transitional and IgM memory B cells expressed higher proportion of IL-10 producing B cells (frequency of IL-10 producing B cells/total B cell subset frequency) n=3 **B.** Sort purified transitional and IgM memory B cells secreted higher levels of IL-10 by ELISA after stimulation with naïve and switched memory B cell subsets (n=3). Bars in A and B represent median values, and upper whisker of error bar represents the range. $P < 0.05$ by nonparametric ANOVA.
This research was originally published in *Blood*. Khoder, A*., Sarvaria, A*., Alsuliman, A., Chew, C., Sekine, T., Cooper, N., Mielke, S., de Lavallade, H., Muftuoglu, M., Curbelo, I F., Liu, E., Muraro, P.A., Alousi, A., Stringaris, K., Parmar, S., Shah, N., Shaim, H., Yvon, E., Molldrem, J., Rouce, R., Champlin, R., McNiece, I., Mauri, C., Shpall, E.J., and Rezvani, K. Regulatory B cells are enriched within the IgM memory and transitional subsets in healthy donors but are deficient in chronic GVHD. *Blood*. 2014; 124(13): 2034-2045. © the American Society of Hematology.

III.2.6 IL-10 producing transitional and IgM memory B cells inhibit CD4+ T cell proliferation

I next examined whether IL-10 producing transitional and IgM memory B cell subsets also exhibited regulatory capacity on CD4+ T cell function. To evaluate this, I sort-purified transitional ($CD19^+CD24^{hi}CD38^{hi}$), naïve ($CD19^+CD24^+CD38^+$), IgM memory ($CD19^+CD38^{-/lo}CD24^{hi}IgM^+$) and switched memory ($CD19^+CD38^{-/lo}CD24^{hi}IgM^-$) B cells by flow cytometry from 20 healthy donors and cultured them with CFSE stained and anti-CD3/anti-CD28 bead-stimulated CD4+ T cells to assess their effect on proliferating $CD4^+$ T cells. The gating strategies and post-sort purity checks of B cell subsets are shown in **figure III-10**. The percentage of proliferating CD4+ T cells was assessed by

flow cytometry using CFSE dilution after 96 h of incubation as detailed in II.8.1. Results illustrated that IgM memory B cells and transitional B cells significantly suppressed $CD4^+$ T cell proliferation [median percent proliferating CD4+ T cells 54.5%; (33%-76.5%)] and 49.8% (25%-66%)], respectively when compared with anti-CD3/anti-CD28 stimulated CD4+ T cells cultured alone (positive control) [87% (77%-92%], or with naïve; [89.0%; (77%-97%] or switched memory B cells [80%; (52%-97%] **(figure III-11)**.

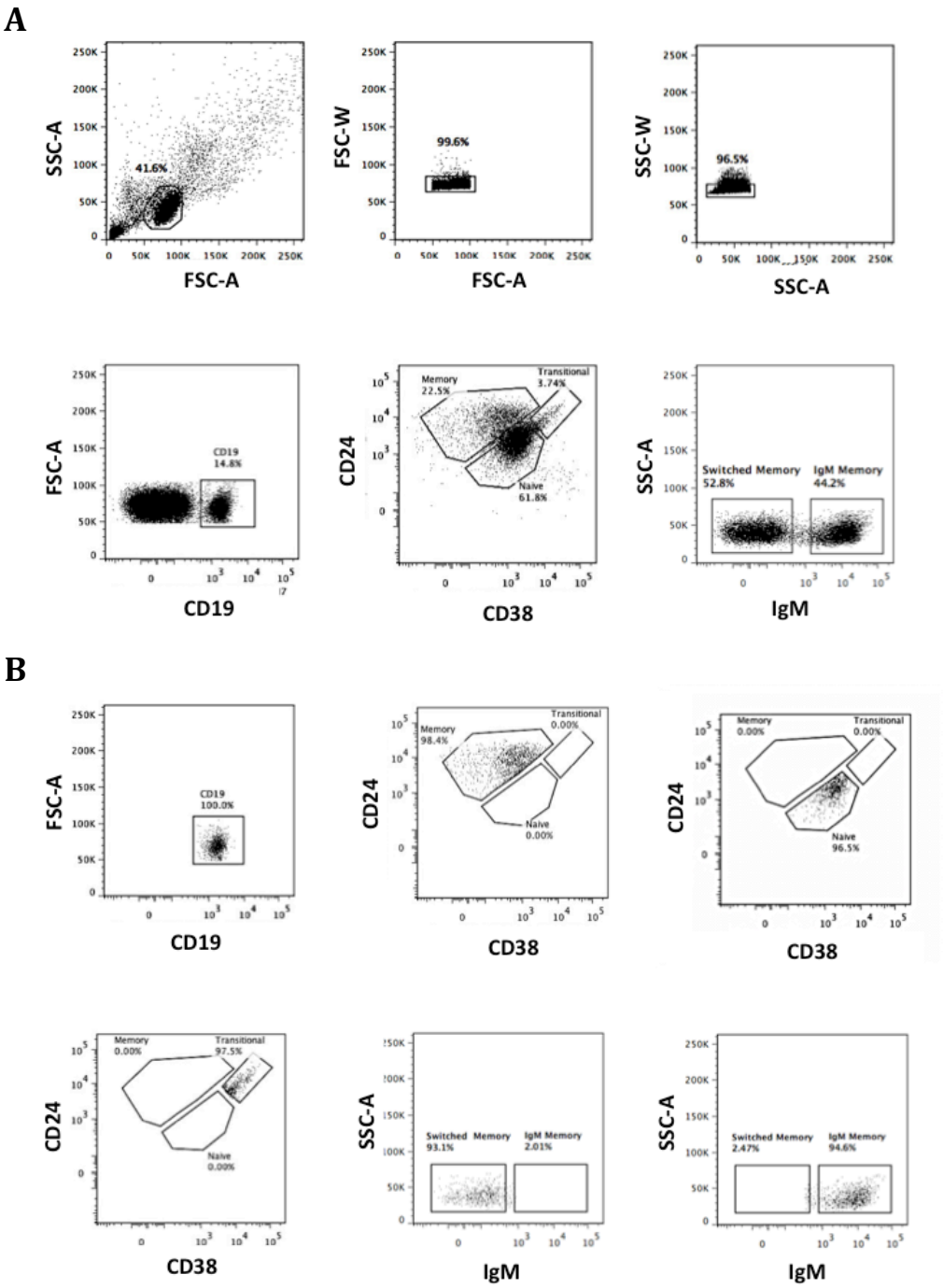

Figure III-10: Gating strategy and purity check of PB B cell sorting
A. Multi-parametric flow cytometric gating strategy for B cell subset sorting on BD FACS ARIA IIIu based on CD24 and CD38 expression **B.** FACS plots illustrating the high purity of sort-purified B cell subsets are shown within the CD19+ gate

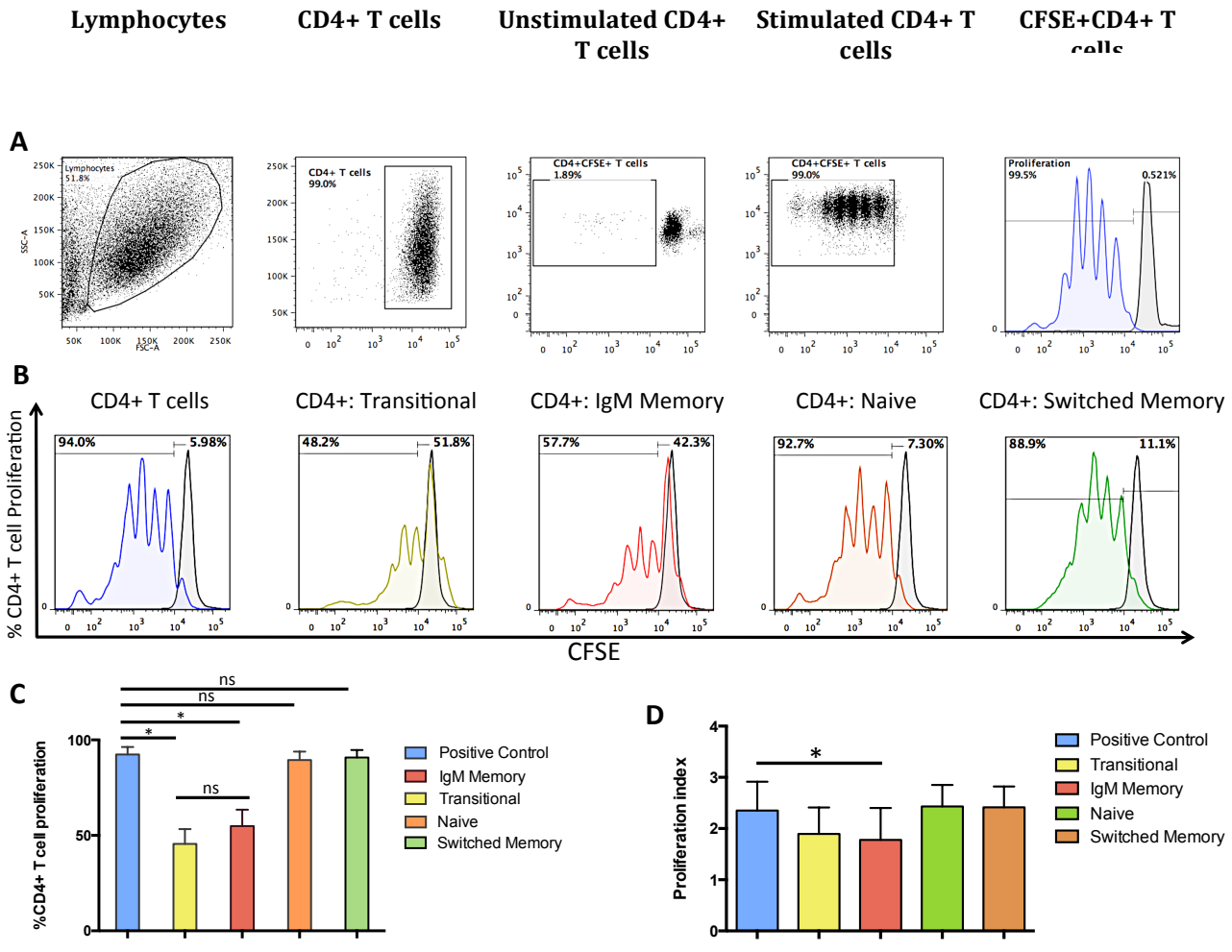

Figure III-11: IL-10 producing Transitional and IgM memory B cells suppress CD4+ T cell proliferation
A. Representative FACS plots illustrating the gating strategy of CD4+CFSE+ T cells. Gated CD4+ T cells from lymphocyte population were assessed for CFSE dilution by flow cytometry after 96 hours. Histogram overlay illustrates CFSE+ CD4+ T cells **(blue)** and negative control **(grey) B**. Magnetically selected and CD3/CD28 activated CD4+ T cells were labeled with CFSE (eBioscience) and plated either alone (positive control (proliferation index 2.27 – **Blue**) or at a 1:1 ratio with transitional (proliferation index 1.24 -**yellow),** IgM memory (proliferation index 1.65-**red),** naïve (proliferation index-2.19-**orange)** or switched memory (proliferation index 2.50-**green)** B cells. CFSE-stained T cells cultured with no stimulation (negative control –grey) were included in each experiment. **C.** Suppressive effects of CD19+ B-cell subsets (1:1 ratio) on frequency of CD4+ T cell proliferation. Bars represent median values, and upper whisker of error bar represents the range for 20 healthy donors. *P < 0.05 by

nonparametric ANOVA. **D**. Suppressive effects of CD19+ B-cell subsets on T cell proliferation index (n=20). Bars represent median values, and upper whisker of error bar represents the range. *$P < 0.05$ vs positive control by nonparametric ANOVA.

The inhibitory effect of transitional and IgM memory B cells on $CD4^+$ T cell proliferation was cell dose dependent, with the highest suppression observed at a ratio of 5:1 **(figure III-12).** I also compared the suppressive ability of IgM+ memory B cells and transitional B cells with that of magnetically selected $CD25^{hi}$ $CD127^-$ CD4 T cells regulatory T cells on autologous CD4+ T cell proliferation at a ratio of 1:1 (n=4). The suppressive ability of transitional and IgM memory was comparable to that of T regulatory cells (% suppression 48.3% vs. 53.6%; 58% respectively;) as shown in **figure III.13** indicating that transitional and IgM memory B cells exert similar suppressive function on CD4+ T cell proliferation as Treg.

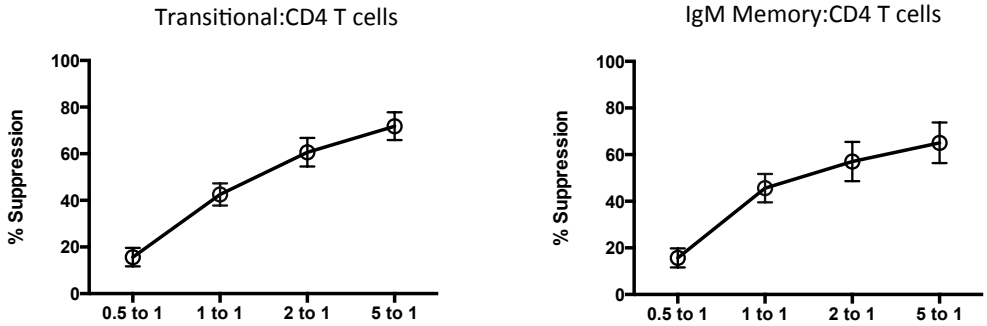

Figure III-12: Suppressive effect of transitional and IgM memory B cells on CD4+ T cell proliferation was dose dependent as indicated at the B cell: T cell ratios (n=4)

Error bars represent mean with standard deviation.

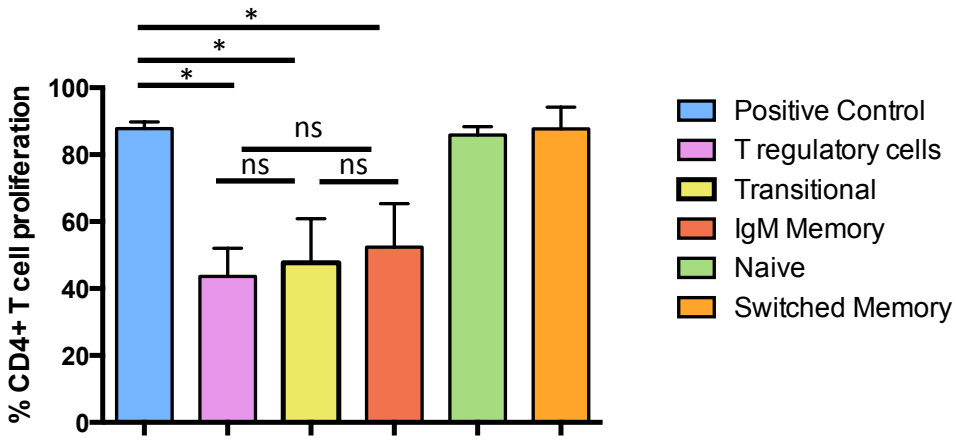

Figure III-13: Suppressive effect of transitional and IgM memory B cells on CD4+ T cell proliferation was comparable to that of T regulatory cells
Results illustrated that IgM memory B cells and transitional B cells significantly suppressed $CD4^+$ T cell proliferation in a manner comparable to T regulatory cells after 96 hours of B cell/T cell co-culture, when compared with anti-CD3/anti-CD28 stimulated CD4+ T cells cultured alone (positive control). Bars represent medians and upper ranges for 4 healthy donors. *P < .05 for individual comparisons with controls by nonparametric ANOVA; ns, not significant. This research was originally published in *Blood*. Khoder, A., Sarvaria, A., Alsuliman, A., Chew, C., Sekine, T., Cooper, N., Mielke, S., de Lavallade, H., Muftuoglu, M., Curbelo, I F., Liu, E., Muraro, P.A., Alousi, A., Stringaris, K., Parmar, S., Shah, N., Shaim, H., Yvon, E., Molldrem, J., Rouce, R., Champlin, R., McNiece, I., Mauri, C., Shpall, E.J., and Rezvani, K. Regulatory B cells are enriched within the IgM memory and transitional subsets in healthy donors but are deficient in chronic GVHD. *Blood*. 2014; 124(13): 2034-2045. © the American Society of Hematology.

III.2.7 IL-10 producing transitional and IgM memory B cells inhibit CD4+ T cell proinflammatory cytokine production

In order to assess the suppressive effect of sort-purified transitional and IgM memory B cell subsets on CD4+ T cell effector function, sort-purified B cell subsets were co-cultured with anti-CD3/anti-CD28 stimulated magnetic-bead purified $CD4^+$ T cells and the relative frequencies of $CD4^+IFN\gamma^+$ and CD4+TNF-α+ cells were assessed by flow cytometry. Both transitional and IgM memory B cells suppressed cytokine production by CD4+ T cells compared with CD4+ T cells cultured either alone or with naïve or switched memory B cell subsets (**figure III-14**). Consistent with these data, using ProcartaPlex Luminex assay, I also found suppression of IFN-γ, TNF-α and IL-2 production in supernatants harvested from cultures of sort purified

transitional and IgM memory B cells cocultured with CD4+ T cells. In contrast, naïve and switched memory B cell subsets failed to suppress production of proinflammatory cytokines by CD4+ T cells. Additionally, the suppressive ability of IgM memory and transitional B cells on CD4+ cytokine production was also comparable to that by T regulatory cells (**figure III-15**). Collectively, these results suggest that the suppressive capacity of transitional and IgM memory B cells on CD4+ T cell proliferation and cytokine production is comparable to T regulatory cells.

III.2.7 Depletion of Treg cells does not influence the suppressive ability of transitional and IgM memory B cells

I then examined if Treg cells are important for the *in vitro* suppressive effect of transitional and IgM memory B cells by depleting CD127- $CD25^{hi}$ Tregs from CD4+ T cells, using magnetic cell purification as detailed in II.4.2.2. Transitional and IgM memory B cell subsets were then cultured with anti-CD3/anti-CD28 stimulated and CFSE-stained Treg depleted-$CD4^{+}$ T cells at a ratio of 1:1. IgM memory and transitional B cells significantly suppressed the proliferation of Treg-depleted CD4+ T cells compared with the positive control; 57.9% (47.5-59) vs. 58.9% (49-62.7%) vs. 89.2 (85.8-92.1) respectively, **figure III-16**, while neither naïve nor switched memory B cells exerted any significant suppressive effect.

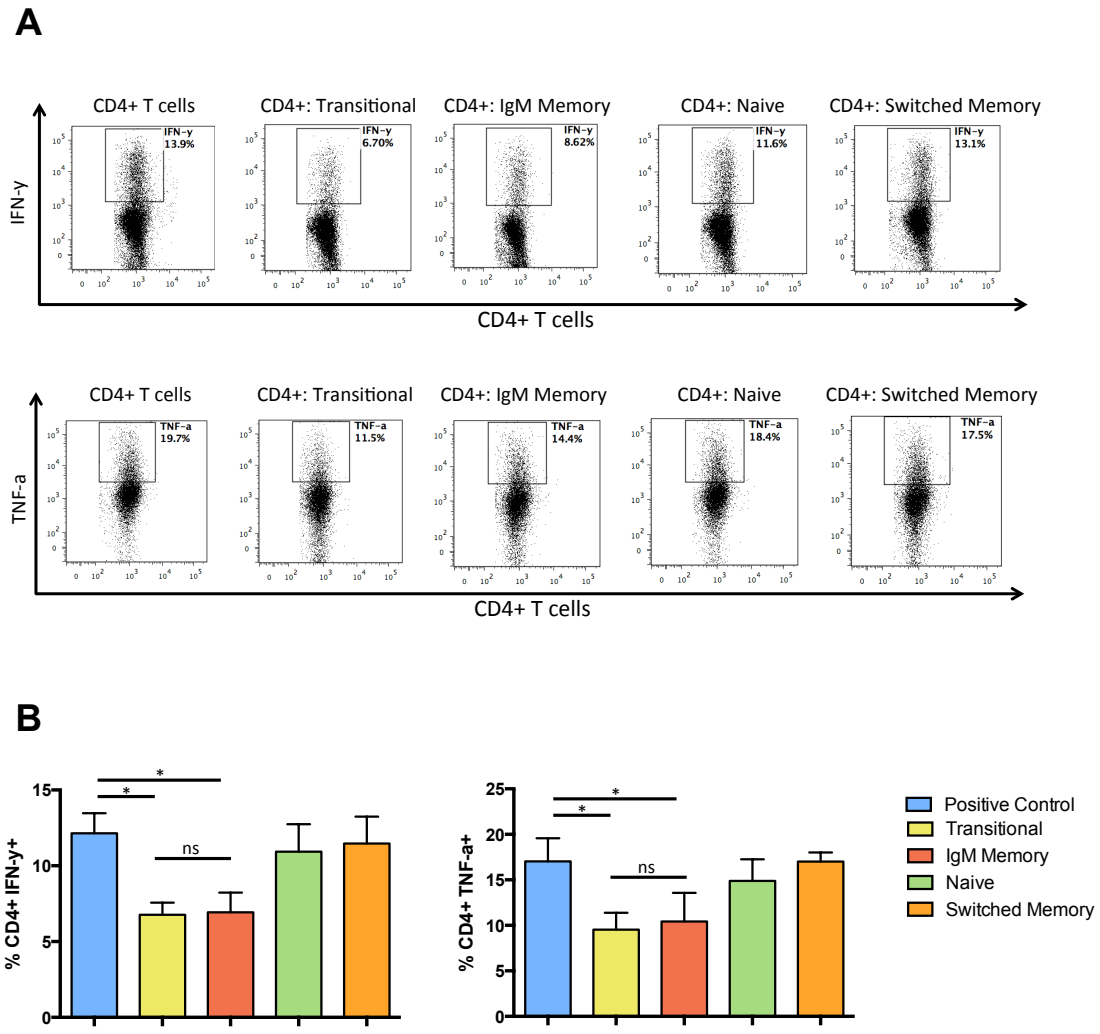

Figure III-14: Suppressive effect of transitional and IgM memory B cells on CD4+ T cell cytokine production.

A. Representative dot plots illustrating *in vitro* suppression of different CD19+ B cell subsets on anti-CD3/CD28 activated CD4+ T cell IFN-y and TNF-**α** production after 48 hours of coculture **B**. Cumulative data summarizing B cell suppression of CD4+ T cell IFN-y and TNF-**α** production assayed by intracellular staining. Bars represent median values, and upper whisker of error bar represents the range for 4 healthy donors. **P* < 0.05 for individual comparisons with controls (anti-CD3/anti-CD28 stimulated CD4+ T cells alone) by nonparametric ANOVA; ns, not significant.

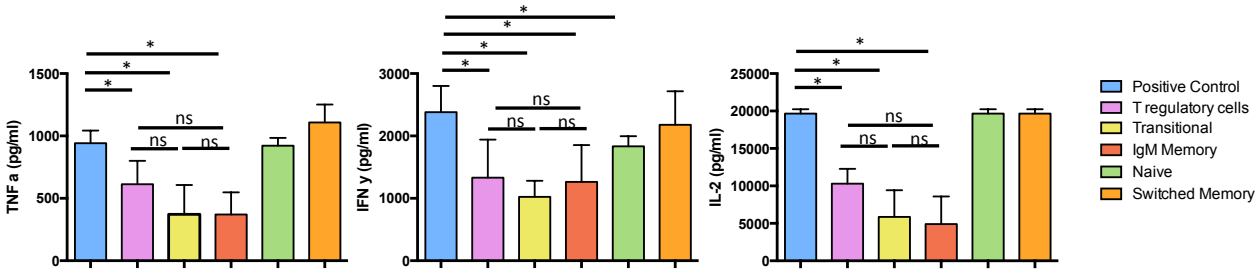

Figure III-15: The suppressive effect of transitional and IgM memory B cells on cytokine production by CD4+ T cells is comparable to that of T regulatory cells
Using Luminex assay, IFN-y, TNF-α and IL-2 levels were measured in supernatants harvested from cocultures of sort purified transitional and IgM memory B cell subsets with CD4+ T cells. Bars represent median values and upper whiskers of error bars represent range for 4 healthy donors. *P<0.05 for individual comparisons with controls (anti-CD3/anti-CD28 stimulated CD4+ T cells alone) by nonparametric ANOVA; ns, not significant.

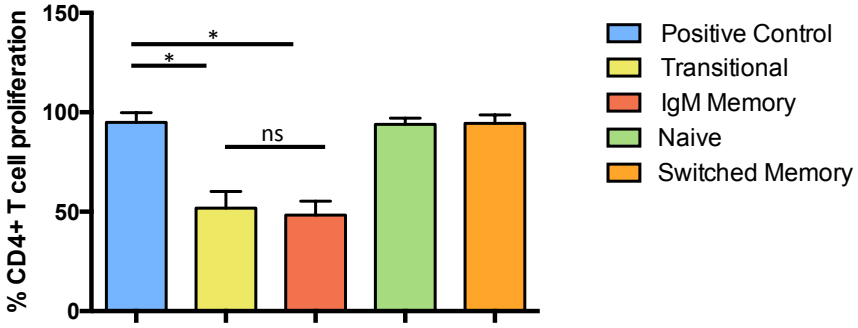

Figure III-16: Depletion of Treg cells does not influence the suppressive ability of transitional and IgM memory B cells
Bars represent median values and interquartile ranges from triplicate experiments. *P<0.05 vs. T- cell control for individual comparisons with controls (anti-CD3/anti-CD28 stimulated CD4+ T cells alone) by nonparametric ANOVA; ns, not significant.
This research was originally published in *Blood*. Khoder, A*., Sarvaria, A*., Alsuliman, A., Chew, C., Sekine, T., Cooper, N., Mielke, S., de Lavallade, H., Muftuoglu, M., Curbelo, I F., Liu, E., Muraro, P.A., Alousi, A., Stringaris, K., Parmar, S., Shah, N., Shaim, H., Yvon, E., Molldrem, J., Rouce, R., Champlin, R., McNiece, I., Mauri, C., Shpall, E.J., and Rezvani, K. Regulatory B cells are enriched within the IgM memory and transitional subsets in healthy donors but are deficient in chronic GVHD. *Blood*. 2014; 124(13): 2034-2045. © the American Society of Hematology.

III.2.8 The suppressive effect of transitional and IgM memory B cells is IL-10 dependent

I next examined the potential mechanism(s) by which transitional and IgM memory B cells suppress CD4+ T cell proliferation and effector function. CD4+ T cells were cultured either alone or at a 1:1 ratio with sort purified transitional and IgM memory B cell subsets in the presence of IL-10 and IL-10 receptor (IL-10R) blocking mAbs. Blockade of IL-10 significantly restored

cytokine production and proliferation of CD4+ T cells co-cultured with transitional and IgM memory B cells, supporting previous data with transitional B cells (Blair et al., 2010), that the regulatory properties of transitional and IgM memory B cells is mediated through IL-10 (**figure III-17A**). However, IL-10 blockade did not fully reverse the suppressive capacity of transitional and IgM memory B cells [median proliferating CD4+ T cells 78.2%; (65.6%-84.7%)]; and 74.6%; (66.7%-82.5%), respectively], (**figure III.17B**), when compared to the corresponding positive control [median 92.3% (90%-94.8%)], suggesting that other mechanisms, most likely other soluble factors or costimulatory molecules, are involved in regulating Breg-mediated suppression of CD4+ T cells. I further illustrated that adding exogenous IL-10 to co-cultures of CD4+ T cells with naïve B cells or switched memory B cells induced minimal suppression of CD4+ T cell proliferation [median proliferating CD4+ T cells 82%; (81%-89%)]; p=0.00649 and 81.5%; (78.8%-82.4%), p=0.0074 respectively] (**Figure III-18A**), when compared with the corresponding positive control, [93.60% (91.6%-95%], but this effect was substantially less than that seen with transitional or IgM memory B cells [51% (25-63%) and 52.5% (33-75%), respectively]. These results strengthen the hypothesis that IL-10 does not by itself confer suppressive capacity to B cells and further support the involvement of other mechanisms in Breg-mediated T cell suppression. Previous reports suggest that the immunoregulatory effects of B regulatory cells may be mediated by TGF-β (Blair et al., 2010). Therefore, I performed additional blocking experiments using TGF-β specific mAbs. TGF-β blockade had no significant impact on transitional and IgM memory B cell mediated suppression on CD4+ T cells (p=0.4585 and p=0.8637, respectively; n=3)

(**figure 18B**), indicating that this cytokine lacks any appreciable role in human Breg-mediated inhibition of T cells.

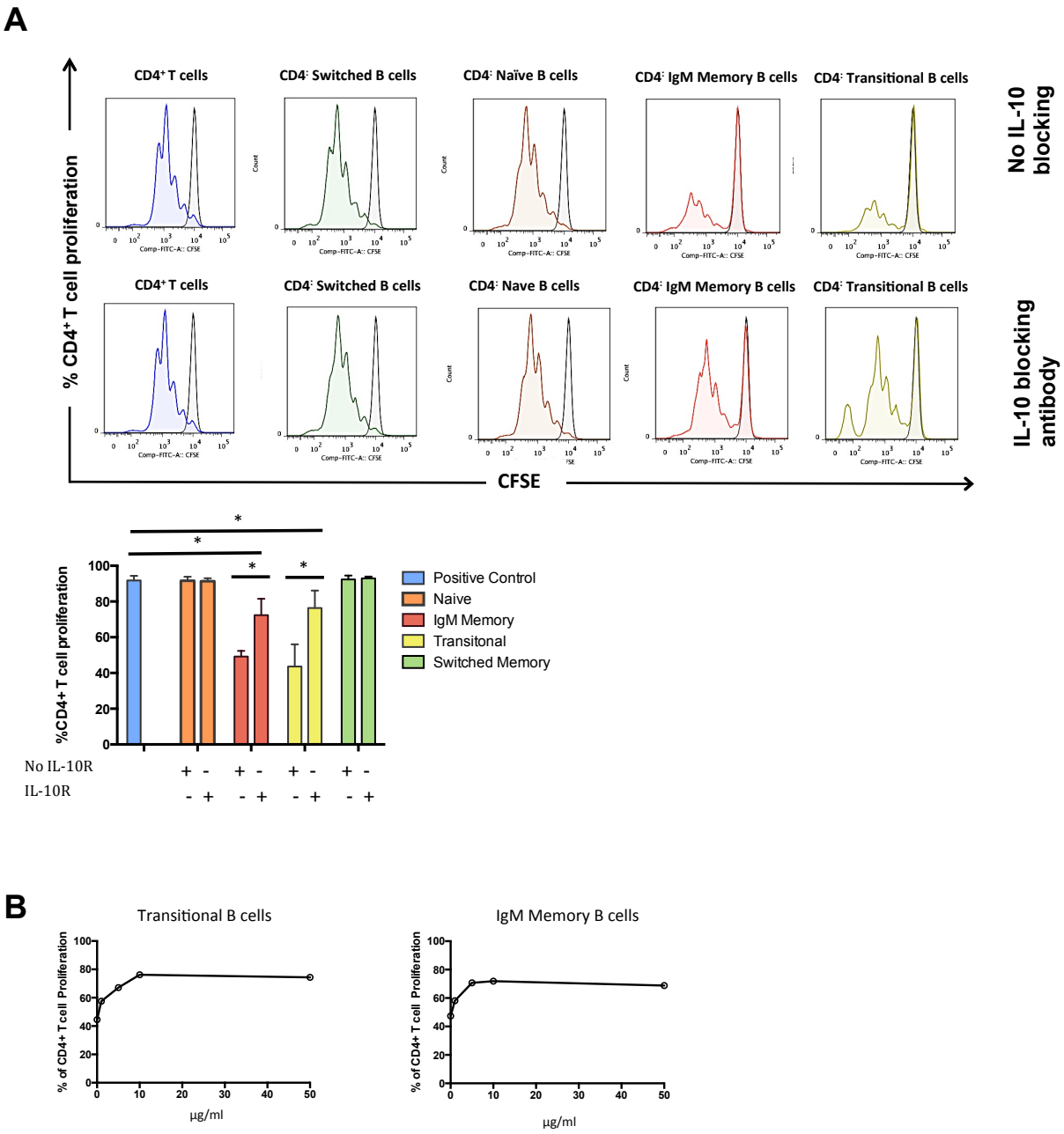

Figure III-17: The suppressive effect of transitional and IgM memory B cells on CD4+ T cells is partially dependent on IL-10.
A. Anti-CD3/anti-CD28 stimulated CD4+ T cells were cultured with FACS-sorted CD19+ B cell subsets in the presence or absence of IL-10 blockade 96 hours. Unstimulated CD4+ T cells (negative control-grey shaded area) were included with each experiment. Flow cytometry histograms show proliferation of CD4+ T cells cultured alone (blue) or with naïve (green), switched memory (orange), IgM+ memory (red) or transitional B cells (yellow). Proliferation index values for histograms without IL-10 blockade are: CD4+T cells – 2.70, with naïve – 2.62, switched –2.91, transitional – 1.31, IgM memory – 1.48.

Proliferation index values for cultures with IL-10 blockade are: CD4+T cells – 2.70, with naïve – 2.55, switched -2.68, transitional -2.05, IgM memory -2.23**.** Bar charts compare the suppressive effect of B cell subsets on proliferating CD4+ T cells with or without IL-10 blockade. Bars indicate medians, and whiskers indicate the upper range for 3 healthy donors. **P* < 0.05 vs positive control or IL-10R blockade by nonparametric ANOVA. **B**. Dose titration graphs for transitional and IgM memory B cells co-cultured with CFSE-stained CD4+ T cells in the presence of varying concentrations of anti-IL-10 and anti-IL-10 receptor blocking antibody (n=3)

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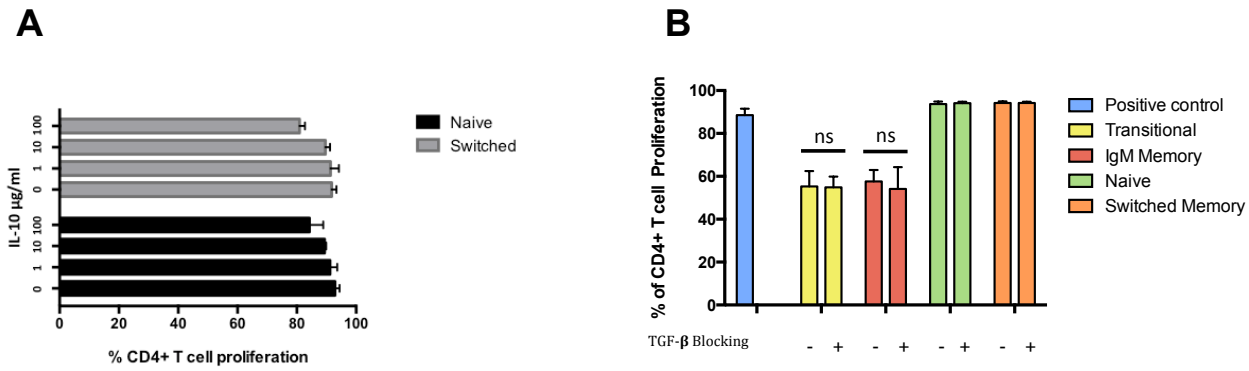

Figure III-18: The suppressive effect of transitional and IgM memory B cells on CD4+ T cells is dependent on IL-10 but not TGF-β.

A. Titrated effects of adding exogenous IL-10 at varying concentrations had minimal effect on the ability of naïve and switched memory B cells to suppress CD4+ T cell proliferation. **B.** TGF-β blocking had no significant effect on the suppressive ability of transitional and IgM memory B cells (n=3). Bars indicate medians, and whiskers indicate the range. **P* <0.05 by nonparametric ANOVA.

This research was originally published in *Blood*. Khoder, A*., Sarvaria, A*., Alsuliman, A., Chew, C., Sekine, T., Cooper, N., Mielke, S., de Lavallade, H., Muftuoglu, M., Curbelo, I F., Liu, E., Muraro, P.A., Alousi, A., Stringaris, K., Parmar, S., Shah, N., Shaim, H., Yvon, E., Molldrem, J., Rouce, R., Champlin, R., McNiece, I., Mauri, C., Shpall, E.J., and Rezvani, K. Regulatory B cells are enriched within the IgM memory and transitional subsets in healthy donors but are deficient in chronic GVHD. *Blood*. 2014; 124(13): 2034-2045. © the American Society of Hematology.

III.2.9 The suppressive effect of IL-10+ IgM memory and transitional B cells also depends on cell-cell contact, mediated through CD80/CD86

Previous studies have postulated that the suppressive capability of murine Bregs is mediated both by the provision of IL-10 and direct contact with $CD4^+$T cells (Blair et al., 2010, Mauri and Bosma., 2012, Khoder et al., 2014). However, it remains to be determined whether a similar direct cell-to-cell contact is involved in human Breg-mediated T cell suppression. To examine this, I performed transwell experiments, in which transitional and IgM memory B cells were either in direct contact or separated from anti-CD3/anti-CD28 stimulated and CFSE-stained $CD4^+$ T cells by a permeable membrane. Proliferation of CD4+ T cells was measured 96 hours after onset of culture. Direct co-culture of $CFSE^+CD4^+$ T cells with transitional or IgM memory B cells resulted in significant suppression of CD4+ T cell proliferation, median frequencies of proliferating CD4+ T cells are 48.9% (43.3%-65.1%) and 47.4% (39.7%-64.7%), respectively n=5]. On the other hand, separation of B cell/T cell direct contact by a transwell membrane partially reversed the suppressive effect of these B cell subsets, [median frequencies of proliferating CD4+ T cells 74% (59.7%-85.5%) p=0.011 and median 67.7% (52.5%-88.7%), p=0.0305, respectively; n=4] as shown in **figure III-19**. However, this reversal was not complete (compared with positive control).

In order to examine whether a combination of IL-10 blockade and abrogation of direct cell-to-cell contact can completely reverse the suppressive effect of proposed regulatory B cell subsets on CD4+ T cell proliferation, I added IL-10 and IL-10 receptor (IL-10R) mAbs to cultures of sort purified transitional and

memory B cells in a transwell setting. Subsequent addition of IL-10 blockade in a transwell setting almost completely abolished transitional B cell and IgM memory B cells mediated inhibition of CD4+ T cell proliferation [% of proliferating CD4+ T cells; median 87.7% (86.5%-88.3%) and median 90.4% (85.7%-91.0%), respectively; n=3] (**figure III-19C**).

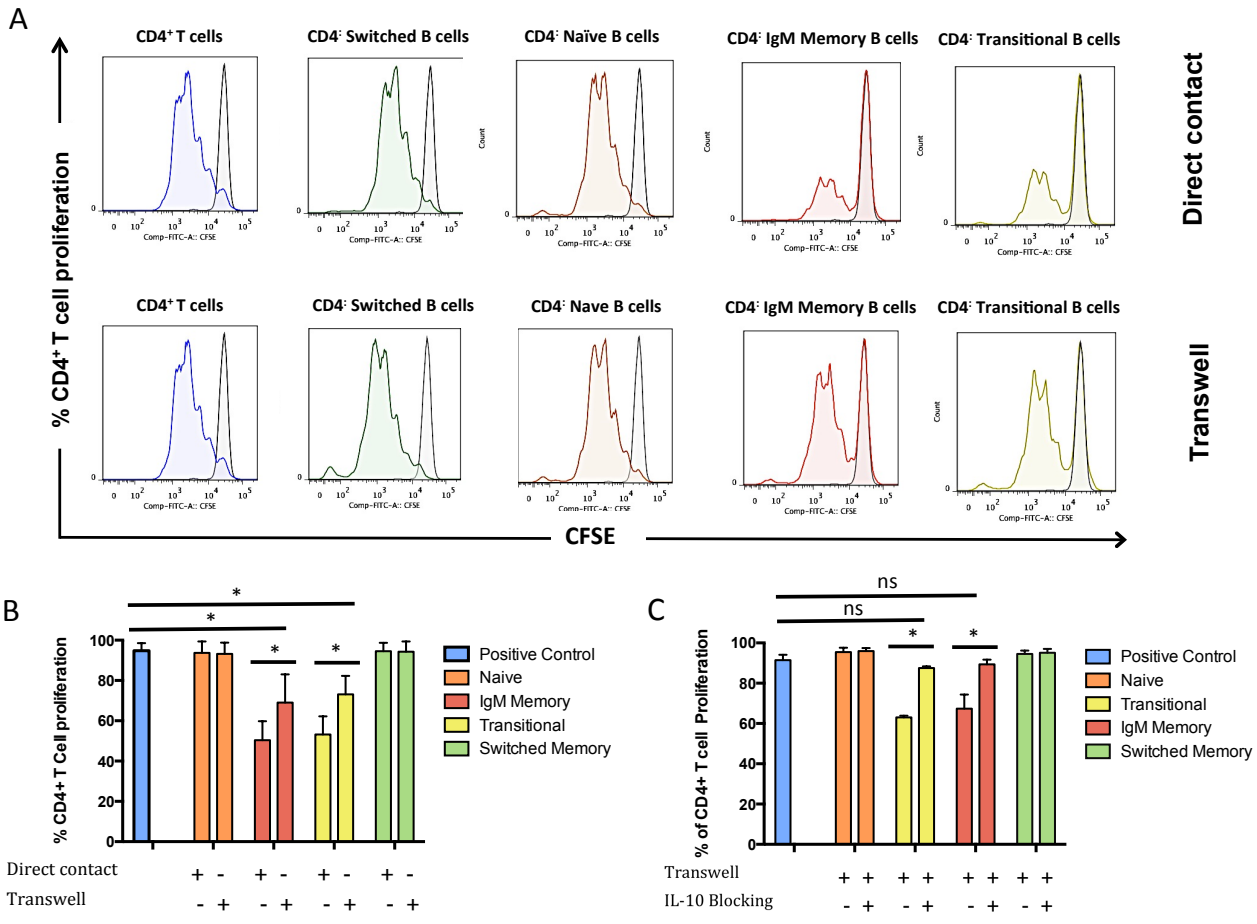

Figure III-19: Effect of B cell:T cell contact on the profiles of CD4+ T cell proliferation

A. Representative histograms illustrating CD4+ T cells either directly added to sorted CD19+ B cell subsets at a ratio of 1:1 or placed in transwell chambers in the same well: CD4+ T cells alone (blue), with naïve B cells (orange), switched memory B cells (green) IgM+ memory B cells (red) and transitional B cells (yellow) at a ratio of 1:1. Unstimulated controls (grey) were included. Proliferation index values for histograms with direct contact are: CD4+T cells – 2.91, with naïve – 2.85, switched –3.00, transitional – 1.60, IgM memory – 1.41. Proliferation index values for cultures with transwell are: CD4+T cells – 2.91, with naïve – 3.94, switched-3.11, transitional - 2.07, IgM memory -2.28**. B.** Collective data (n=5) compare the effect of suppressive B cells on the proliferation of CD4+ T cells with or without direct cell to cell contact. The data are shown as medians and upper whisker of error bars are range. *$P < 0.05$

by nonparametric ANOVA; ns, not significant. **C.** The suppressive capacity of IgM+ memory B cell and transitional B cell suppression is dependent on IL10 and cell-to-cell contact (n=3). The data shown are medians and upper whisker of error bars are range. *P < 0.05 by nonparametric ANOVA; ns, not significant.

Thus, both direct cell-to-cell contact and IL-10 production are necessary for Breg cells to achieve their full suppressive potential. To determine the mechanism by which Bregs produce IL-10 in the transwell setting, we measured the levels of soluble CD40L in the supernatant by ELISA assay. Soluble CD40L is naturally secreted by activated T cells (van Kooten et al., 2000) and can induce IL-10 production by B cells (Iwata et al., 2011). As shown in **figure III-20**, soluble CD40L was present in co-cultures with CD3/CD28-activated CD4+ T cells. Hence, I propose that soluble CD40L can cross the membrane and induce IL-10 production by transitional and IgM memory B cells to suppress CD4+ T cell proliferation.

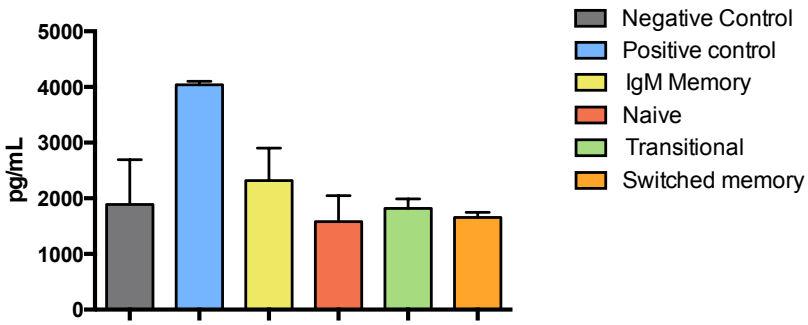

Figure III-20: Anti-CD3/anti-CD28 stimulated CD4+ T cells release soluble CD40L, measured using the Human CD40 Ligand Quantikine ELISA kit (R&D) in the transwell supernatant (pg/mL) (n=4). Bars represent median values and error bars represent range.

I next tested the contribution of CD80 and CD86 costimulatory signalling to the suppressive ability of transitional and IgM memory B cells. Although the addition of blocking antibodies against CD80 or CD86 molecules individually did not impact on the suppressive activity of IgM memory and transitional B cells, addition of blocking antibodies against both molecules together partially inhibited the ability of transitional and IgM memory B cells to suppress the proliferation of CD4+ T cells (**figure III-21**). While blockade of IL-10/IL-10R and CD80/CD86 individually was not sufficient to completely reverse the ability of transitional and IgM memory B cells, a combination of mAbs Blocking mAbs against all three molecules completely reversed the inhibitory effect of these B cell subsets. (**figure III-22**).

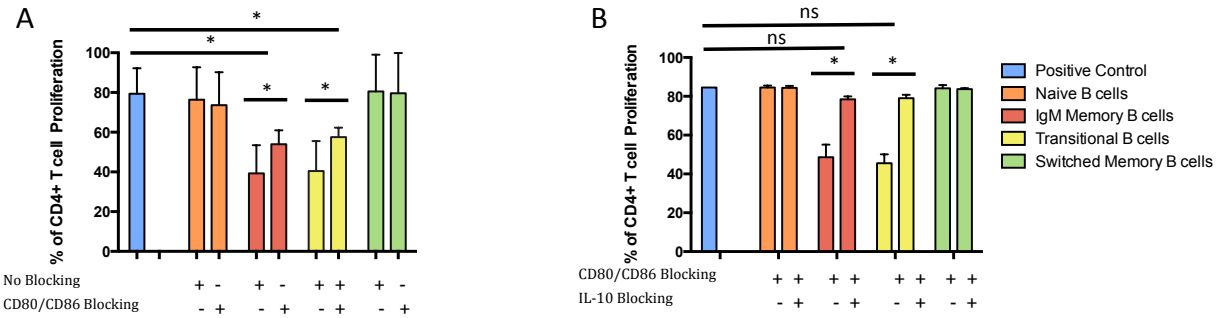

Figure III-21: CD80 and CD86 co receptor signalling and IL-10 are both prerequisites for the suppressive effect of Bregs.
A. Cumulative data representing co-receptor blockade in cultures of purified CD4+ T cells and sorted CD19+ B cell subsets, which did not fully reverse the suppressive capacity of Bregs as compared to the corresponding positive control B. Bar charts compare between the effect of co-receptor blocking and IL-10 blocking in CD4+ T cell and B cell co cultures. All bars in (a) and (b) represent median values, and upper whiskers indicate the range from 3 independent experiments. *$P < 0.05$ by nonparametric ANOVA; ns, not significant. This research was originally published in *Blood*. Khoder, A., Sarvaria, A., Alsuliman, A., Chew, C., Sekine, T., Cooper, N., Mielke, S., de Lavallade, H., Muftuoglu, M., Curbelo, I F., Liu, E., Muraro, P.A., Alousi, A., Stringaris, K., Parmar, S., Shah, N., Shaim, H., Yvon, E., Molldrem, J., Rouce, R., Champlin, R., McNiece, I., Mauri, C., Shpall, E.J., and Rezvani, K. Regulatory B cells are enriched within the IgM memory and transitional subsets in healthy donors but are deficient in chronic GVHD. *Blood*. 2014; 124(13): 2034-2045. © the American Society of Hematology.

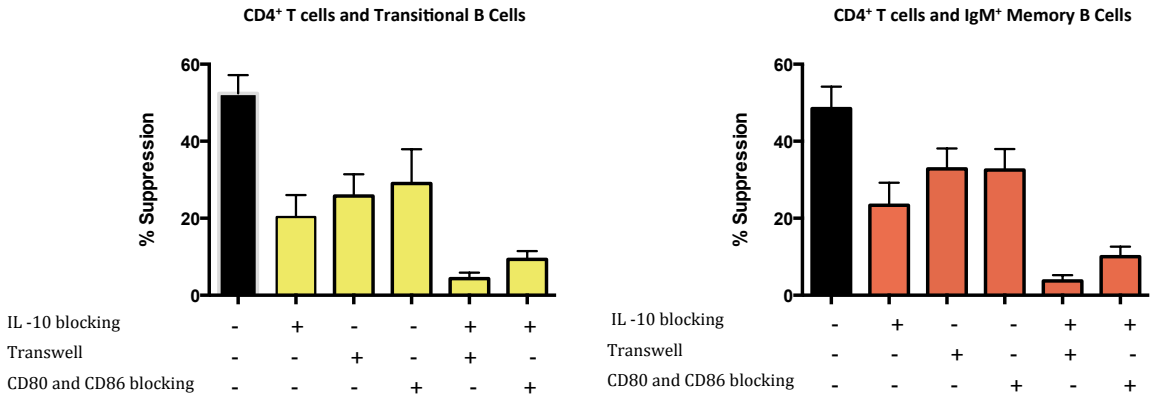

Figure III-22: A combination of blocking antibodies to IL-10, IL-10R, CD80 and CD86 in the presence or absence of cell-to-cell contact reversed the ability of Breg subsets to suppress CD4+ T cell proliferation *in vitro*

The bars represent median values and upper whisker of error bars indicate the range from 3 independent experiments *$P < 0.05$ by nonparametric ANOVA

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However, the suppressive effect of human transitional and IgM Memory B cells was independent of CD80 co-interaction with the inhibitory receptor CTLA-4 expressed on T cells (**figure III-23**). Thus, analogous to previous studies [Blair et al., 2010], the suppressive effect of human Breg cells is mediated by a number of mechanisms including the release of IL-10, cell-to-cell contact and CD80 and CD86 costimulatory signaling.

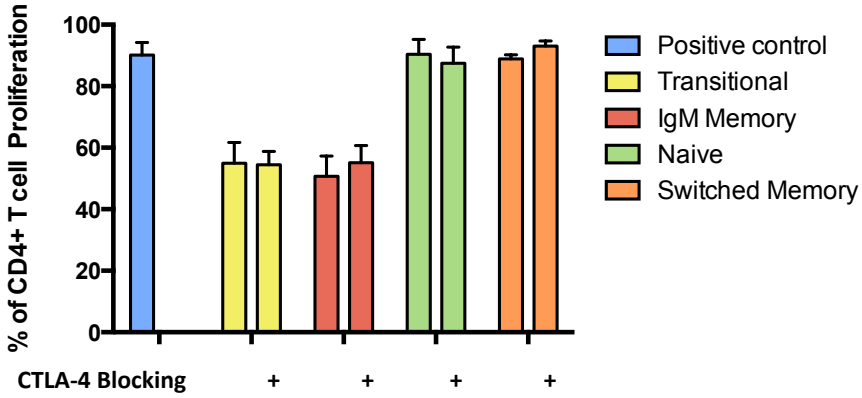


Figure III-23: CTLA-4 blocking had no significant impact on the ability of Breg subsets to suppress CD4+ T cell proliferation *in vitro*

The bars represent median values and upper whisker of error bars represent the range from 3 independent experiments

III.3 Discussion

The delicate balance between effector T-cell function and immunoregulatory networks in tolerance to self- and environmental antigens has been largely ascribed to regulatory T cells (Rezvani et al., 2006, Mielke., 2007). Recent research has shed light on at least one functional group of PB-derived B cells in humans, $CD19^{+}CD24^{hi}CD38^{hi}$ transitional B cells, that appear to exert robust immune regulatory capacity (Blair et al., 2010). On the other hand, other studies have shown that CD40L-stimulated $CD24^{hi}CD27^{+}$ memory B cells can release IL-10 and exert suppression of monocyte activation and cytokine production *in vitro*; (Iwata et al., 2011, Bouaziz et al., 2010). My results support the immunoregulatory function of transitional B cells and identify IgM+ memory B cells as a new candidate Breg subset in healthy individuals. Both transitional and IgM memory B cells can secrete IL-10 and suppress both the proliferation and cytokine production of CD4+ T cells in a manner that was comparable with that of Tregs. This suppressive effect was also found to be dose dependent. Although I also found the presence of IL-10 producing B cells within naïve and switched memory B cell subsets, albeit at much lower frequencies, they lacked any suppressive effect on CD4+ T cell function. This is in agreement with other studies that have also shown lack of suppressive activity in naïve B cells ($CD27^{-}$ IgM+) (Blair et al., 2010, Iwata et al., 2011). However, as we have no means of purifying exclusively those B cells that express IL-10 in sufficient quantities to perform functional studies, it is also possible that a number of distinct regulatory B cell subsets may exist. It is therefore important to continue looking for more specific markers of IL-10 producing B cells, given that they might play a significant role in the

maintenance of the balance between tolerance and autoimmunity. Furthermore, this discovery broadens the proportion of PB-derived regulatory B cell subset within circulating total CD19+ B cells from <5% ($CD19^{+}CD24^{hi}CD38^{hi}$ transitional cells only) to 20%-30% (transitional plus $CD19+CD24hiCD38^{-/lo}IgM+CD27+$ memory B cells), suggesting a prominent role for Bregs in the maintenance of immune tolerance.

The mechanism through which transitional and IgM memory B cell subsets suppressed CD4+ T cell proliferation and effector function was partially via the provision of IL-10. IL-10 blockade led to partial reversal of CD4+ T-cell proliferation in the presence of either IgM memory or transitional B cells, which supports the role of IL-10 in mediating the suppressive effect of Bregs. However, as this reversal was not complete, these findings suggest that the regulatory function of Breg subsets is at least partly IL-10 dependent. Recombinant IL-10 has been tried in some autoimmune diseases such as Crohn's disease, rheumatoid arthritis and psoriasis. In the majority of clinical trials, systemic administration of recombinant IL-10 was not associated with clinical improvement with the exception of psoriasis (Sanz et al., 2008). Similarly in my experiments, the addition of exogenous IL-10 to co-cultures of naïve or switched memory B cells failed to suppress CD4+ T cells to the same extent as seen with transitional and IgM memory B cell subsets, suggesting that cellular contact may be required for IL-10 to deliver immune regulation. Indeed, transwell experiments and CD80/CD86 blockade confirmed that cell-to-cell contact was needed for IgM memory and transitional B cells to exert their full suppressive activity on CD4+ T cell function. In addition, whereas in mice TGF-β has been shown to mediate Breg cell-suppressive activity in

experimental diabetes (Tian et al., 2001), in my study transitional and IgM memory suppressive capacity was not dependent on TGF-β production. Previously published work in murine models has highlighted an important role for Bregs in protection against cGVHD (Huu et al., 2013, Weber et al., 2014) and a role for IL-10 deficiency in cGVHD pathogenesis (Barak et al., 1995, Korholz et al., 1997). In keeping with previous studies, previous work performed by Dr. Khoder, a previous member of Dr. Rezvani's laboratory has also provided evidence of a deficiency in IL-10 producing B cells in cGVHD patients that were also refractory to CD40 activation (Khoder et al., 2014). These findings are in keeping with reports in SLE where Bregs were refractory to CD40 engagement, associated with reduced phosphorylation of STAT3 downstream of CD40 (Blair et al., 2010). Additionally, as a result of the impaired IL-10 production, Dr. Khoder in our group (2014) observed a significantly lower ratio of IL-10+ B cells to IFN-γ+CD4+ T cells in cGVHD patients compared with the control group, which implies an imbalance between the regulatory B-cell and effector T-cell compartments, analogous to that recognized with Tregs during the development of cGVHD (Yamashita et al., 2004). My findings in this chapter may provide support for future investigations of regulatory B cell–based therapy to tip the scales in favor of immune regulation. It also highlights the need for more specific markers to define IL-10 producing B cells, given that they might play a significant role in the maintenance of the balance between tolerance and autoimmunity.

Chapter IV IL-10 producing regulatory B cells are enriched in cord and may play a role in protection against GVHD after cord blood transplantation

IV.1 Introduction

Allogeneic hematopoietic SCT (HSCT) is a potentially curative option for many patients with high-risk hematological malignancies (Daikeler et al, 2009, Barrett and Battiwala, 2010). However, approximately 70% of patients who require an allograft will lack an HLA-identical sibling donor, and many in this group will lack a suitably matched unrelated donor (Koh and Chao, 2008).

Human cord blood (CB) is widely used as a source of hematopoietic stem cells (HSC) for many patients who lack a fully matched related or unrelated donor due to their less stringent requirement for human leukocyte antigen (HLA) matching (Beaudette-Zlatanova et al., 2013, Komanduri et al., 2007, Stanevsky et al, 2009). Most adult and larger adolescent patients receive two CB units matched at ≥4/6 HLA alleles in order to overcome the limited cell dose in a single CB graft and to accelerate engraftment (Komanduri et al, 2007, Stanevsky et al., 2009, Newell et al., 2013). Although an increased incidence of acute GVHD after double-unit CBT (DUCBT) compared with single CBT has been described (Cutler et al., 2011, Ballen et al, 2013) a lower incidence of extensive chronic GVHD has been reported after both single and double CBT compared with other stem cell sources, despite broader HLA disparity (Beaudette-Zlatanova et al., 2013, Komanduri et al., 2007, Stanevsky et al., 2009).

Alloreactive reactions between donor-derived CD4+ and CD8+ T lymphocytes have typically been considered to be the chief effector cells arbitrating the pathogenesis of acute and chronic GVHD (Shimabukuro-Vornhagen et al., 2009, Rezvani et al., 2006). Several independent lines of evidence clearly demonstrate a critical breakdown in peripheral B-cell tolerance and insufficient immune regulation after allogeneic HSCT (Kapur et al., 2008, Khoder et al, 2014). Indeed, evidence of typically activated B cells by increased signaling networks through ERK and AKT pathways have been reported in B cells isolated from patients with cGVHD (Allen et al., 2012, Sarantopoulos et al., 2015). IL-10 producing B cells (B10 cells) with regulatory capacity, commonly known as regulatory B cells (Bregs) are a newly identified subset of suppressor B cells, shown to exhibit inhibitory function on the immune system. Mizoguchi and collaborators, first introduced the term “regulatory B cells” in 2002 (Mizoguchi and Bhan., 2006). Since these seminal observations, a considerable body of evidence has conclusively demonstrated the significance of IL-10-producing regulatory B cells in divergent models of autoimmunity, infection, and cancer (DiLillo et al., 2010, Yang et al., 2013, Tedder et al., 2015, He et al., 2014), and more recently cGVHD (Sarantopoulos et al., 2015, Khoder et al., 2014., de Masson et al., 2015). To date, discrepancies in the cell surface antigens studied and a lack of consensual definitions of the Breg subset phenotypes limit the direct comparison of human B cell subsets with regulatory function. In murine studies, B cells with regulatory function were found within B10 cells, MLN B cells, marginal-zone B cells, T2-MZP cells, and Tim-1+ Bregs (Mauri and Blair et al., 2014, Blair et al., 2010, He et al., 2014). The phenotype of Breg cells in

humans has yet to be fully elucidated. Blair and coworkers (2010) have described human Bregs as $CD19^{+}CD24^{hi}CD38^{hi}$, a phenotype that is usually used to define a population of human transitional B cells. Furthermore, recent evidence showed human Bregs, identified through IL-10 intracellular staining, to be contained within the $CD24^{hi}CD27^{+}$ B cell subset (de Masson et al., 2015, Iwata et al., 2011).

I previously illustrated in Chapter III, that Bregs are enriched within both the transitional and IgM memory B cell subsets and mediate T cell suppression in an IL-10 dependent, as well as contact-dependent manner (mainly through CD80/CD86). Moreover, Khoder et al (2014) showed that Bregs are deficient in recipients of HLA-matched sibling or matched unrelated donor HSCT with chronic GVHD.

Cord blood is a rich source of transitional B cells. Whereas CD19+CD24hiCD38hi transitional B cells represent approximately 4% of peripheral blood B cells in healthy adults, they are abundant in cord blood (near 50% of B cells), with their frequency progressively decreasing during infancy (Sims et al., 2005, Marie-Cardine et al., 2008, Cuss et al., 2006, Ha et al., 2008). In contrast to PB, $CD24^{hi}CD38^{-}$ $CD27^{+}$ memory B-cells are absent in CB and only become detectable in the first year of life (Cuss et al., 2006, Ha et al., 2008). Thus, I hypothesized that the higher frequencies of B cells with regulatory phenotype in CB may contribute to the lower rates of chronic GVHD post-CBT.

Here, I show that IL-10-producing B cells with Treg-independent immunosuppressive properties are enriched in CB. They suppress T cells through production of IL-10, as well as by cell-cell contact-mediated mechanisms involving CTLA-4. Moreover, we found a robust recovery of IL-10-producing B cells by 6 months post CBT, with significantly greater frequencies than seen in the peripheral of healthy donors or in patients prior to CBT. Furthermore, Breg reconstitution in patients with GVHD was significantly impaired in comparison to patients without GVHD. Taken together, these studies suggest a protective role for CB-derived B cells in the development of cGVHD in CBT recipients and support the development of strategies to exploit CB as a source regulatory B cells in the treatment of this disease.

IV.2 Results

IV.2.1 Cord Blood B cell Phenotype and subset frequencies

Phenotypic characterization of CB revealed the presence of two distinct B cell populations: $CD19^{+}CD38^{hi}CD24^{hi}$ transitional B cells (a population that includes immature B cells) and $CD19^{+}CD38^{int}CD24^{int}$ naïve B cells (primarily mature B cells) (**figure IV-1**). In contrast to the phenotypic analysis described for peripheral blood B cells in chapter III, $CD24^{hi}CD38^{-}$ $CD27^{+}$ memory B-cells are almost absent in CB. In keeping with previously published studies, further phenotypic characterization confirmed that the majority of $CD19^{+}CD38^{hi}CD24^{hi}$ transitional B cells are also $IgM^{hi}IgD^{hi}CD10^{+}CD27^{-}$, whereas $CD19^{+}CD38^{int}CD24^{int}$ naive B cells are $IgM^{int}IgD^{+}CD10^{-}CD27^{-}$ (Sims et al., 2005, Marie-Cardine., 2008, Ha et al., 2008, Palanichamy et al., 2009).

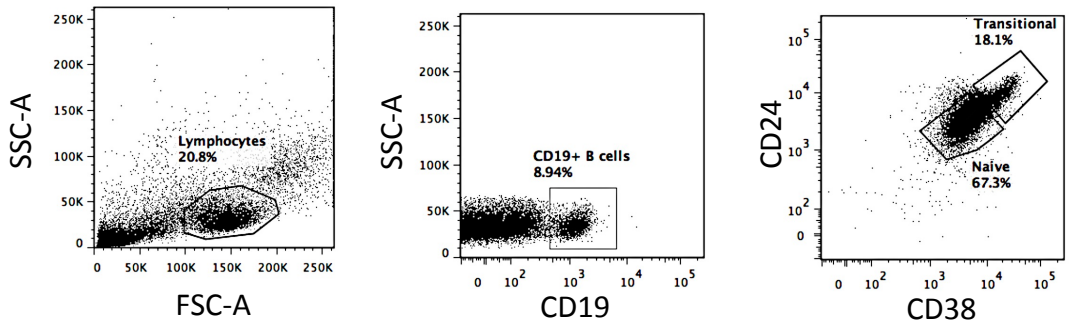


Figure IV-1: Phenotypic characterization of cord blood B cell subsets
Representative FACS plots illustrating gating strategy on lymphocyte population, total CD19+ B cells and $CD19^{+}CD38^{hi}CD24^{hi}$ transitional B cells and $CD19^{+}CD38^{int}CD24^{int}$ naive B cells

The frequency of $CD19^{+}$ B cells in healthy cord blood mononuclear cells (CBMCs) was 6.0% (3.21%-13.1%); 22.2%(11.42%-28.0%) of total $CD19^{+}$ B cells comprised of CD24hiCD38hi transitional, 63.2%(58.8%-72.8%) of $CD24^{+}$

CD38^{+} naïve and 2.14%(1.38%-3.64%) of CD24^{hi} CD38^{lo} CD27^{+} memory B cells (**figure IV-2**).

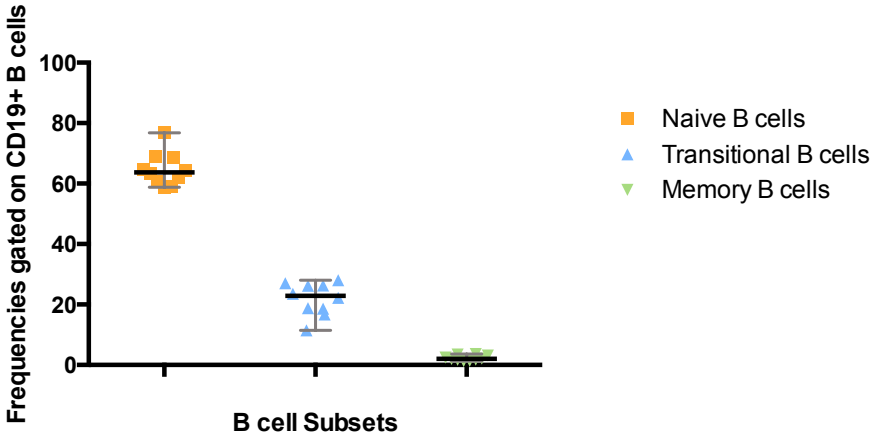


Figure IV-2: Frequency of B cell subsets in healthy control CBMCs
Frequency of B cell subsets (n=10) out of total CD19+ B cells in healthy controls was determined through flow cytometric analysis of B cells subsets with respect to the CD24 and CD38 parameters. The line on the scatter dot plot represents median and error bars represent range

I next validated the effect of freezing on the signal intensity and the frequency of CD19+ B cell populations against the CD24 and CD38 axis in paired fresh and frozen samples (stored at -80°c for a minimum of 8 days) from 10 CB units. No significant differences were found in the signal intensities or B cell subsets in paired fresh and frozen CBMC CD19+ B cell populations (**figure IV-3**), which validates my panel to study frozen patient samples that have been collected and cryopreserved.

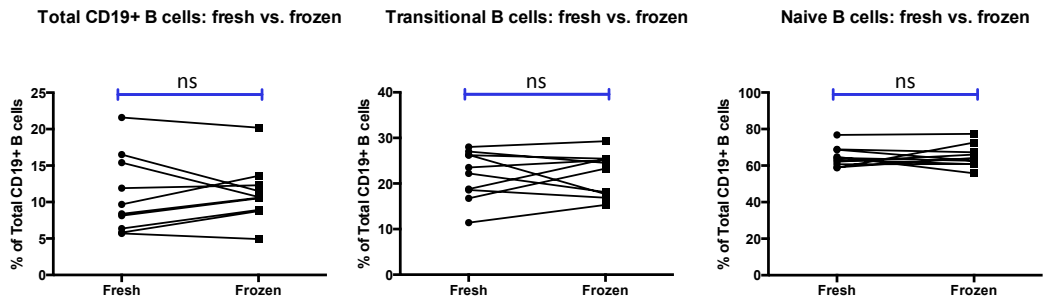


Figure IV-3: Effect of cryopreservation on B cell phenotype for each subset is illustrated as bivariate plots representing data acquired from 10 paired fresh and frozen samples

Data was analyzed using a paired t-test for total CD19+ B cells (p=0.9064), transitional B cells (p=0.9225) and naïve B cells (p=0.9104)

IV.2.2 Human cord blood is enriched in IL10-producing CD19+ B cells

IL-10 production has long been considered a defining trait of Breg cells (Tedder., 2015, Mauri and Blair., 2014, Iwata et al., 2011). I first determined whether freshly isolated CB-derived CD19+ B cells produced IL-10 by magnetically purifying CD19+ B cells from CBMCs and co-culturing them with either irradiated fibroblasts transfected with CD154 (CD40L cells), CpG or BCR ligation for 24, 48 or 72 hours to study the kinetics of IL-10 production as described in section II.7.3. Resting B cells were also cultured alone as negative control. I found that that CB-derived CD19+ B cells had the capacity to produce IL-10 in response to stimulation by BCR ligation, CD40L or CpG in a time-dependent manner [**figure IV-4A**]. Human IL-10^{+} B cells from peripheral blood have been previously shown to be enriched within the $CD19^{+}CD24^{hi}CD38^{hi}$ transitional and CD24hiCD27+ and CD19+CD27+IgM+ memory cells (Khoder et al., 2014, Blair et al., 2010, Iwata et al., 2011). To discover the source of IL-10-producing B cells in CB, I magnetically isolated total CD19+ B cells and also sort-purified naïve and transitional B cells from healthy CB units and stimulated them with either CD40L, CpG, BCR ligation or a combination of all three for 48 hours and measured the concentrations of

IL-10 in the culture supernatants measured by ELISA. Based on the previous ELISA results, I chose to stimulate CB-derived B cell subsets for 48 hours, which allowed sufficient production of IL-10 detection by ELISA without compromising CB B cell viability staining with trypan blue (data not shown). Interestingly, stimulation of transitional and naive B cells with a combination of CD40 ligation, CpG and BCR engagement resulted in significantly more IL-10 production than when cultured with each stimulus alone (**figure IV-4B**).

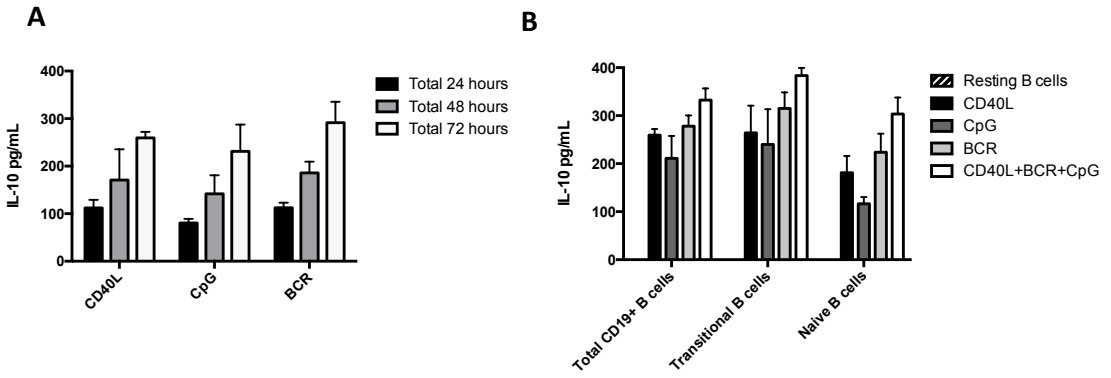


Figure IV-4: IL-10 production in CB-derived B cells after stimulation with CD40L, CpG or BCR ligation
A. Bar graphs showing cumulative data of IL-10 production from CB-derived CD19+ B cells in response to stimulation with CD40L, CpG and BCR ligation in a time dependent manner (n=10) **B**. Cumulative data illustrating IL-10 production from CB total CD19+ B cells, and sort purified naïve and transitional B cell subsets after stimulation with CD40L, CpG, BCR ligation or a combination of all. Resting B cells (un-stimulated) were used in each experiment as a negative control.
The bars in A and B represent mean and the error bars represent range

I next determined the effect of cryopreservation on IL-10 production by B cells. Paired CBMC from the same cord blood units were frozen at -80° C as described in II.2.3 for a minimum of 3 weeks. Cryovials were thawed as described in II.2.4 and rested for a minimum of 4 hours at 37° C before B cell selection was performed (II.4.1). Cells were activated as described previously for 48 hours and supernatant was assayed for IL-10 by ELISA. Minimal differences were observed in IL-10 levels between fresh and frozen paired

samples and overall no significant differences were found (**figure IV-5**). The results validate the use of frozen samples for the detection of IL-10 for future studies.

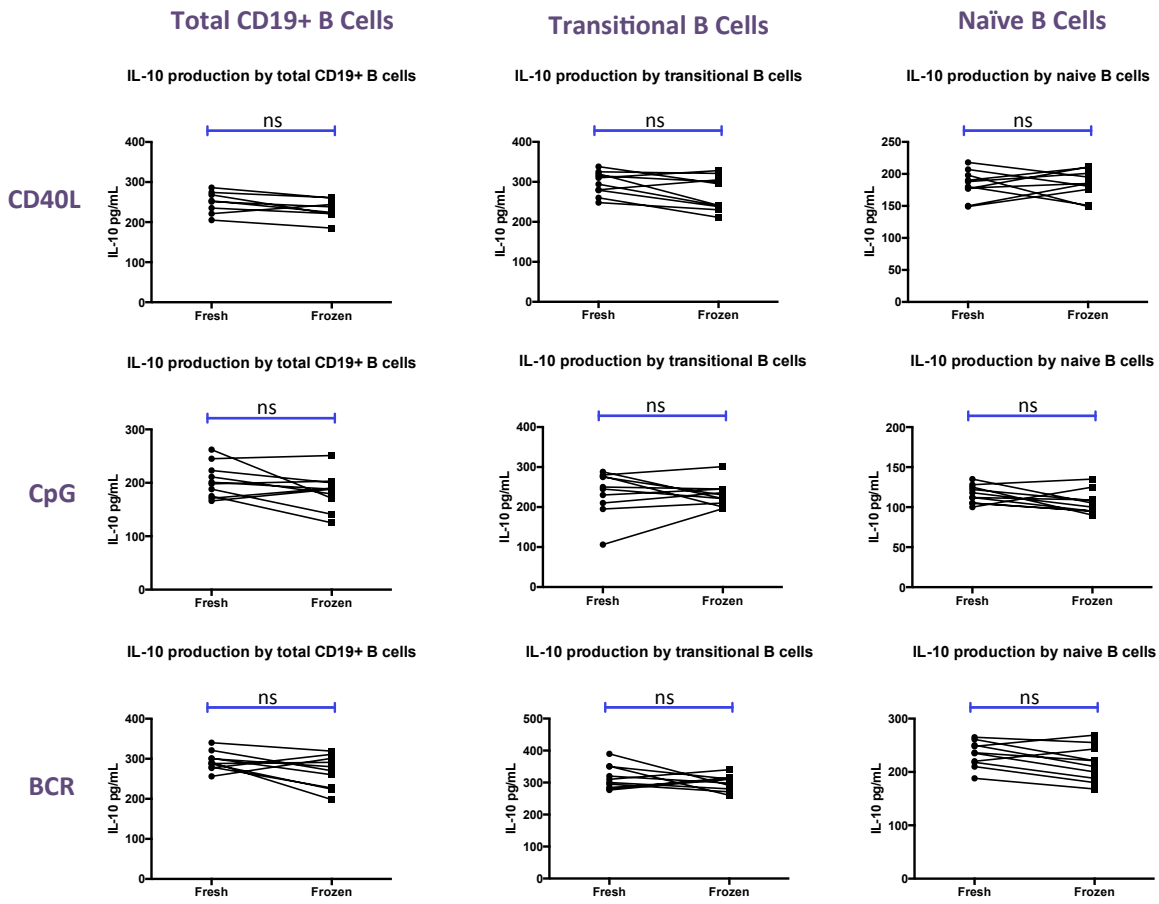


Figure IV-5: Effect of cryopreservation on IL-10 production by total CD19+ B cells and sort-purified transitional and naïve B cell subsets activated with CD40L, CpG and BCR ligation.
Data was acquired from 10 paired fresh and frozen samples and analyzed using a paired t-test; ns, no significant difference.

These results are in agreement with previous studies that have also described transitional B cells as IL-10 producing suppressor cells (Khoder et al., 2014, Mauri and Blair., 2014, Blair et al., 2010). However, I also propose a novel and previously undescribed attribute of naïve CB B cells as an IL-10 producing B cell subset. These findings are in contrast to Breg studies in PB, reporting that naive B cells do not possess suppressive function (Khoder et

al., 2014). However, my results underscore the regulatory capacity of immature transitional B cells (CD19+CD24^{hi}CD38^{hi}), as previously shown by our group and others (Khoder et al., 2014, Mauri and Bosma., 2012, Blair et al., 2010) and support CB-derived naïve B cells as a candidate Breg subset.

IV.2.3 Sort purified naïve and transitional B cells from CB inhibit proliferation and pro-inflammatory cytokine production by peripheral CD4+ T cells in a dose-dependent manner

To gain further insight into the suppressive capacity of CB-derived IL-10-producing transitional and naive CB B cells on CD4+ T cell function, I sort-purified transitional (CD19^{+}CD24^{hi}CD38^{hi}) and naïve (CD19^{+}CD24^{+}CD38^{+}) B cell subsets as well as total CD19+ B cells from CB units (n=10) and evaluated their suppressive effects on PB CD4^{+} T cell proliferation and cytokine production by flow cytometry as detailed in section II.8.1 and II.8.3 respectively. The gating strategies and post-sort purity checks are outlined in **figure IV-6**. Results illustrated that following 96-hour of co-culture with peripheral CFSE-stained CD4+ T cells (stimulated with anti-CD3/anti-CD28 beads) at a B cell: T cell ratio of 1:1, total CD19+ B cells as well as both naive and transitional B cells significantly suppressed CD4^{+} T cell proliferation [median percent proliferating CD4+ T cells 71.8%; (64.9%-78.9%) and 68.0% (63.4%-77.9%) and 64.1% (58.7%-69.2%), respectively], when compared with anti-CD3/anti-CD28 stimulated CD4+ T cell alone (positive control) [94.6% (86.7%-97.5%] (**figure IV-7**). These effects were cell dose-dependent, with the highest suppression observed at a Breg: CD4+ T cell ratios of 5:1 and 10:1 for CD19+ total B cells as well as transitional and naïve B cell subsets **(figure IV-8).** In order to assess the suppressive effect of CB-derived B cells

on CD4+ T cell effector function, total CD19+ B cells and sort-purified transitional and naïve B cell subsets were co-cultured with anti-CD3/anti-CD28 stimulated magnetic-bead purified $CD4^+$ T cells. Similarly, I also found that CD19+ total B cells and both transitional and naïve B cell subsets suppressed IFN-γ, TNF-α and IL-2 production by ex vivo stimulated PB CD4+ T-cells in supernatants harvested from B cell/T cell cultures through ELISA assays as detailed in section II.8.3 (**figure IV-9).**

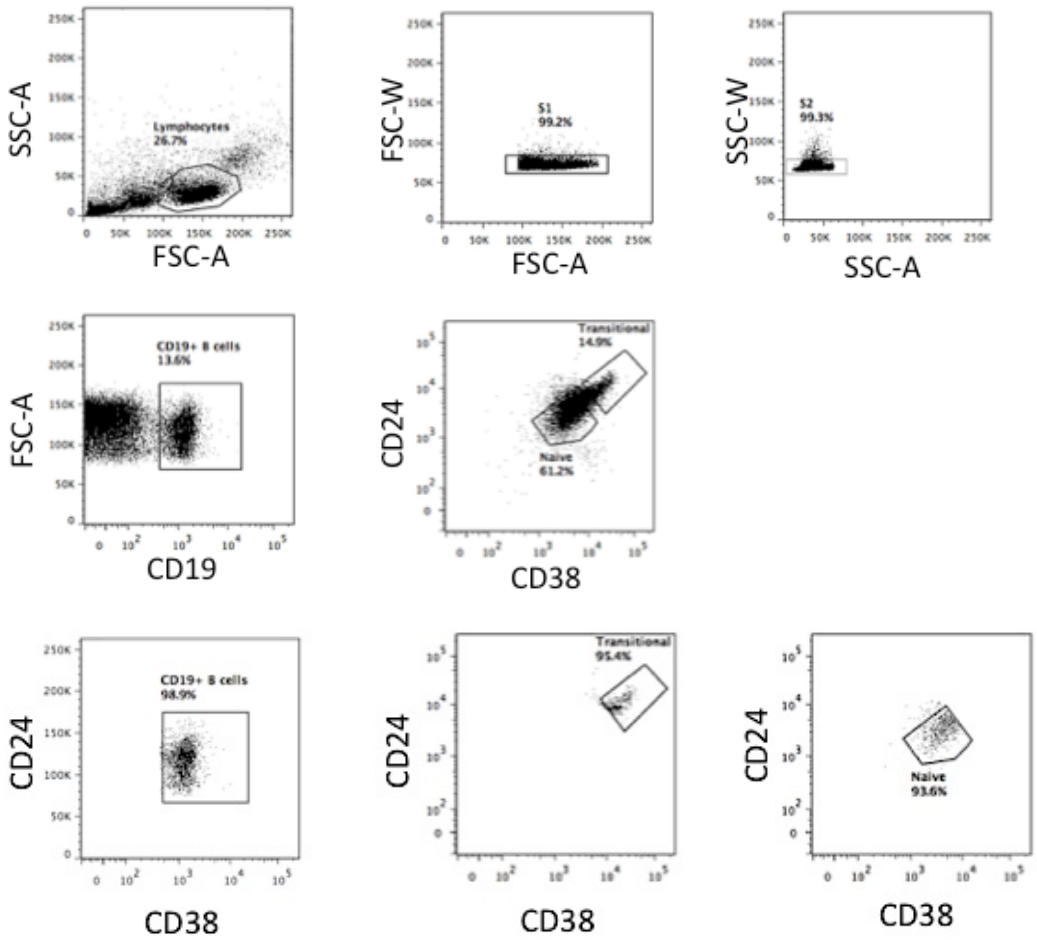


Figure IV-6: Gating strategy and purity checks of CB B cell sorting
Multi-parametric flow cytometric gating strategy for sorting B cell subsets on BD FACS ARIA IIIu. Following lymphocyte gate and cell doublet discrimination, CD19+ B cells are then sort-purified based on CD24 and CD38 expression into 2 subsets, $CD19^+CD38^{hi}CD24^{hi}$ transitional B cells and $CD19^+CD38^{int}CD24^{int}$ naïve B cells. FACS plots illustrating the high purity of sorted B cell subsets are shown within the CD19+ gate

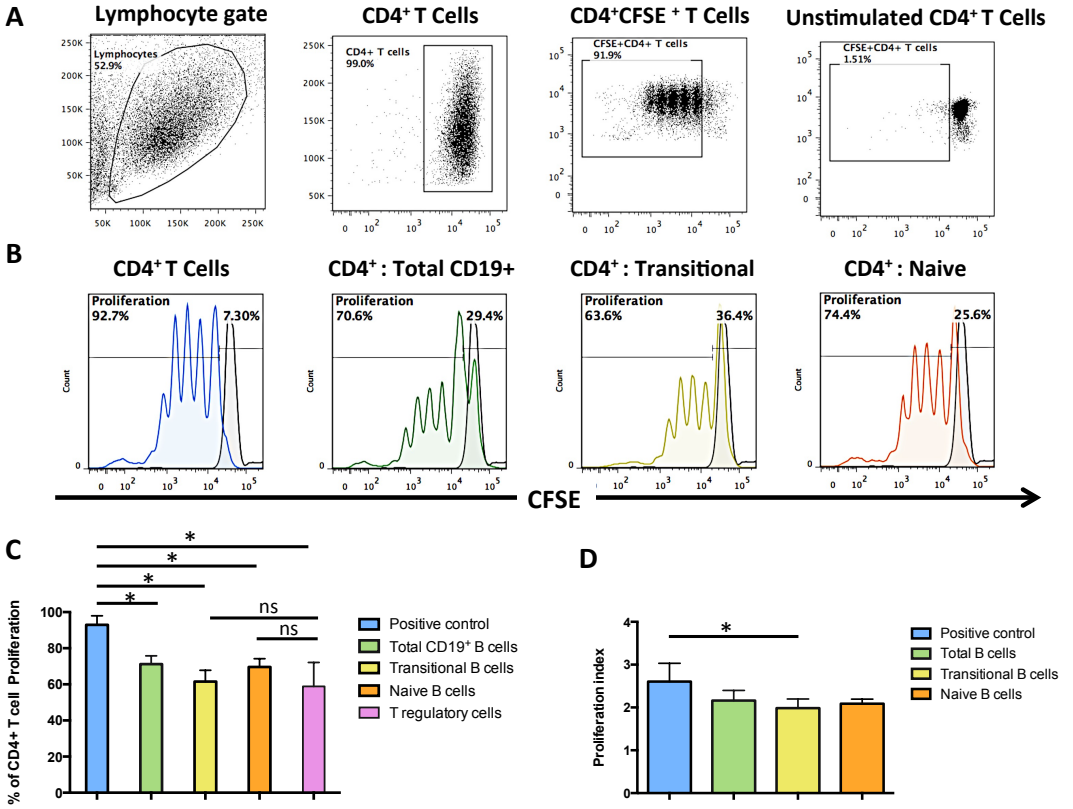


Figure IV-7: Cord Blood derived B cell subsets suppress allogeneic CD4+ T cell proliferation

A. Representative dot plots showing the gating strategy of CD4+CFSE+ T cells. Gates were made on the lymphocyte population, CD4+ T cells followed by dot plots of CD4+CFSE+ T cells. Gate was determined based on CFSE intensity of unstimulated CD4+ T cells. **B**. Magnetically selected and CD3/CD28 activated PB-CD4+ T cells were labeled with CFSE (eBioscience) and plated either alone (positive control – **Blue**) or at a 1:1 ratio with total CD19+ B cells **(green),** transitional **(yellow) or** naïve **(orange)**. CFSE-stained T cells cultured with no stimulation (negative control –**grey**) were included in each experiment. Proliferation index values for histograms are: CD4+T cells – 2.87, with total CD19+ B cells- 1.94, naïve – 2.23, and transitional – 1.79 **C.** Suppressive effects of CB derived CD19+ B-cell subsets (1:1 ratio) on CD4+ T cell proliferation index *in vitro* (n=14).
In (C) and (D), bars represent median values and upper whiskers indicate the range. **P* < 0.05 by nonparametric ANOVA; ns, not significant

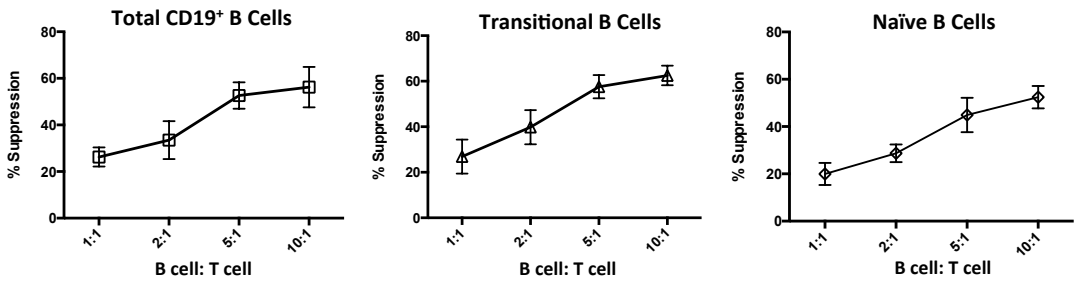


Figure IV-8: The suppressive effect of CB derived CD19+ B cells and transitional and naïve B cell subsets on CD4+ T cell proliferation is dose dependent as indicated at the B cell: T cell ratios (n=4)

Error bars represent mean with standard deviation.

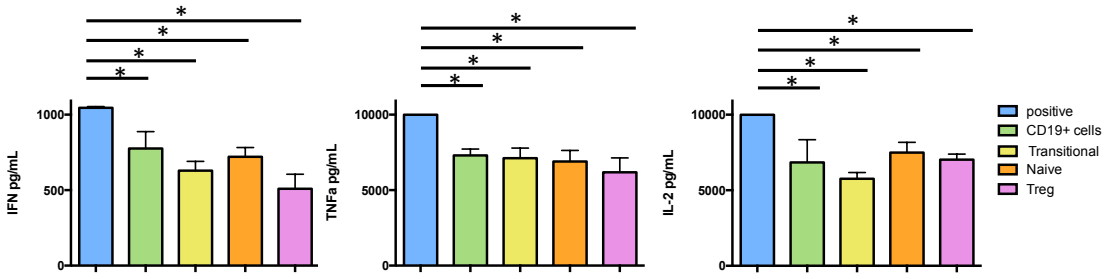


Figure IV-9: CB-derived CD19+ B cells and both transitional and naïve B cell subsets suppressed IFN-γ, TNF-a and IL-2 production by ex vivo stimulated PB CD4+ T-cells in supernatants harvested from cultures of CB-derived B cells or Tregs cocultured with CD4+ T cells
Bars represent median values and upper whisker of error bars represent range from 6 independent experiments. *$P < 0.05$ by nonparametric ANOVA.

I further compared the suppressive capacity of naive and transitional B cells with that of cord blood derived Tregs, defined as CD4+CD25^{hi}CD127^{-} T cells. In experiments in which magnetically purified cord blood derived Tregs were cocultured with peripheral CD4^{+} T cells at a 1:1 ratio, and the cultured cells stimulated with anti-CD3/anti-CD28 beads, the inhibition of T-cell proliferation and IFN-γ, TNF-α and IL-2 production by CB-Bregs was comparable to that achieved by Tregs (**figure IV-7 and IV-9).** Collectively, these results suggest that the suppressive capacity of total CD19+ B cells and transitional and naive CB-derived B cell subsets on CD4+ T cell proliferation and cytokine production is comparable to that of T regulatory cells.

I next determined whether pre-treatment of CB CD19+ B cells with CpG, CD40 and BCR ligation to induce IL-10 production could potentiate their suppressive capacity. These results indicate that 'pre-activated' CB-derived total CD19+ B cells as well as pre-activated sort-purified transitional and naïve B cell subsets suppress CD4+ T cell proliferation and cytokine production significantly more potently than their 'non-activated' counterpart (**figure IV-10).**

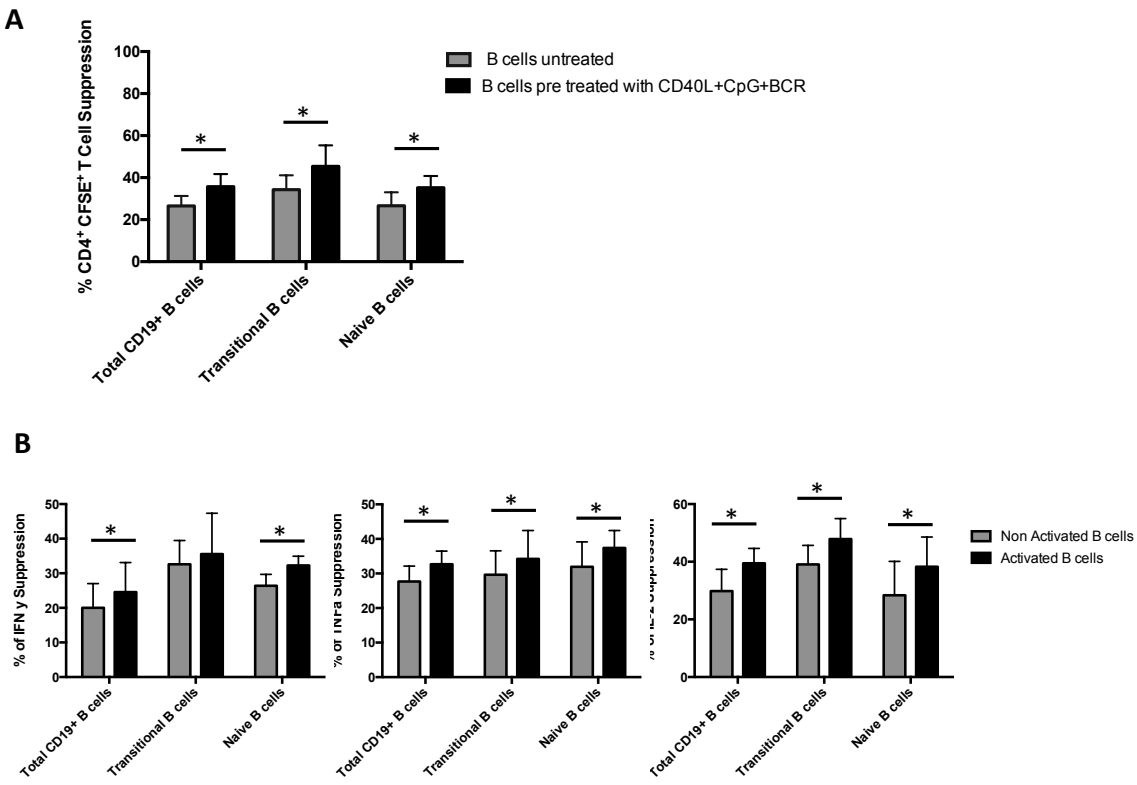


Figure IV-10: Pre-treatment of CB CD19+ B cells with CpG, CD40 and BCR ligation potentiated the suppressive capacity of CB-derived B cells
A. CD4+ T cell proliferation and **B**. Effector function, when compared to their non-induced counterparts**.** In (A) and (B), bars represent mean and upper whiskers indicate the range. *$P < 0.05$ by paired t-test.

IV.2.4 IL-10 contributes to the suppressor function of cord blood derived transitional and naïve B cells

To clarify the mechanism(s) by which CB derived naive and transitional B cells suppress CD4+ T cell proliferation and effector cytokine function, I cultured PB CD4+ T cells alone or at a 1:1 or 5:1 B : T cell ratio with total CD19+ B cells or with naïve or transitional B cell subsets purified from CB units in the presence or absence of mAbs against IL-10 and IL-10 receptor (IL-10R) in 4 independent experiments. IL-10 blockade partially restored the proliferation of CD4+ T cells cocultured with the CB-derived total CD19+ B cells, naïve or transitional B cell subsets at both a 1:1 (**figure IV-11)** and 5:1 B

: T cell ratios **(figure IV-12**)**.** These results indicate that the regulatory property of CB-derived Bregs is at least partially IL-10-mediated. Our finding that IL-10 blockade could not fully suppress CB-B cell-mediated T cell suppression suggests that other mechanisms may be involved in mediating the suppressive function of CB-derived regulatory B cells. Consistent with these data, I also found that IL-10 blockade did not fully reverse the suppressive capacity of either transitional or naïve CB B cell on CD4+ T cell cytokine production (**figure IV-13),** These data further support the involvement of other mechanisms in CB-Breg mediated T cell suppression.

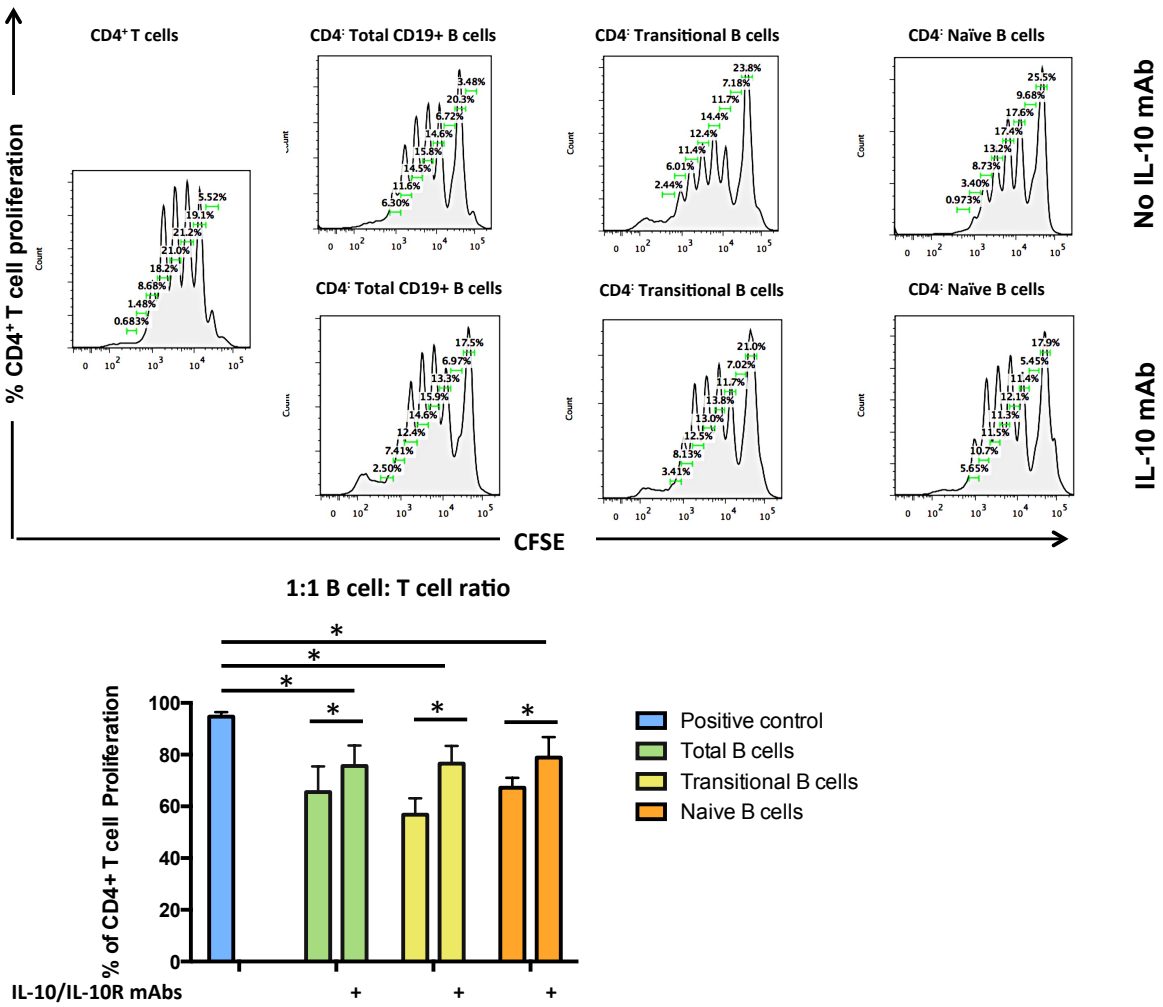


Figure IV-11: IL-10 blockade partially reversed the suppressive effect of CB derived B cells on T cell proliferation and effector function.
Representative histograms show CFSE-stained anti-CD3/anti-CD28 stimulated proliferating CD4+ T cells when cultured alone (positive control) or at a 1:1 ratio with CB derived total B cells or sort purified naïve and transitional B cell subsets with and without IL-10 blockade. Proliferation index values for histograms without IL-10 blockade are: CD4+ T cells alone-3.09, with total CD19+ B cells-2.24, naïve-2.10, transitional-1.90. Proliferation index values for histograms with IL-10 blockade are: CD4+ T cells alone-3.09, with total CD19+B cells-2.55, naïve-2.33, transitional 2.27. Data are representative of 4 independent experiments. Bars represent median values and upper whisker of error bar represent range. *P < 0.05 by nonparametric ANOVA.

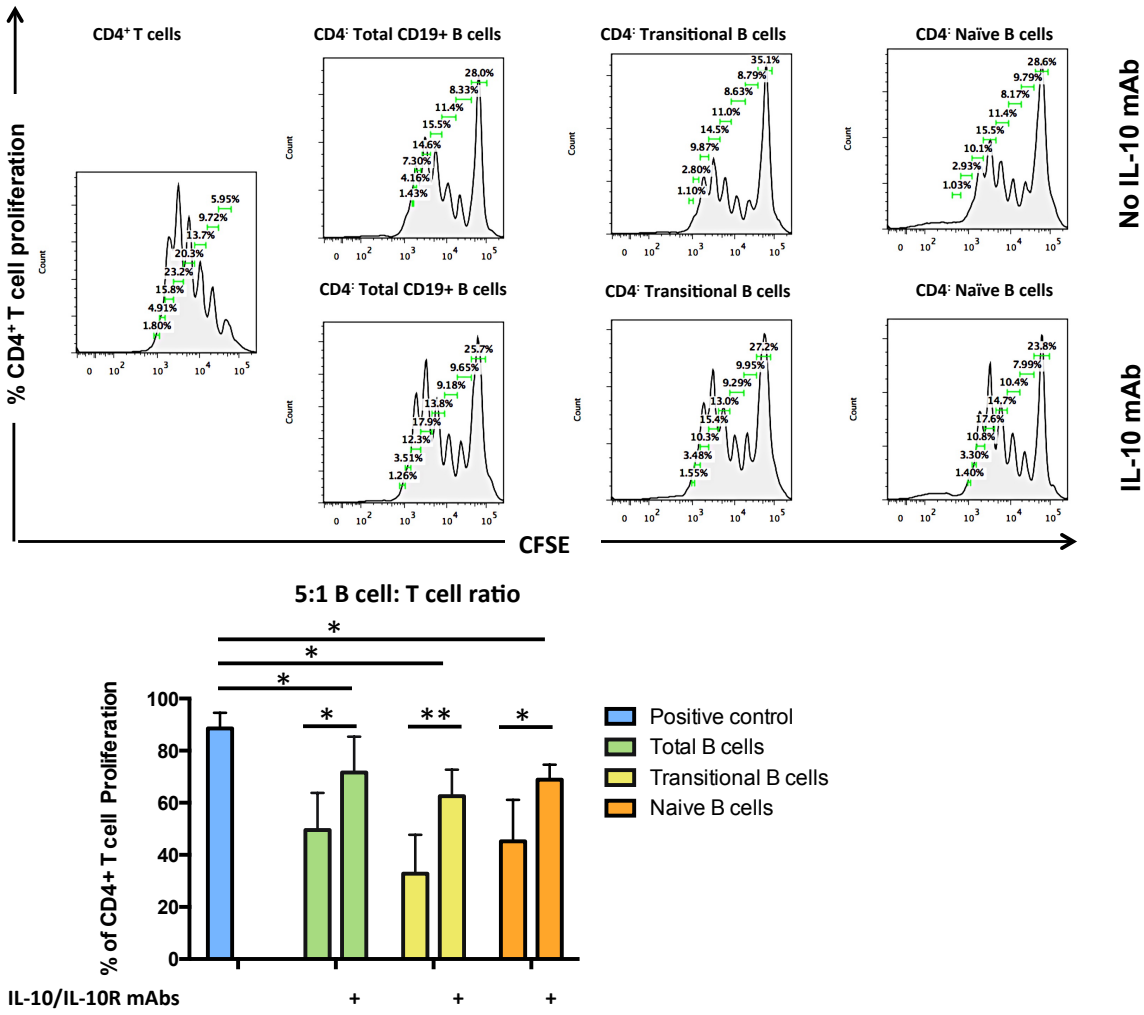


Figure IV-12: IL-10 blockade partially reversed the suppressive effect of CB derived B cells at a 5:1 (B cell: T cell) ratio

Representative histograms show CFSE-stained anti-CD3/anti-CD28 stimulated proliferating CD4+ T cells when cultured alone (positive control) or at a 5:1 ratio with CB derived total B cells or sort purified naïve and transitional B cell subsets with and without IL-10 blockade. Data are representative of 4 independent experiments. Bars represent median values and upper whisker of error bar represent range. **P* < 0.05 ** P<0.01 by nonparametric ANOVA.

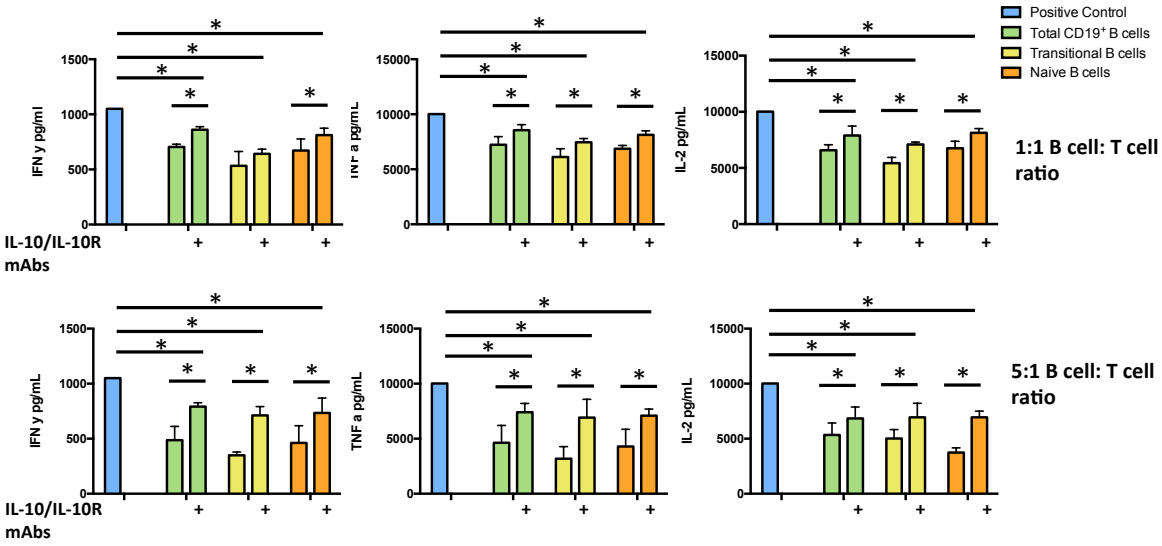


Figure IV-13: IL-10 blockade partially reversed the suppressive effect of CB derived B cells on CD4+ T cell cytokine production at a 1:1 and 5:1 (B cell: T cell) ratio

Bar graphs represent the suppressive activity of CB-derived total B cells, transitional and naïve subsets on anti-CD3/anti-CD28 stimulated CD4+ T cell cytokine production when cultured at a 1:1 or 5:1 B cell to T cell ratio in the presence or absence of IL-10 blockade. Supernatants were harvested from B cell/T cell co-cultures and assayed for the presence of IL-2, IFN-γ and TNF-α production by ELISA. Data is representative of 4 independent experiments. Bars represent median values and upper whisker of error bars represent range. *$P < 0.05$ by nonparametric ANOVA.

Previous reports suggest that the immunoregulatory effects of B regulatory cells may be mediated by TGF-β (Blair et al., 2010). To pursue the notion that TGF-β might at least partly mediate the immunoregulatory capacity of CB regulatory B cells, I performed additional blocking experiments using TGF-β-specific mAbs. The failure of TGF-β blockade to alter the suppression of CD4+ T cells by either CD19+ total B cells, transitional or naive B cells **(figure IV-14)**, indicates that this cytokine lacks any appreciable role in human CB derived Breg-mediated inhibition of peripheral T cells.

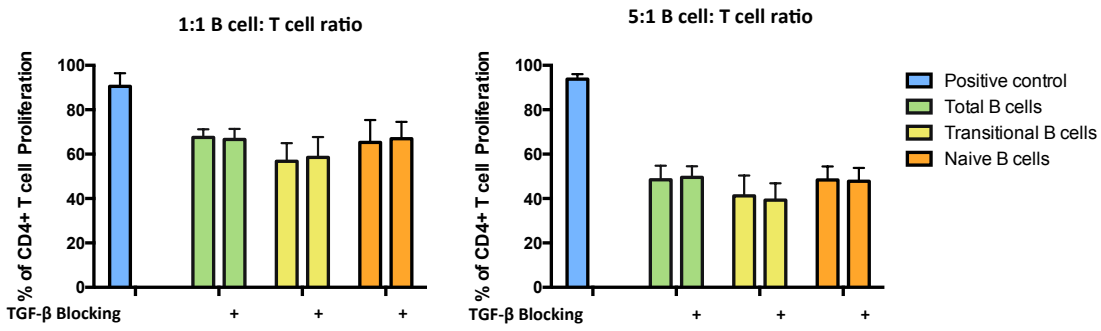


Figure IV-14: TGF-β blockade has no significant effect on the suppressive function of CB derived Bregs
Cumulative bar graphs histograms show CFSE-stained anti-CD3/anti-CD28 stimulated proliferating CD4+ T cells when cultured alone (positive control) or at a 1:1 or 5:1 ratio with CB derived total B cells or sort purified naïve and transitional B cell subsets with and without TGF-β blockade. Data are representative of 4 independent experiments. Bars represent median values and whiskers indicate the upper ranges. No significant differences were found between B cell subsets with or without TGF-β blockade by nonparametric ANOVA.

IV.2.5 The suppressive activity of CB Bregs is partially dependent on cell-to-cell contact, mediated through CD80/86 and CTLA-4

The suppressive capability of Bregs has previously been shown to be mediated by both the secretion of IL-10 and direct contact with CD4+ T cells in both murine and human studies (DiLillo et al, 2010, Khoder et al., 2014, Mauri and Blair., 2014, Blair et al., 2010). It is unclear, however, whether direct cell-cell contact contributes to human CB-derived Breg-mediated T-cell suppression. To examine this mechanism, I performed transwell experiments, in which CB-derived total CD19+ B cells or sort-purified transitional and naive B cell subsets were either in direct contact or separated from anti-CD3/anti-CD28-stimulated and CFSE-stained PB CD4+ T cells by a permeable membrane. The proliferation of CD4+ T cells was measured at 96 hours after the culture was initiated. Separation of CB-derived B cells from anti-CD3/anti-CD28-activated CD4+ T cells by a transwell membrane partially reversed the

suppressive effect of these CB-Bregs at a B cell: T cell ratio of 1:1 (**figure IV-15**). This suppressive effect was also evident at higher B cell to T cell ratios of 5:1 (**figure IV-16**). However, this reversal was not complete (compared with positive control).

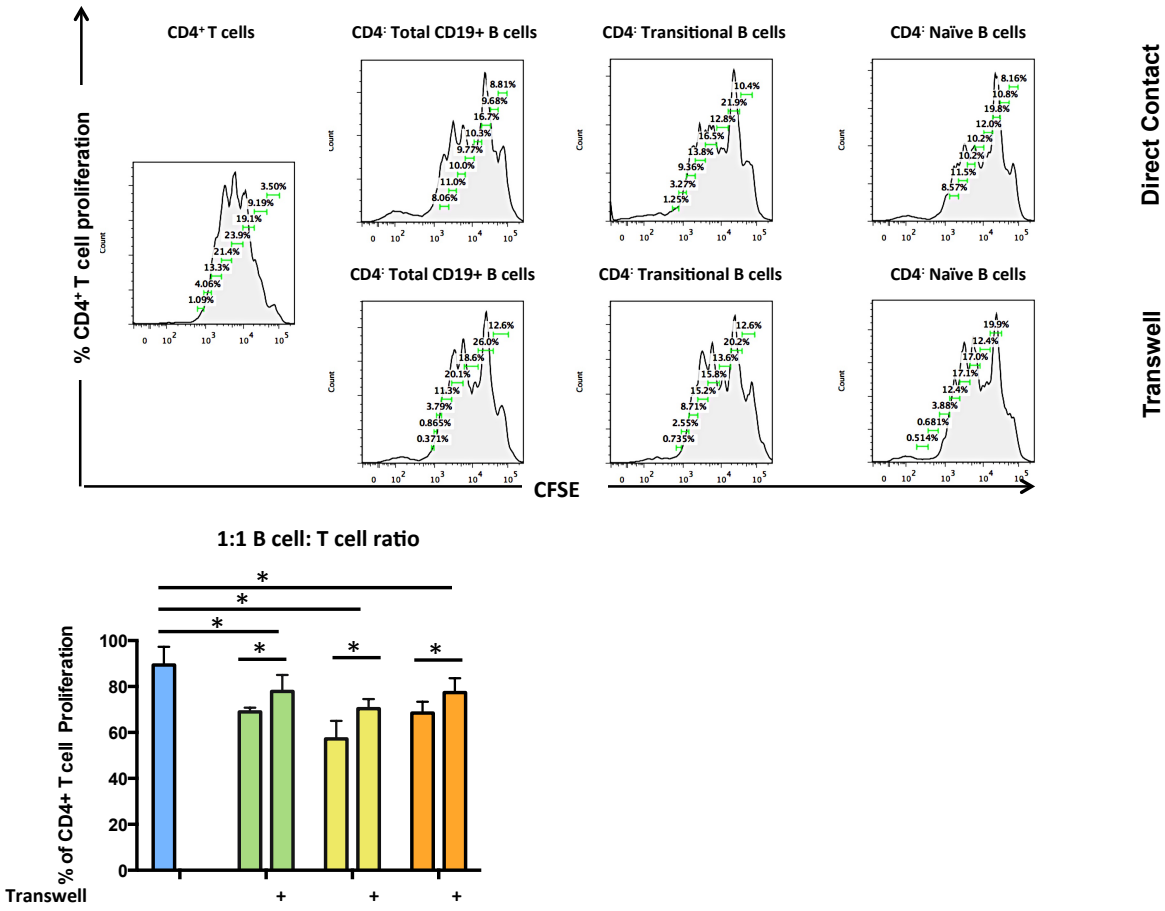


Figure IV-15: Effect of direct B cell: T cell contact on CD4+ T cell proliferation (cocultured at at a 1:1 ratio)
Representative histograms show CFSE-stained anti-CD3/anti-CD28 stimulated proliferating CD4+ T cells when cultured alone (positive control) or in direct cell-cell contact or separated using transwells at a ratio of 1 B cell :1 T cell; we studied CB derived total B cells or sort purified naïve and transitional B cell subsets. Bar graphs illustrate collective data representative of 4 independent experiments, which compare the suppressive activity of CB-derived B cells on T cell proliferation in the presence or absence of direct cellular contact. Bars represent median values and upper whisker of error bars represent the range. *P < 0.05 by nonparametric ANOVA.

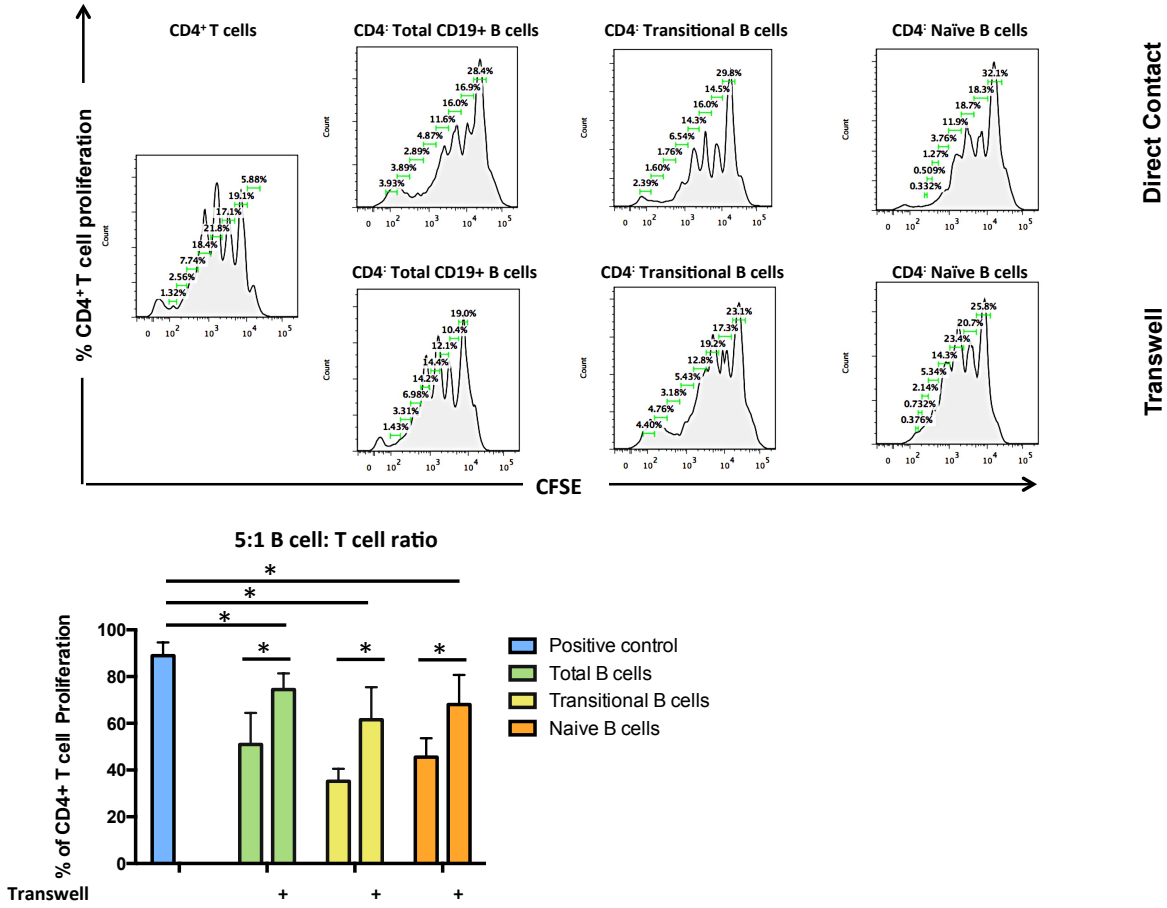


Figure IV-16: Effect of direct B cell: T cell contact on CD4+ T cell proliferation (cultured at 5:1 ratio)
Representative histograms show CFSE-stained anti-CD3/anti-CD28 stimulated proliferating CD4+ T cells when cultured alone (positive control) or cultured in direct cell-cell contact or separated using transwell chambers (transwell) at a ratio of 5 B cells :1 T cell; we studied CB derived total B cells or sort purified naïve and transitional B cell subsets. Bar graphs illustrate collective data representative of 4 independent experiments, which compare the suppressive activity of CB-derived B cells on T cell proliferation in the presence or absence of direct cellular contact. Bars represent median values and upper whisker of error bars represent the range. *P < 0.05 by nonparametric ANOVA

Similarly, separation of CB-derived total CD19+ B cells and sort-purified transitional and naïve subsets from CD4+ T cells by transwell only partially reversed their ability to suppress IFN-γ, TNF-α and IL-2 production by ex vivo activated PB CD4+ T-cells at both a 1:1 and 5:1 B cell to T cell ratios (**figure IV-17).** Thus, I next asked whether a combination of IL-10 blockade and abrogation of direct cell-cell contact could completely reverse the suppressive

effect of CB candidate regulatory B cells on CD4+ T-cell proliferation and cytokine production. The addition of IL-10/IL-10R blocking mAbs to either purified total CB CD19+ B cells or transitional or naive B cells in the transwell setting completely abolished the suppressive effect of CB B cells on CD4+ T-cell proliferation and cytokine production (**figure IV-18**).

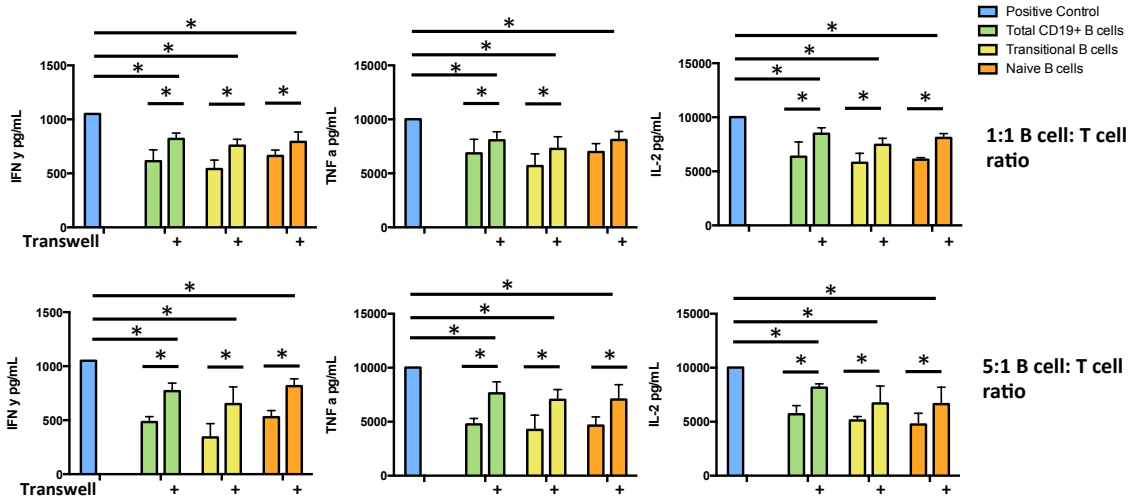


Figure IV-17: Effect of cell-to-cell contact on CB B cell-mediated suppression of CD4+ T cell cytokine production
I compared the suppressive activity of CB-derived B cells on T cell cytokine production in the presence or absence of direct cellular contact. Anti-CD3/anti-CD28 stimulated CD4+ T cells were cultured either alone (positive control) or at a 1:1 or 5:1 cell ratio with CB derived total B cells or sort purified naïve or transitional B cell subsets. The B and T cells were either in direct cell contact or separated by transwell membrane. Bars represent median values and upper whisker of error bars represent the range. *$P < 0.05$ by nonparametric ANOVA

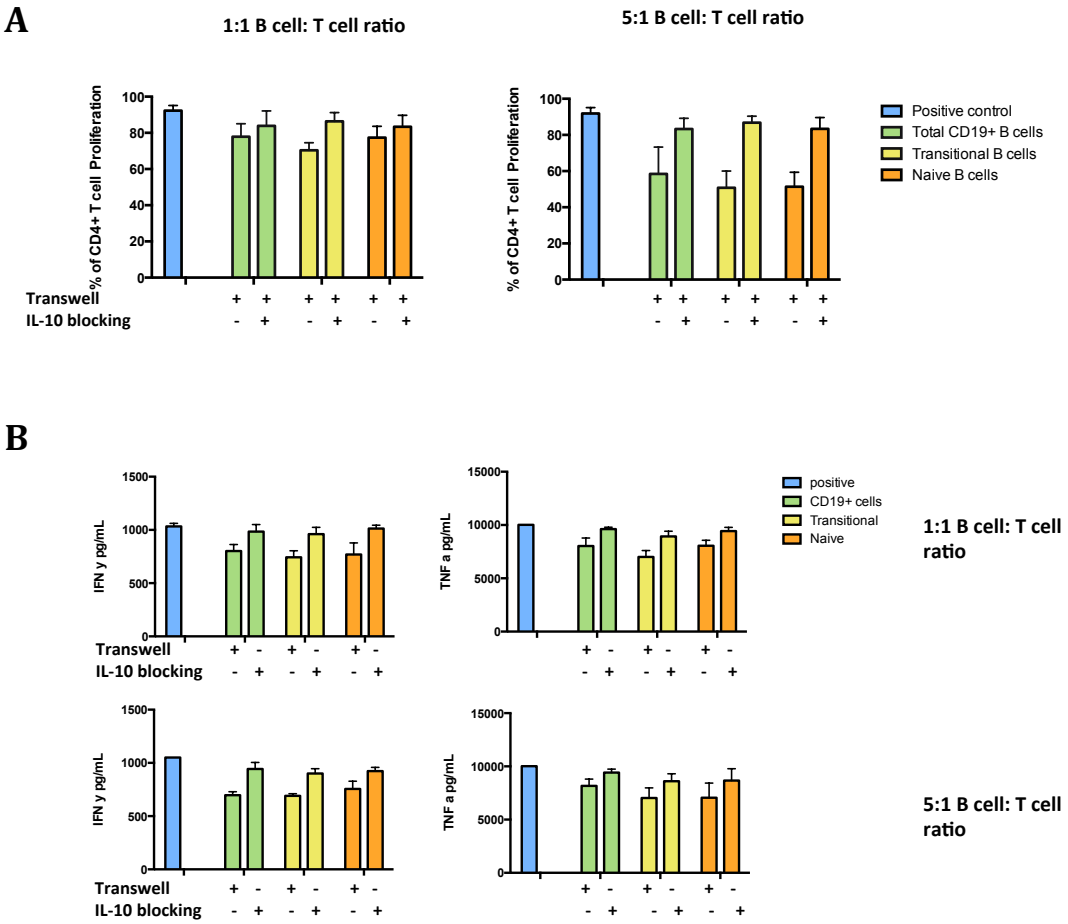


Figure IV-18: The suppressive capacity of CB-derived B cells is dependent on IL10 and cell-to-cell contact
Bars represent median values and upper whiskers indicate the range from 3 independent experiments. No significant differences were found between a combination of transwell and IL-10 blockade in B cell:T cell co-cultures vs. positive control (anti-CD3/anti-CD28 stimulated CD4+ T cells alone) by nonparametric ANOVA at both a 1:1 and 5:1 B cell:T cell ratio.

I next examined whether soluble CD40L is the trigger for IL-10 production in the transwell setting. I measured the levels of soluble CD40L that is naturally secreted by activated T cells (van Kooten and Banchereau., 2000) in the supernatant harvested from the transwell cultures by ELISA, to determine whether soluble CD40L can induce IL-10 production by B cells (Iwata et al., 2011). As is shown in **figure IV-19**, soluble CD40L was present in the cocultures with CD3/CD28-activated CD4+ T cells. For this reason, I propose

that soluble CD40L can cross the membrane and induce IL-10 production by CB-derived transitional and naive B cells to mediate T-cell suppression.

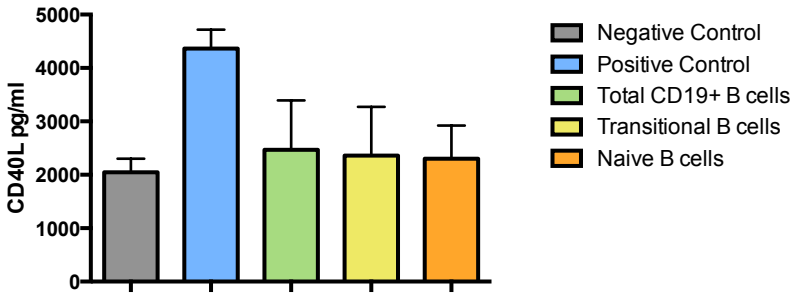


Figure IV-19: Anti-CD3/anti-CD28 stimulated CD4+ T cells release soluble CD40L, measured using the Human CD40 Ligand Quantikine ELISA kit (R&D) in the transwell supernatant (pg/mL).
Bars represent medians and upper whiskers represent range from 3 independent experiments.

Prompted by evidence from both murine and human B-cell experimental systems (Chapter III, Khoder et al., 2014., Blair et al., 2010, Mauri et al., 2010), I next tested the contribution of CD80 and CD86 costimulatory signaling to the suppressive capacity of sort-purified CB derived transitional or naïve B cell subsets. Although the addition of blocking antibodies against CD80 or CD86 molecules individually was not sufficient to reverse the suppressive activity of total CD19+ B cells or naïve and transitional B cell subsets (**figure IV-22**), addition of blocking antibodies against both molecules partially inhibited the ability of CB B cell subsets to suppress the effector function and proliferation of PB CD4+ T cells at both T cell: B cell ratios of 1:1 and 5:1 (**figure IV-20**). Thus, the suppressive effect of CB-Breg cells is at least partially mediated by CD80/CD86 costimulatory signaling, consistent with findings in human and murine experimental models (Khoder et al., 2014, Blair et al., 2010, Mauri and Bosma., 2012).

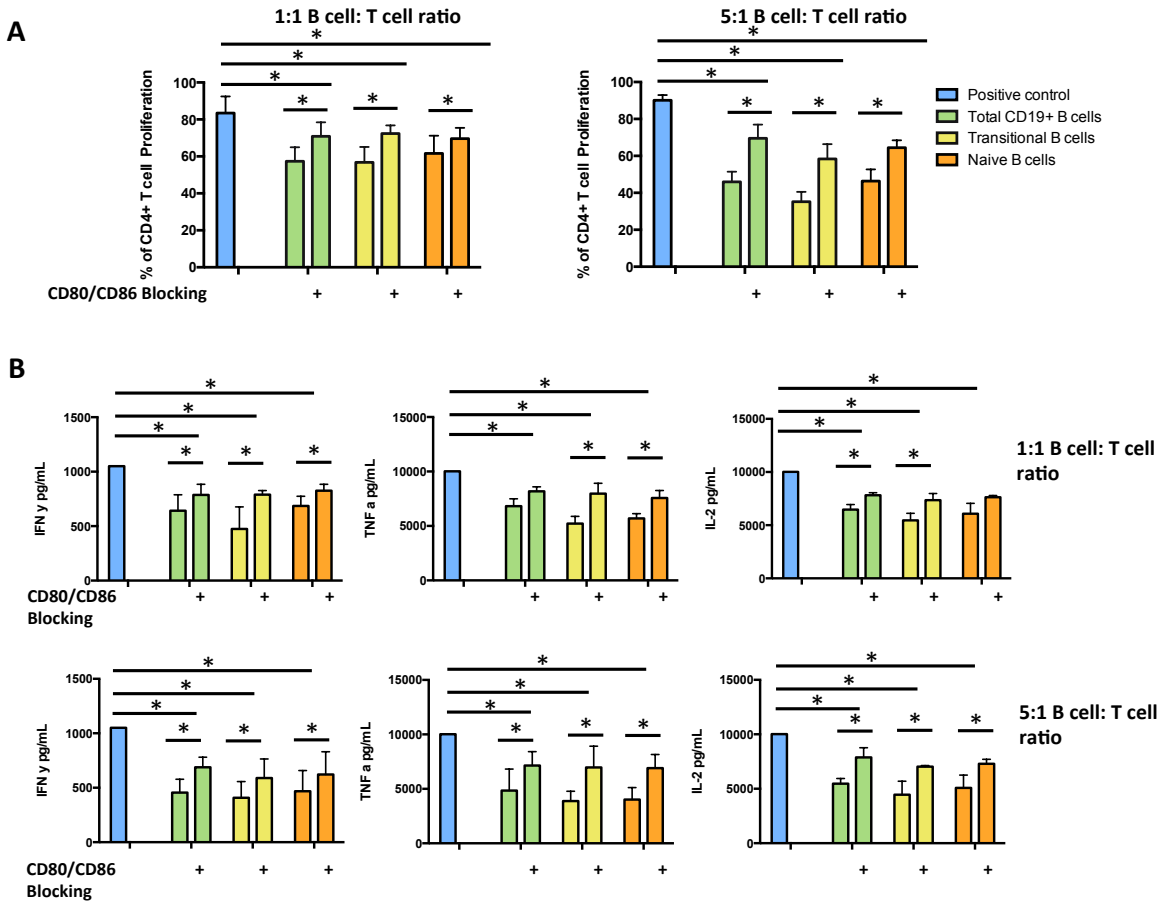


Figure IV-20: CD80 and CD86 co receptor signalling is a prerequisite for the suppressive effect of Bregs.
A. Cumulative data representing CD80 and CD86 co-receptor blockade in cultures of purified CFSE-stained proliferating CD4+ T cells and sorted CB-derived CD19+ B cell subsets, which partially reversed the suppressive capacity of Bregs on CD4+ T cell proliferation as compared to the corresponding positive control at a 1:1 and 5:1 B cell to T cell ratios (n=4) **B.** Bar charts compare the effect of CD80 and CD86 co-receptor blocking on CD4+ T cell IFN-γ, TNF-α and IL-2 production at a 1:1 and 5:1 B cell to T cell ratios (n=4). Bars represent median values and upper whisker of error bars represent the range. *P < 0.05 by nonparametric ANOVA

Previous evidence has illustrated that CD80/CD86 molecules co-interact with the CTLA-4 inhibitory receptor on T cells. (Minguela et al., 2000, Jago et al., 2004). I next determined whether the suppressive effect of CB derived regulatory B cells was dependent on CD80 and CD86 co-interaction with CTLA-4. Addition of blocking antibody against CTLA-4 significantly inhibited the ability of CB-B cell subsets to suppress the effector function and

proliferation of peripheral CD4+ T cells at both T cell: B cell ratios of 1:1 and 5:1 **(figure IV-21).** However, this reversal was not complete when compared to CD4+ T cell alone (positive control). Consequently, the reversal of CB-derived regulatory B cell suppression by CTLA-4 blockade was further enhanced when combined with CD80/CD86 blockade **(figure IV-22)**. These results provide evidence for an important interaction between CD80/CD86 on CB-derived Bregs and CTLA-4 on CD4+ T cells in Breg mediated T cell suppression. While the blockade of IL-10, CD80/CD86 and CTLA-4 individually was not sufficient to completely reverse the suppressive capacity of CB-derived transitional and naïve B cells, this endpoint was achieved with a combination of mAbs against all molecules (**figure IV-22**).

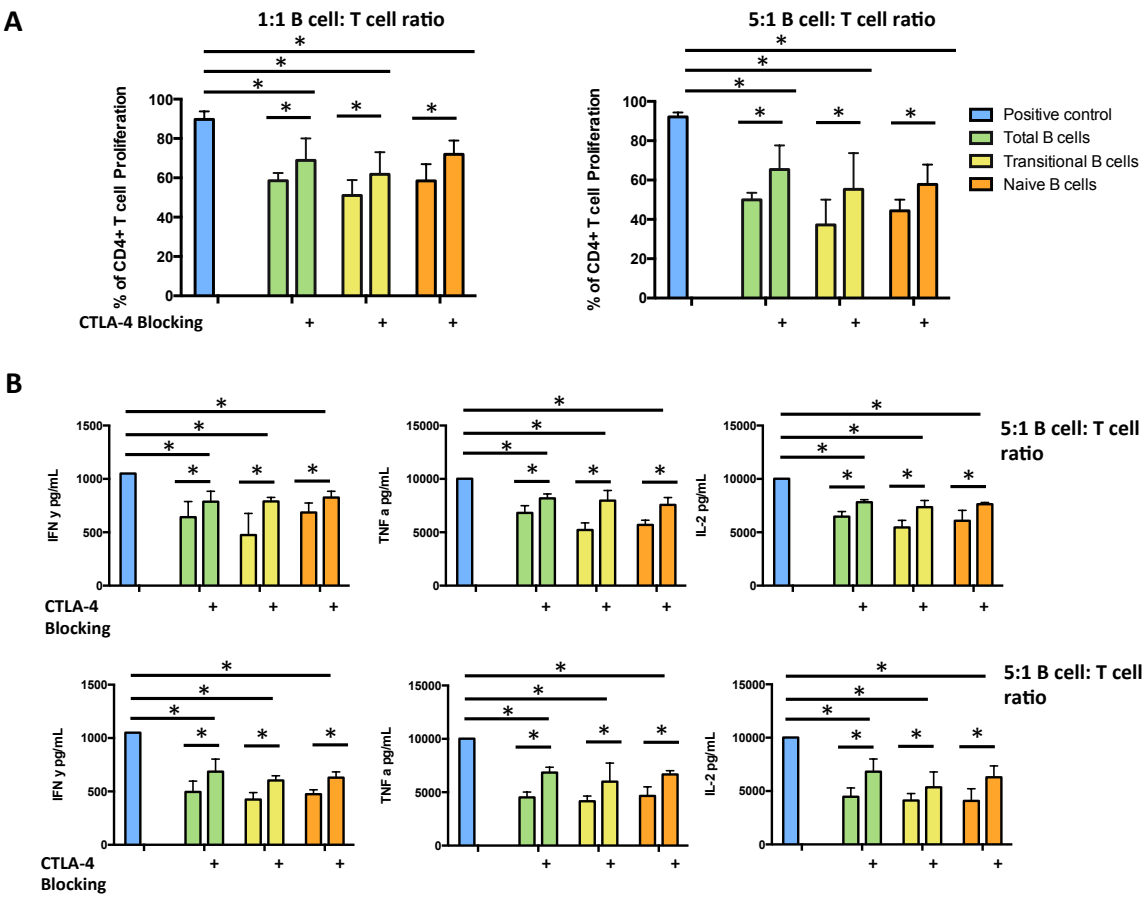


Figure IV-21: CTLA-4 blockade significantly inhibited the ability of CB B cell subsets to suppress the effector function and proliferation of peripheral CD4+ T cells at both 1:1 and 5:1 ratios

A. Cumulative data representing CTLA-4 blockade in cultures of purified CFSE-stained proliferating CD4+ T cells and sorted CB-derived CD19+ B cell subsets, which partially reversed the suppressive capacity of Bregs on CD4+ T cell proliferation as compared to the corresponding positive control at a 1:1 and 5:1 B cell to T cell ratio (n=4) **B.** Bar charts compare the effect of CTLA-4 blocking on CD4+ T cell IFN-γ, TNF-α and IL-2 production at a 1:1 and 5:1 B cell to T cell ratio. Bars represent median and whiskers indicate the range from 4 independent experiments. **P* < 0.05 by nonparametric ANOVA

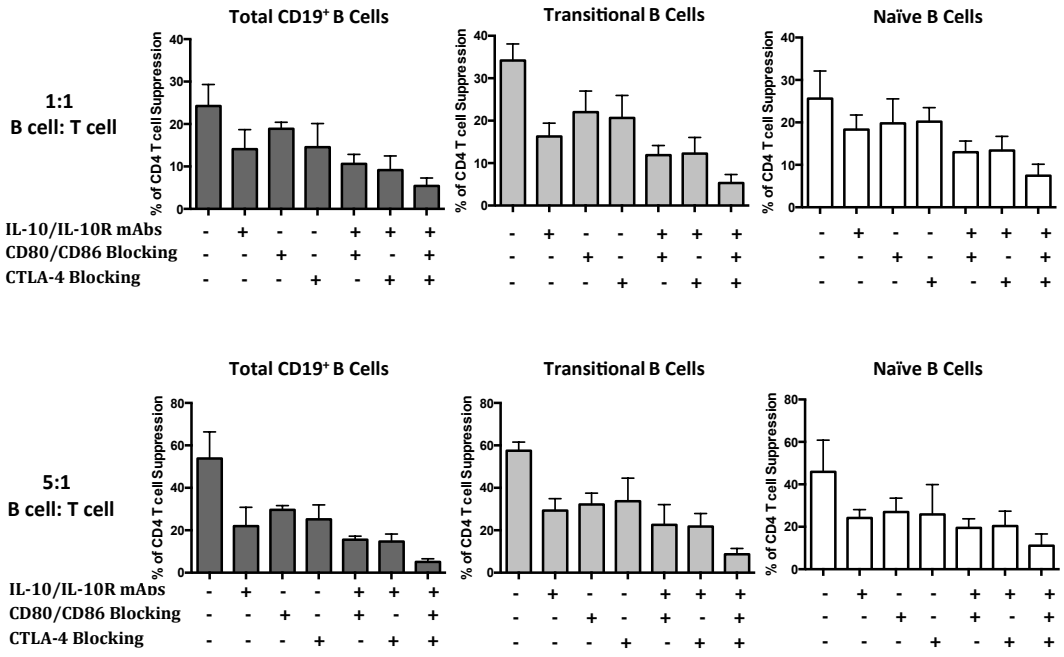


Figure IV-22: A combination of blocking antibodies to IL-10, CTLA-4, CD80 and CD86 fully reversed the ability of CB-Breg subsets to suppress CD4+ T cell proliferation *in vitro*

Bars represent median values and upper whiskers of error bars represent the range from 3 independent experiments.

IV.2.6 Depletion of Treg cells does not influence the suppressive ability of transitional and naïve CB B cells

To test whether the *in vitro* suppressive effects of IL-10 producing CB derived transitional and naive B cells are partly mediated by Treg cells, I depleted CD4+ T cells of CD127- $CD25^{hi}$ Tregs by using magnetic bead cell purification as described in section II.4.2.2. CB-derived total CD19+ B cells and naive and transitional CB B cell subsets were cultured with anti-CD3/ anti-CD28-stimulated and CFSE-stained Treg-depleted CD4+ T cells at a T cell: B cell ratio of 1:1 and 5:1. Both subsets and total CD19+ B cells significantly suppressed the proliferation of Treg-depleted CD4+ T cells when compared with the corresponding positive control (**figure IV-23**).

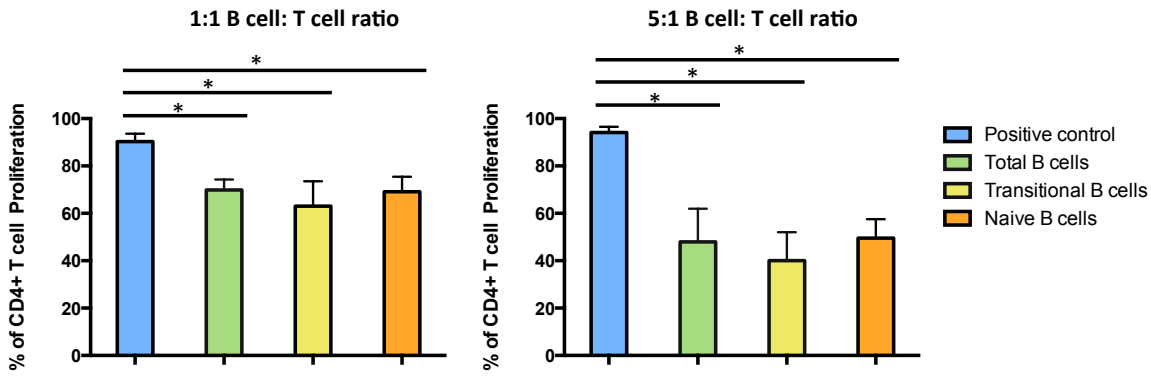


Figure IV-23: Depletion of Treg cells does not influence the suppressive ability of transitional and naïve CB B cells
Bars represent median values and upper whisker of error bars represent the range from 3 independent experiments. *$P < 0.05$ by nonparametric ANOVA

IV.2.7 Regulatory B cells in cord blood may account for lower rates of GVHD after CBT

Rapid B-cell recovery following allo-HSCT has been reported to correlate with lower rates in cGVHD (Beaudette-Zlantanova et al, 2013, Shimabukuro-Vornhagen et al., 2009, Sarantopoulos et al., 2015). Thus, given the ability of CB derived CD19+ B cell subsets to control CD4+ T cell function, and the low incidence of severe cGVHD in CB recipients (Beaudette-Zlantanova et al, 2013, Komanduri et al., 2007, Stanevsky et al., 2009), I hypothesized that the higher frequencies of B cells with regulatory properties in CB grafts may contribute to the lower rate of GVHD post-CBT.

To test this hypothesis, I first determined the frequency and proportion of total CD19+ B cells in sequential blood samples collected pre-transplant and at intervals of 90 days for up to 1 - 2 years post-CBT (**Table IV-1)**. Total CD19+ B cells from healthy cord blood units were also analyzed as the control group. CD19+ B cells could be detected at low frequencies as early as 1-month post-CBT, and increased in frequencies and absolute numbers per μL of CD19+ B cell populations between 3-9 months post transplant **(figure IV-24)**, after

which the B cell population progressively decreased. By 1-year post-CBT there were no significant differences in the frequency and numbers of circulating B cells in CBT recipients, CB units and healthy donor PB.

To further determine the kinetics of IL-10 producing Breg recovery after CBT, I next determined the relative frequencies of IL-10+CD19+ B cells in PB samples collected from 17 post CBT recipients before and at intervals of 90 days for up to 1 year post CBT and at 2 years. In the first 1-3 months post CBT B cells activated through CD40L followed by PMA+ionomycin stimulation had low IL-10 production; however, by 3-9 months post CBT there was a significant increase in the ability of B cells to produce IL-10, with high frequencies and absolute numbers of CD19+IL-10+ B cells **(figure IV-25).** During these months, the frequency of CD19+IL-10+ B cells was significantly higher than the frequency of IL-10 producing B cells found in the PB of healthy individuals or fresh cord blood **(figure IV-26),** but progressively reduced after 9 months post transplant, to levels comparable to that of healthy individuals. The early and robust reconstitution of both CD19+ B cells and the IL-10+ B cell pool post CBT supports an important role for donor CB-B cells in protection against development of cGVHD.

Table IV-1: Clinical characteristics of patients

	N=17
Age in years Median (range)	42 (21-64)
Sex	
Females, n (%)	12 (70.6)
Males, n (%)	5 (29.4)
Race, n (%)	
White	11 (64.7)
Black	2 (11.8)
Hispanic	3 (17.6)
Asian	1 (5.9)
HLA matching, n (%)	
4+4	11 (64.7)
4+5	2 (11.8)
5+5	2 (11.8)
Conditioning	
Flu/Cy/TBI	5 (29.4)
Flu/Mel/Thio	9 (52.9)
Flu/Mel140	2 (11.8)
Bu/Flu/Clo/TBI	1 (5.9)
Diagnosis, n (%)	
Primary AML	9 (52.9)
Secondary AML	3 (17.6)
CML	2 (11.8)
CLL/NHL	3 (17.6)
Cytogenetics, n (%)	
Favorable	1(5.9)
Intermediate	8 (47.1)
Unfavorable	8 (47.1)
Disease Status at transplant, n (%)	
CR1	8 (47.1)
CR2/CR3	4 (35.3)
Active Disease	3 (17.6)
ALC (k/μL) Median, range	0.87 (0.20 – 5.28)
Day 30 ALC,(x 10^6/L) Median(range	406 (70-931)
Acute GVHD, n (%)	8 (47.1)
Chronic GVHD, n (%)	7 (41.2)
Relapse, n (%)	5 (29.4)
PFS, days Mean, (95% CI)	1578.3 (1139.4-2017.3)
Overall Survival, days Mean, (95% CI)	1606 (1131.7-2080.4)
Follow-up, median (range), days	1219 (165-2283)

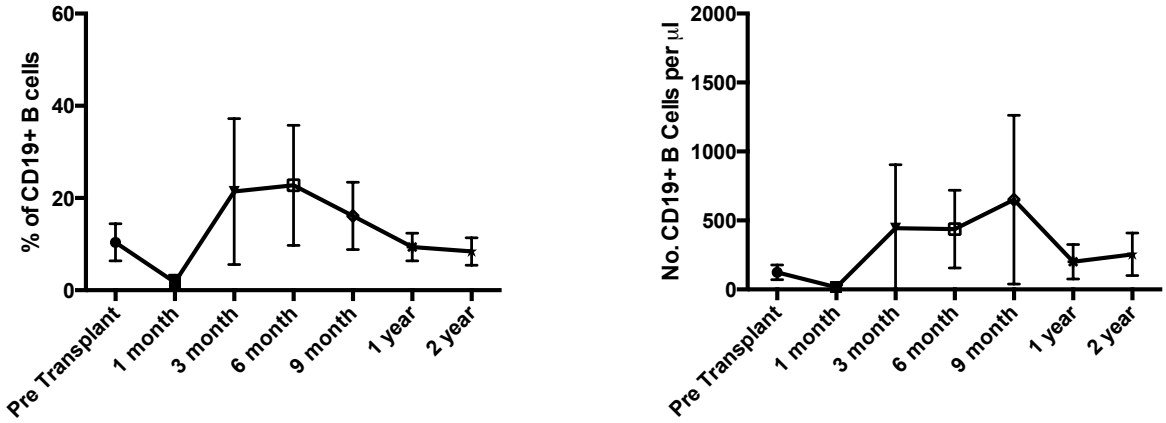


Figure IV-24: B cells from CBT recipients exhibit an early and robust reconstitution of CD19+ B cells
Total CD19+ B cell counts and frequencies were analyzed in sequential blood samples collected from CBT recipients and were significantly elevated in CB recipients at 3-9 months post transplant. The figures represent mean values and errors bars represent range (n=17)

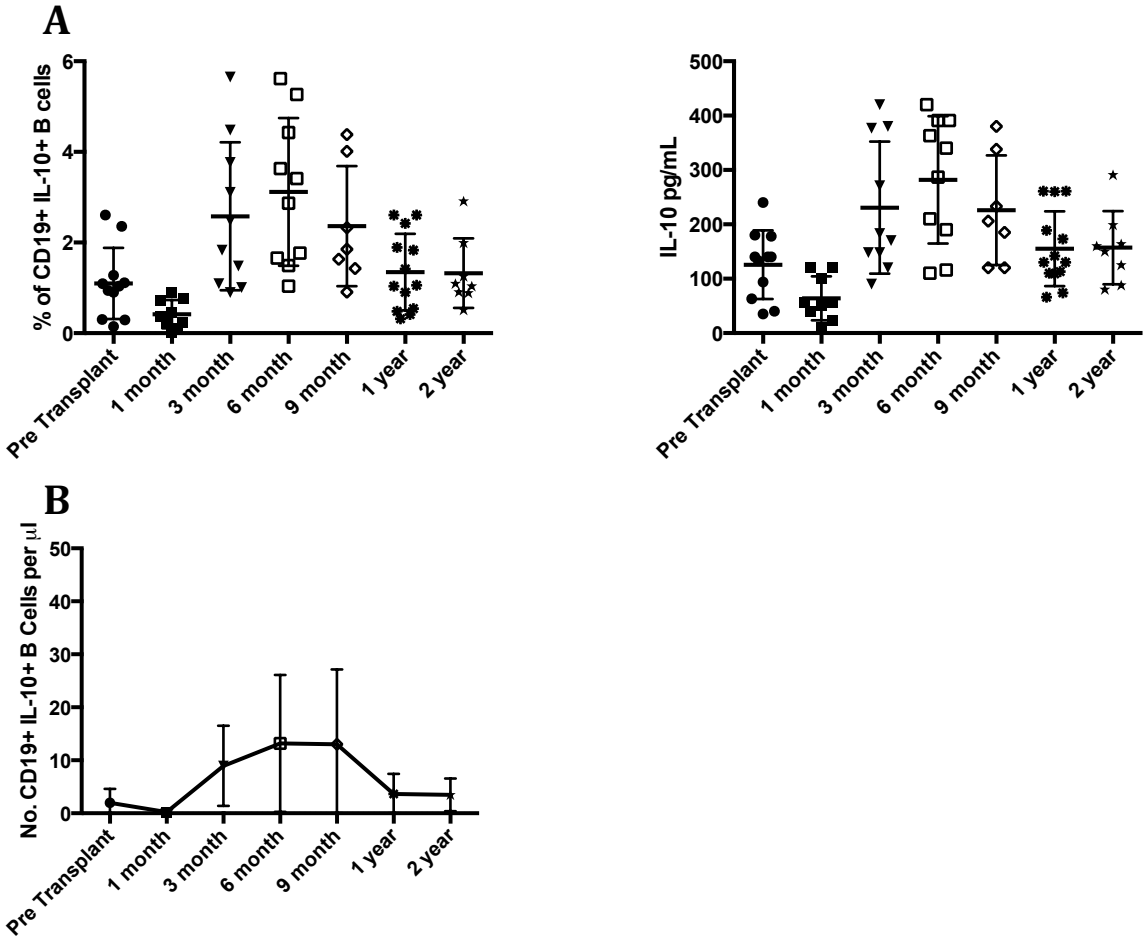


Figure IV-25: B cells from Cord Blood transplant recipients exhibit an early and robust reconstitution of IL-10 B cell pool
Thawed CBMC were used to isolate total CD19+ B cells and cultured with CD40L for 48 hours. **A.** The relative frequencies and secretion (pg/ml) of IL10+CD19+ B cells in 17 post CBT patients were significantly elevated **B.** The absolute counts of IL10+CD19+ B cells in CBT recipients were elevated at 3-9 months post transplant. A and B figures represent mean values and errors bars represent range (n=17)

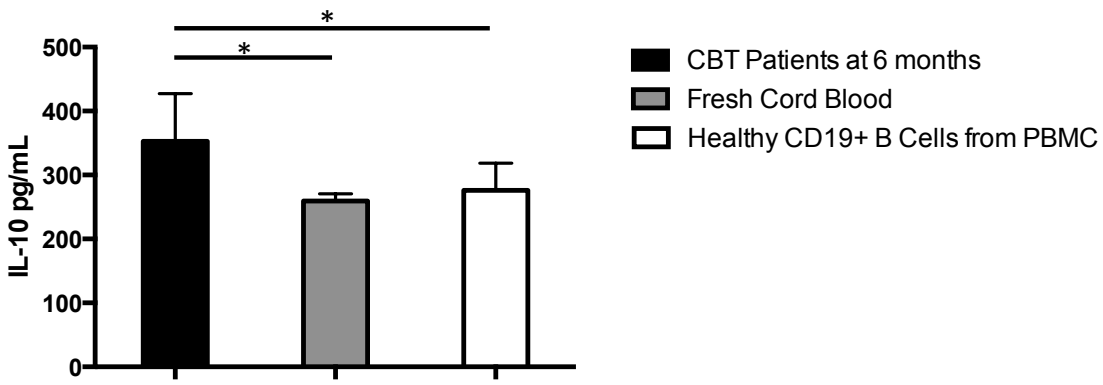


Figure IV-26: Bar graph compares IL-10 secretion in supernatants of activated total CD19+ B cells in healthy PB, healthy cord blood units and patients 6 month post CBT assayed by IL-10 ELISA.
The figures represent median values and errors bars represent range. *P < 0.05 by nonparametric ANOVA.

I next examined the frequencies and absolute numbers of recovering CD19+ B cells and CD19+IL-10+ B cells at multiple time-points post CBT in patients who developed GVHD [acute (n=8) and chronic (n=7)] (n=9) compared to patients without GVHD (n=8) to further elucidate the protective role for CB-derived donor Breg in GVHD after CBT.

Patients with GVHD had significantly lower frequencies and absolute counts (per µl) of CD19+ B cells in the first 3-9 months post CBT compared to patients without GVHD (**figure IV-27)**. Similarly, CBT recipients with GVHD had lower frequencies and absolute numbers of IL-10 producing-CD19+ B cells compared to those without cGVHD (**figure IV-28**). Our results are in line with previous studies that have also reported lower IL-10 producing Bregs in patients with GVHD than in healthy donors after allo-HSCT (Rowe et al., 2006, Khoder et al., 2014, Weber et al., 2014). No significant differences were observed in the transitional and naïve B cell compartments in patient with or without GVHD.

Our data support our hypothesis that the robust recovery of B cells with regulatory properties in CB grafts observed during the first 3-9 months post CBT may contribute to the low incidence of GVHD post-CBT. Collectively, these data suggest a novel and protective role for IL-10 producing Bregs in the regulation of human cGVHD after CBT.

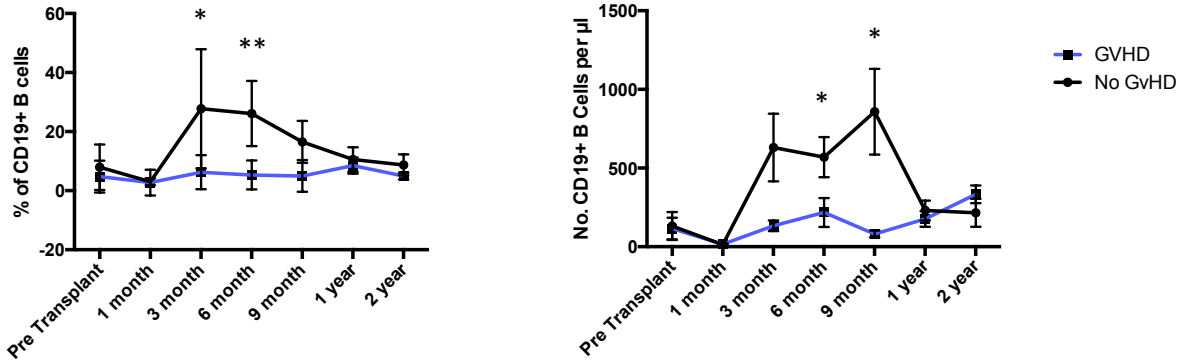


Figure IV-27: CD19+ B cell frequencies and absolute counts per µl in patients with GVHD. Patients with cGVHD (n=9) had significantly decreased CD19+ B cell reconstitution when compared to patients who did not develop cGVHD (n=8)
The figures represent mean values and errors bars represent range. *$P < 0.05$ and ** P<0.01 by unpaired t-test.

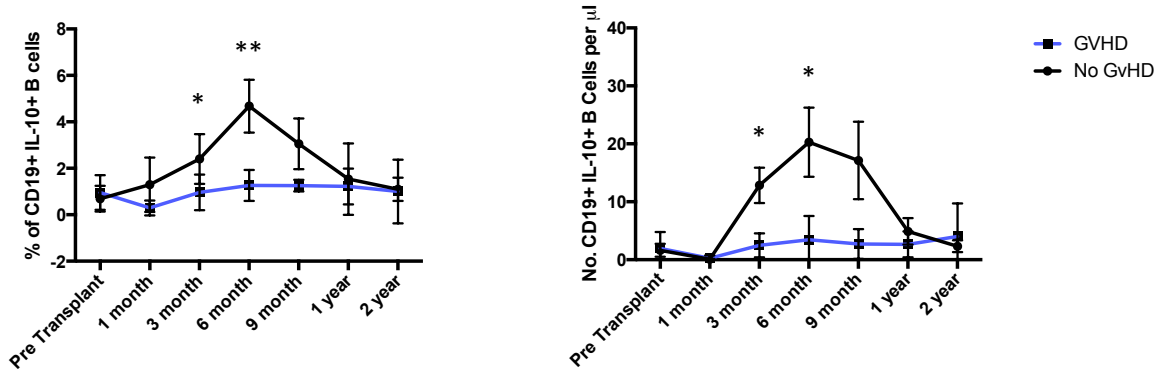


Figure IV-28: CD19+IL-10+ B cell frequencies and absolute numbers in patients with GVHD. Patients with cGVHD (n=9) had significantly decreased CD19+IL-10+ Breg cell reconstitution when compared to patients who not develop cGVHD (n=8)
The figures represent mean values and errors bars represent range. *$P < 0.05$ and ** P<0.01 by unpaired t-test.

To further confirm that the recovering B cells after CBT have regulatory capacity, CD19+ B cells were magnetically isolated from sequential PB samples collected and cryopreserved from post CBT recipients (as described above) and cultured with anti-CD3/anti-CD28-activated allogeneic CFSE+CD4+ T cells from healthy individuals for 96 hours. B cells from patients at 6 and 9 months had a significantly greater suppressive capacity on CD4+ T cell proliferation when compared to fresh cord blood B cells **(figure IV-29)**. My result indicate the presence of an expanded population of IL-10 producing regulatory CD19+ B cells at 6-9 months, which posses greater suppressive function on T cell proliferation, supporting an important role for Bregs in limiting or preventing the severity of cGVHD.

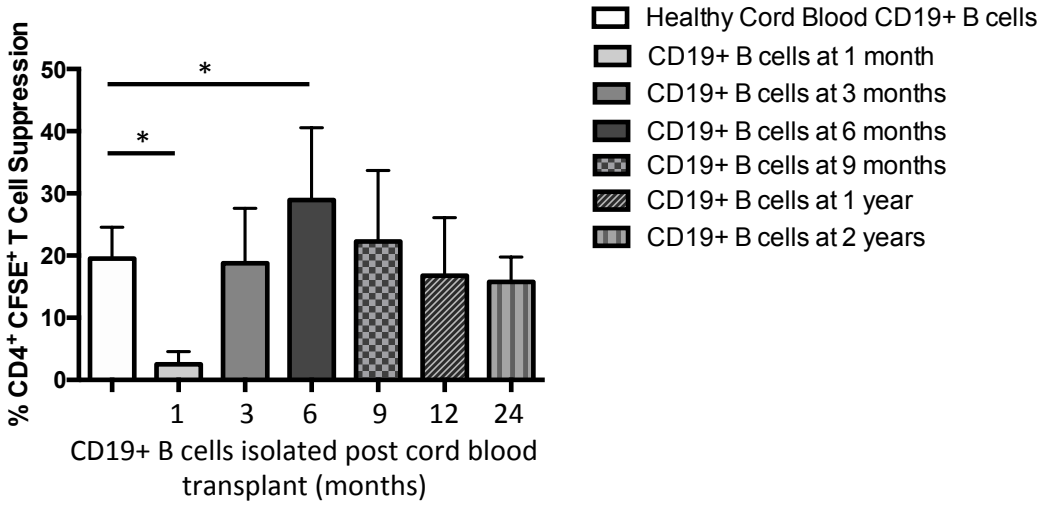


Figure IV-29: Expanded population of IL-10 producing regulatory CD19+ B cells posses greater suppressive function on T cells as compared to healthy cord blood B cells.
Magnetically isolated CD19+ B cells from post CBT patients collected at varying time points after CBT were cultured with anti-CD3/anti-CD28-activated CFSE+CD4+ T cells from healthy individuals for 96 hours at a 1:1 B cell: T cell ratio. Cells were harvested and stained for CD4+ CFSE+ proliferating T cells. The bars represent median values and errors bars represent range. *P < 0.05 by nonparametric ANOVA.

IV.3 Discussion

Regulation of the delicate balance between effector T cell activities against invading pathogens and the tolerance of self- and environmental antigens has been largely ascribed to regulatory T (Treg) cells (Rezvani et al., 2006). The pathophysiology of GVHD involves impaired regulatory mechanisms of tolerance between recipient tissues and donor-derived immunity (Blazar et al., 2012). Our understanding of the function of Bregs in this disease is limited. Recent research has described a functional group of IL-10 producing $CD19^{+}CD24^{hi}CD38^{hi}$ transitional B cells in human PB that possess immune regulatory capacity (Blair et al., 2010, Khoder et al., 2014). Conversely, other studies have identified human IL-10 producing B cells to be contained mainly within the $CD24^{hi}CD27^{+}$ memory B cell compartment (de Masson et al., 2015, Iwata et al., 2011). In addition to these studies, Khoder *et al* reconciled the results of previous studies and demonstrated that regulatory subsets of IL-10-secreting CD19+IgM+CD27+ memory B cells coexist with IL-10+$CD24^{hi}CD38^{hi}$ transitional B cells in healthy human donors and play a role in protection against cGVHD after HSCT (Khoder et al., 2014).

Recently published work has also established the presence of $CD19^{+}CD38^{hi}CD24^{hi}$ immature transitional B cells that are abundant in human cord blood (Ha et al,. 2008, Cuss et al., 2006). Here, we underscore the regulatory capacity of CB-derived immature transitional B cells, and identify naïve B cells as new candidate Breg subset that can be identified in CB. I propose that within the CB-CD19+ B cell pool there exist 2 distinct subsets; $CD19^{+}CD38^{hi}CD24^{hi}$ transitional B cells and $CD19^{+}CD38^{int}CD24^{int}$ naïve B

cells, both of which are functionally regulatory. My results signify the suppressive capacity of CB-derived Bregs on peripheral CD4+ T cell proliferation and effector function.

Despite several phenotypic and functional similarities between human PB- and CB-derived Bregs, (Cuss et al., 2006, Marie-Cardine et al., 2008), our study revealed a number of key differences. In contrast to previous studies that ascribed regulatory capacity to PB-derived CD24hiCD27+ or CD27+IgM+ memory cells (Khoder et al., 2014, Iwata et al., 2011), memory B-cells are absent in CB (Ha et al., 2008). Further, unlike PB-naïve B cells that failed to suppress CD4+ T cells (Khoder et al., 2014), our discovery presents a novel suppressive role for naïve B cells in CB and broadens the proportion of Bregs in CB, suggesting a prominent role for these functional CB-derived B cells in the maintenance of immune tolerance.

My data further demonstrate that the suppressive capacity of CB-Breg was augmented in the presence of pre-activated B cells co-cultured with CD4+T cells, suggesting that in human PB, Breg designation may not be limited to the memory and transitional B cell subsets described previously and it is likely that discrete subsets of naïve and switched memory B cells could also be induced to exert regulatory function in response to CD40-ligand signaling provided by activated T cells, analogous to reports of inducible Tregs during inflammation (Feuerer et al., 2009).

Similar to previous reports of PB-Bregs, the mechanism by which CB-derived total CD19+ B cells and naïve and transitional B cell subsets suppress CD4+T

cell function was mediated synergistically through IL-10 production and cell-to-cell contact (Blair et al., 2010, Khoder et al., 2014). IL-10 blockade partially reversed CD4+ T cell proliferation and effector cytokine secretion in the presence of total CD19+, naïve or transitional B cells. In contrast to reports in murine Breg studies (Mizoguchi and Bhan., 2006, Mauri and Bosma et al., 2012), I did not find a significant role for TGF-β in CB B cell-mediated T cell suppression. However, exogenous addition of IL-10 to CD4+ T cells alone failed to induce significant CD4+ T cell suppression, supporting the existence of additional mechanisms of CB-B cell-mediated T cell suppression. Additional mechanistic studies using transwell and CD80/86 blockade confirmed that CD80 and CD86 interactions between B cells and $CD4^+$ T cells work synergistically with IL-10 production for Bregs to exert their full suppressive effect on CD4+ T cell function. Our findings are in agreement with previous studies with human PB Bregs that describe the involvement of CD80 and CD86 as an important feature of their suppressive capacity (Blair et al., 2010, Khoder et al., 2014) and with murine studies of intestinal inflammation where CD86 especially has been noted to facilitate B cell suppression (Mann et al., 2007, Mizoguchi et al., 2000). Conversely, the involvement of CD80 and CD86 co-interaction with CTLA-4 on T cells in CB-Breg-mediated T cell suppression is in contrast to findings with PB-Breg, where their suppressive activity was found to be independent of CTLA-4 (Khoder et al. 2014). However, a similar mechanism is employed by CD80 expressed on DCs, which acts preferentially as a ligand for CTLA-4 and mediates Treg cell suppression (Zheng et al., 2004). The differences in CTLA-4 mediated suppressive mechanisms between CB-B cells and PB-B cells found in my

study may be attributed to different compositions of B cells subsets, as CB cells are largely composed of naïve cells that may be more susceptible to CTLA-4 blocking than PB-B cells.

Additionally, while the blockade of IL-10/IL-10R, CD80/CD86 and CTLA-4 individually was not sufficient to fully reverse the suppressive activity of CB-Bregs, this endpoint was achieved with a combination of mAbs against all molecules mediating the prevention of IL-10 and cell-cell contact.

The role of Bregs in cord blood transplantation and GVHD has not been previously studied. Several studies have reported that immune reconstitution after CBT is characterized by an expansion in B cells during the first year post transplant (Komanduri et al., 2007, Nakatani et al., 2014, Beaudette-Zlatanova et al., 2013, Saliba et al., 2015, Lucchini et al., 2015).

Here, I report on B cell reconstitution in 17 CBT recipients; B cells could be detected at low frequencies as early as 1-month post-CBT, where the majority of B cells had a $CD24^{high}$ $CD38^{high}$ transitional profile. Further I observed expanded frequencies and absolute numbers of CD19+ B cell populations between 3-9 months, after which the B cell population progressively decreased. By 1-year post CBT there were no significant differences in the frequency and numbers of circulating B cells in CBT recipients and healthy donors. My data further illustrate a similar pattern in the kinetics of IL-10 producing CD19+ B cell reconstitution after CBT.

I further demonstrated that CBT recipients that developed GVHD (acute n=8 and chronic n=7) had a reduced CD19+ B cell recovery when compared to

CBT recipients that did not develop GVHD (n=8). In addition, CD19+ B cells isolated from CBT recipients with GVHD were refractory to stimulation and had significantly impaired reconstitution of CD19+IL-10+ B cells 3-9 months after CBT when compared to patients without GVHD. These results are in accord with similar studies that have also found low frequencies of IL-10 producing B cells in cGVHD patients following AHSCT when compared to AHSCT recipients without cGVHD (Rowe et al., 2006, Khoder et al., 2014, Weber et al., 2014). Collectively, these results postulate that the early recovery of B cells post-CBT may define a protective role for Bregs in GVHD setting. However, limiting factors associated with the heterogeneous patient group of CBT recipients, in terms of disease background, conditioning regimen, cytogenetics that differ amongst the patient cohort, may affect the data derived and require further consideration.

However, at present we have no means of purifying exclusively those B cells that express IL-10 in sufficient quantities for functional studies. Thus, given the potential role of Bregs in the maintenance of the balance between tolerance and autoimmunity, it imperative to search for a unique Breg signature for the identification of IL-10-producing B cells with immunoregulatory capacity.

This study defines a novel role for CB derived donor Bregs in protection against cGVHD, and support the development of strategies to evaluate novel B-cell directed therapies for the prevention or treatment of cGVHD, specifically targeting B cell reconstitution and function after transplant. Hence, strategies to selectively target B effector cells following B-cell depletion

therapy whilst preferentially sparing regulatory B cells are required. Thus, to counterbalance immune regulation and induce protection against GVHD, so that GVHD is either prevented or attenuated, infusion of donor-derived Bregs early in the patient's post-transplant regime may be an attractive approach. The potential of in-vitro expanded CB-derived IL-10 producing Bregs as an invaluable source of off-the-shelf treatment of human GVHD merits further investigation.

Chapter V. Control of NK functions by Human IL-10 producing $CD19^{+}CD24^{hi}CD38^{hi}$ Transitional and $CD19^{+}CD27^{+}IgM^{+}$ memory cells in Acute Myeloid Leukemia

V.1 Introduction

In recent years, IL-10-producing B cells, a distinct newly recognized subset of B regulatory (Breg) cells that maintain immune tolerance and are critical in host suppression of autoimmune diseases have been the focus of intense research in immunology (Mauri *et al*., 2013). Breg cells have been shown to exert significant immunoregulatory functions both *in vitro* and *in vivo* (Qian *et al*., 2012) through the production of the immunosuppressive cytokine interleukin IL-10 and TGF-beta (Mauri *et al*., 2013), and appear to play important roles in autoimmunity and in cancer (DiLillo *et al*., 2010, Bouaziz *et al*., 2008).

The identification of suppressor B cells that could down-regulate the immune response, originated in 1974 however, the term 'regulatory B cells', was first introduced by Mizoguchi and Bhan nearly 30 years later (Mizoguchi *et al*., 2006, Yang *et al*., 2013). Despite the extensive body of evidence accumulating in the ensuing years since these studies were published, reinforcing the notion of B cells as potential regulatory cells, some controversy over the paucity of markers that can unequivocally identify Bregs, particularly in humans, still exists. Blair and colleagues (2010) have elegantly described human Bregs as $CD19^{+}CD24^{hi}CD38^{hi}$, a phenotype that typically delineates human transitional B cells. Conversely, Iwata *et al* (2011) described human IL-10 producing B cells to be contained mainly within the $CD24^{hi}CD27^{+}$ memory B cell compartment.

In addition to these studies, recently published data from my work (Khoder *et al*., 2014), syndicates previous studies and shows that IL-10-secreting CD19+IgM+CD27+ memory B cells coexist with IL-10+CD24^{hi}CD38^{hi} transitional B cells in healthy human donors and significantly suppressed the proliferation and cytokine production of anti-CD3/anti-CD28-stimulated autologous CD4+ T cells through both IL-10-dependant and cell-to-cell contact mediated mechanisms (Chapter III). Khoder *et al* (2014) further highlighted that IL-10 producing B cells are deficient in patients with chronic graft-versus-host disease (cGVHD), supporting their role in the pathogenesis of cGVHD.

A new wave of research provides evidence that Bregs may play a role in cancer progression. A first indication that B cells and antibodies could be tumor-promoting came from studies conducted almost 60 years ago. In these early works, Kaliss (1958) found that transfer of tumor-specific antibodies augmented growth of transplanted tumor cells, whereas absence of B cells limited tumor formation (Brodt and Gordon, 1982). In addition, recent animal studies have demonstrated that Bregs support tumor growth in mouse models of malignancies though IL-10-mediated suppression of effective anti-tumor T cell responses (Visser *et al*, 2005., Inoue *et al*, 2006, Horikawa *et al*., 2011., Shao *et al*., 2014) . Further, Dililio *et al* (2013) has also highlighted IL-10-producing B cells to be expanded in patients with chronic lymphocytic leukemia (CLL), suggesting that Bregs might support cancer progression in humans. Despite mounting evidence supporting a role for Breg cells in promoting tumor growth through inhibition of T cell proliferation and effector

function, there are currently no data on the interaction of Bregs with other immune effectors important in tumor-immune surveillance such as natural killer (NK) cells.

NK cells are important components of the innate immune system and play an important role in tumor immune surveillance (O'Hanlon, 2004, Ljunggren and Malmberg, 2007, Waldhauer and Steinle, 2008). NK effector function is dictated by the integration of signals received through germ-line-encoded receptors that can recognize ligands on their cellular targets. Functionally, NK cell receptors are classified as activating or inhibitory (Ljunggren and Malmberg, 2007). In cancer patients, NK cell activation can be hampered by downregulation of major histocompatibility complex (MHC) class I molecules on target cells (missing self theory) (Smyth *et al*, 2002, Ljunggren and Karre, 1990) and/or upregulation of proteins such as NKG2D ligands on 'distressed' cells (induced self theory) (Vivier *et al*, 2008), but Giringhelli *et al* (2005) suggested that other mechanisms may also play a role in blunting NK cell responses against cancer. Indeed, increased frequencies of regulatory T cells (Tregs) have been shown to correlate with cancer progression and hamper NK cell function (Ghirenghelli *et al*, 2005, Pedrozo-Pacheo *et al*, 2013). Recent studies have shed light on the existence of NK-B cell bidirectional cross-talk, through the interaction of CD48 on B cells with, 2B4, on NK cells (Yuan *et al*, 2010., Gao *et al*, 2006., Gao *et al*, 2005, Lee *et al*, 2006). NK cells can influence the ability of B cells to secrete antibodies, to present antigens to T cells and activate switch recombination while pre-activated B cells in turn upregulate IFN-y production by NK cells (Gao *et al*., 2006 and

Yuan *et al*, 1992). Signaling lymphocyte activation molecule–associated protein (SAP) is essential for the activating function of 2B4 and signalling, and can bind to all 4 immuno-receptor tyrosine-based switch motifs (ITSMs) of 2B4 (Assarsson *et al*, 2005., Stark and Watzl, 2006). Phosphorylated ITSM can also recruit the inhibitory phosphatases SHP-1, SHP-2 and SHIP (Assarsson *et al*, 2005). SAP can inhibit the interaction between 2B4 and these inhibitory signalling molecules and in its absence, 2B4 can bind to the negative signaling molecules and mediate an inhibitory signal (Eissman *et al*, 2005 and Endt *et al*., 2007). Therefore 2B4 can also function as an inhibitory receptor when engaged by CD48-expressing cells or tumor targets (Lee *et al*., 2006).

Here I report that IgM memory (CD19+IgM+CD27+) and transitional (CD19+CD24hiCD38hi) regulatory B-cell subsets from human PB suppress NK cell effector function and proliferation. This suppressive effect was mediated by predominantly cell-cell contact mediated through 2B4-CD48 signaling. Moreover, I demonstrate that the same populations of B cells are expanded in the PB of patients with AML and exert potent suppression of NK effector function and proliferation. My findings have important clinical implications, as they suggest that Bregs may impact effector anti-tumor NK cell responses in AML and other cancers and support strategies to target these cells for optimal anti-cancer immunotherapy.

V.2 Results

V.2.1 Human IL-10 producing CD19+CD24hiCD38hi transitional and CD19+CD27+IgM+ memory cells suppress NK cell cytotoxic function

The pathological relevance of the Treg-NK cell interaction has been shown in a number of tumor models (Smyth et al., 2006) and in patients with cancer (Pedrozo-Pacheo et al., 2013, Ralainirina et al., 2007) however the interaction between Bregs and NK cells is unknown. Khoder et al (2014) has recently reported that IL-10 producing B cells are enriched within the CD19+CD24+CD38+ transitional and $CD19^{+}IgM^{+}CD27^{+}$ memory B cell subsets (Chapter III) and possess regulatory properties through inhibition of CD4+T cell proliferation and effector function. To determine if Bregs also suppress NK function, I sort-purified IgM memory B cells ($CD19^{+}CD24^{-}CD38^{hi}IgM^{+}$), switched memory B cells ($CD19^{+}CD24^{-}CD38^{hi}IgM^{-}$), naïve B cells ($CD19^{+}CD24^{+}CD38^{+}$), and transitional B cells ($CD19^{+}CD24^{hi}CD38^{hi}$) as described previously (section II.5) from the PB of healthy donors and co-cultured them at a 1:1 ratio with magnetically purified autologous CD56+CD3- NK cells (section II.4.3) for 48 hours, in the presence of IL-15 (15ng/ml). The gating strategies and post-sort purity checks of B cell subsets are shown in **figure III-10**. Effector function of NK cells co-cultured with B cell subsets was assessed against K562 target cells by measuring the frequencies of degranulated (CD107a+) CD56+ NK cells as a marker of cytotoxicity and TNF-α and IFN-γ producing CD56+ cells by flow cytometry as described in section II.9.2. Transitional and IgM memory B cells suppressed CD107a degranulation, and expression of IFN-γ and TNF-α by NK cells in response to K562 targets compared to CD56+CD3-NK cells cultured alone. In contrast,

naïve and switched memory B cells had no significant effect on the NK effector function **(figureV-1).** The inhibitory effect of transitional and IgM memory B cells on NK effector function was cell dose dependent with the highest suppression observed at a ratio of 5:1**.** (**figure V-2)**. We further found that Bregs can suppress the effector function of autologous and allogeneic NK cells to a similar extent **(figure V-3).**

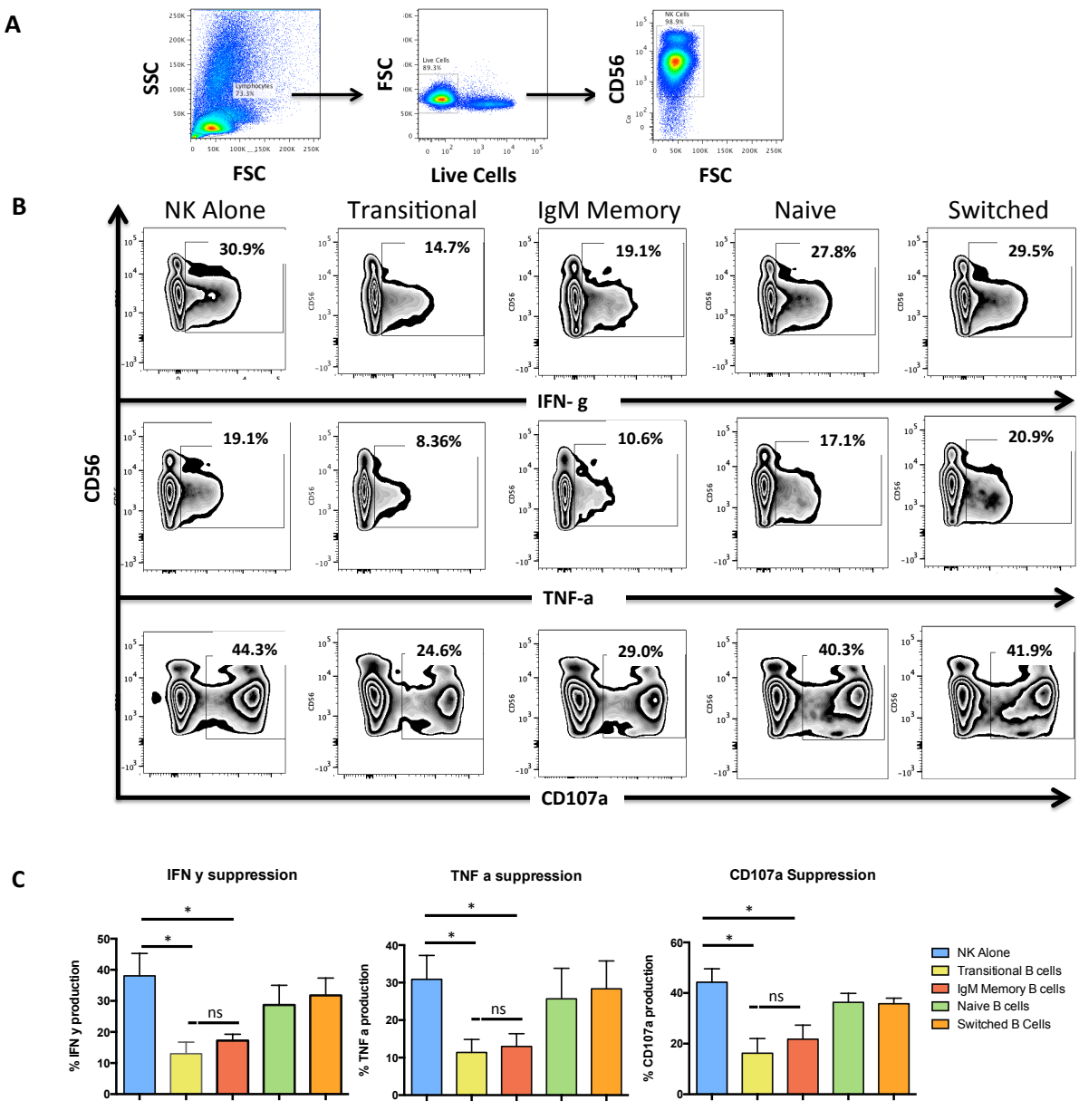


Figure V-1: Transitional and IgM memory B cells suppress NK effector function Sort purified B cell subsets were co-cultured with selected NK cells at a 1:1 ratio in the presence of K562 target cells. **A**. Gating strategy illustrating gated lymphocyte population, live cell gate and CD56+ NK cells **B.** Representative FACS plots illustrating the inhibitory effect of both transitional and IgM memory B cell subsets that significantly suppressed the frequencies of CD107a degranulation, TNF-α and IFN-γ producing CD56+ cells. Naïve and switched memory B cells had no significant effect on the NK effector function **C**. Cumulative data summarizing the suppressive effect of Breg subsets on NK effector function (n=14). Bars represent median values and upper whiskers represent the range. *$P < 0.05$ by nonparametric ANOVA; ns, not significant

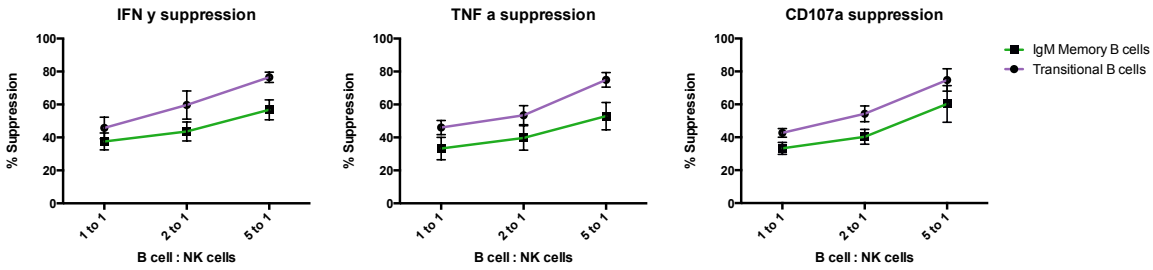


Figure V-2: The suppressive effect of transitional and IgM memory B cells on NK effector function was cell dose dependent (n=4)
Each symbol represents mean and errors bars represent range

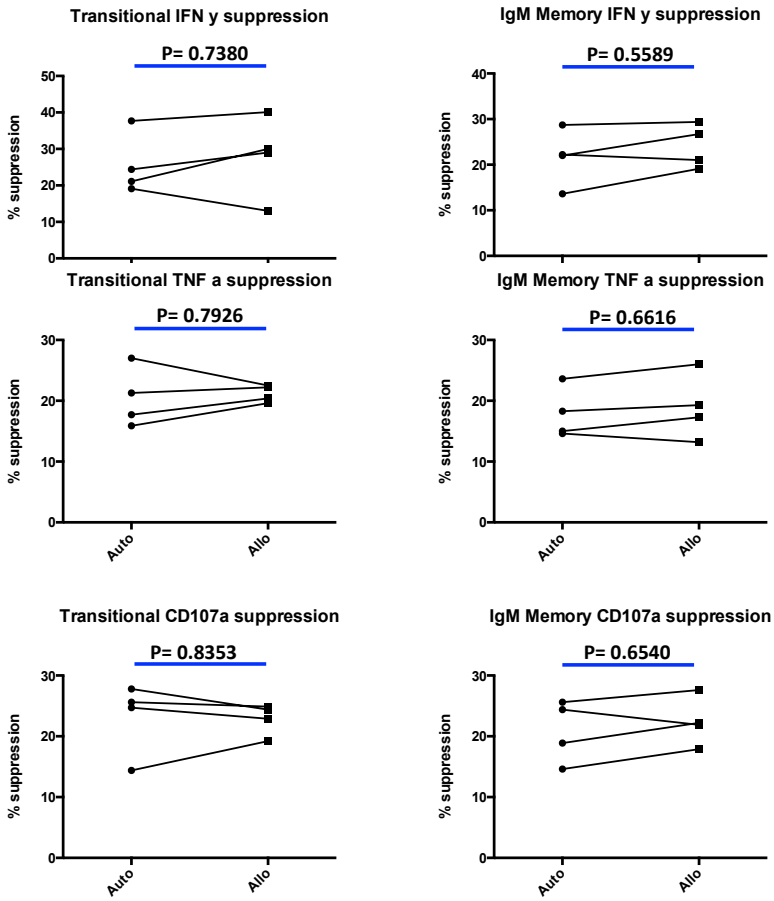


Figure V-3: The suppressive effect of transitional and IgM memory B cells on NK effector function was comparable on both autologous (Auto) and allogeneic (Allo) NK cells n=4
All comparisons were made using a paired t-test.

Granzyme B and perforin are important for the ability of NK cells to kill their targets (Ralainirina *et al*., 2007). Transitional and IgM memory B cells significantly suppressed perforin production by NK cells, when compared with NK cells alone **(figure V-4A)**. Interestingly, only transitional B cells could suppress granzyme B production by NK cells in response to K562 targets **(figure V-4B)**.

The suppressive ability of transitional and IgM memory B cells on NK cytotoxicity was further confirmed using chromium (51Cr) release assay, which measures target cell lysis (Shah et al., 2013). The cytotoxicity of NK cell co-cultured with transitional and IgM memory B cell subsets against K562 cells was significantly lower than NK cells co-incubated with naïve and switched subsets or NK cells cultured alone (representative from n=14 experiments) **(figure V-4C).**

We then compared the suppressive capacity of transitional and IgM memory B cells with that of regulatory T cells (Treg), defined as $CD25^{hi}$ $CD127^{-}$ CD4 T cells.

Magnetically purified Treg were co-cultured with autologous NK cells at 1:1 ratio in 4 independent experiments and cultured cells were stimulated with target K562 cells as described previously. The suppression of NK effector function and cytotoxicity by Tregs, and transitional and IgM memory B cells was comparable, **(Figure V-5),** indicating that transitional and IgM memory B cells can suppress NK effector function to a similar extent as Treg.

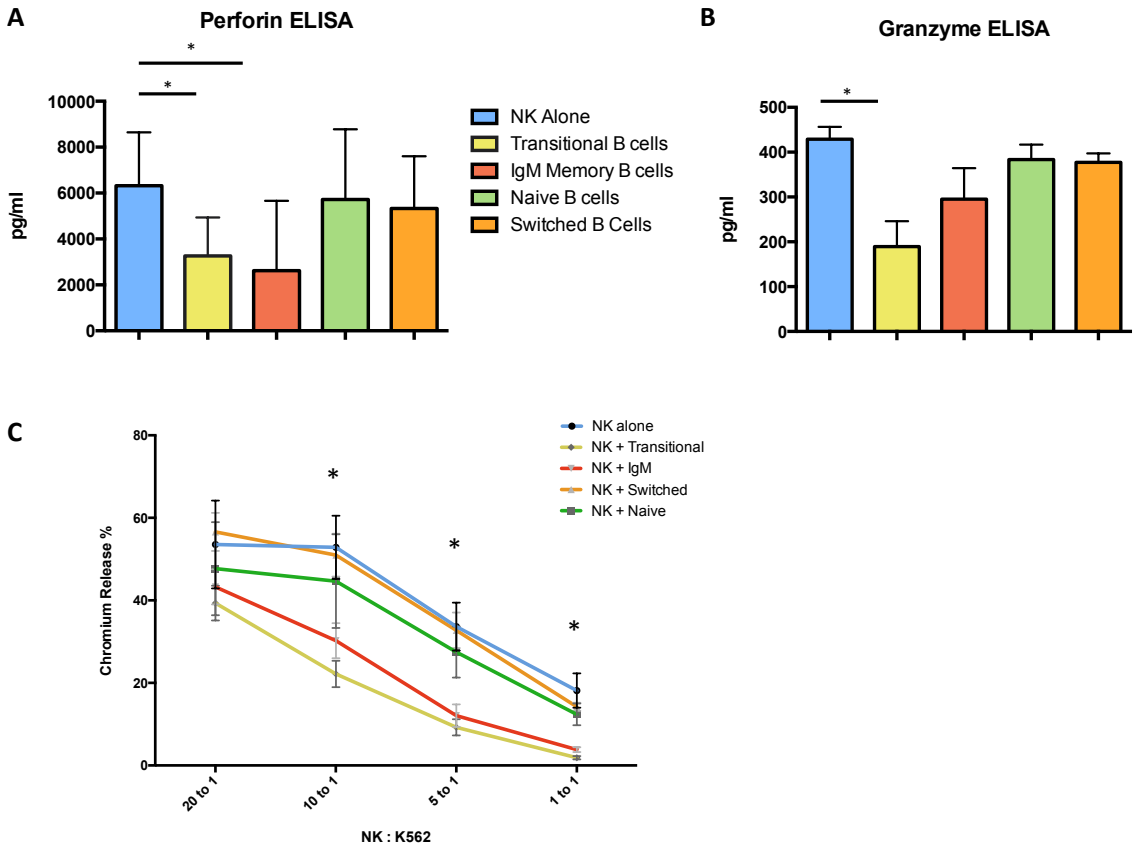


Figure V-4: Transitional and IgM memory B cells suppress NK cytotoxicity
Cumulative data representing the suppressive effect of Bregs **A**. on NK perforin production (n=3). Bars represent median values and upper whisker of error bars represent the range **B.** granzyme B production (n=3). Bars represent median values and upper whisker of error bars represent the range and **C** Chromium release (n=14)**.** This figure reports median values and error bars indicate interquartile ranges. In A-C, **P* < 0.05 by nonparametric ANOVA was used to compare between different individual groups.

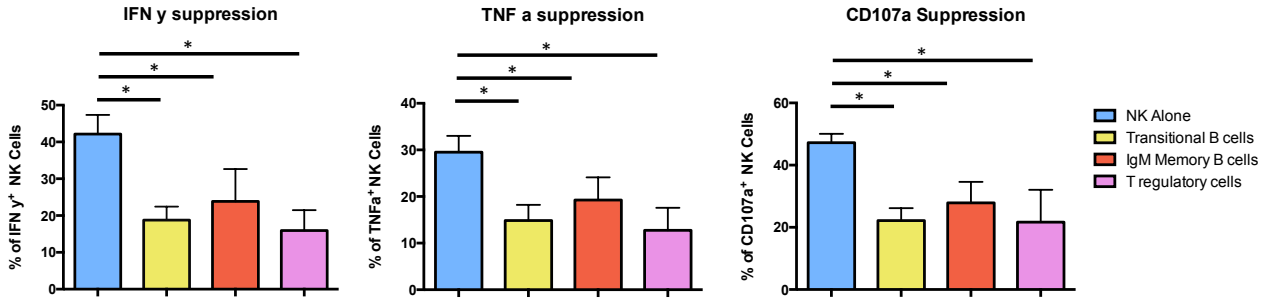


Figure V-5: The suppressive effect of transitional and IgM memory B cells on NK effector function was comparable to Tregs (n=4)
Bars represent median values and upper whisker of error bars represent the range. **P* < 0.05 by nonparametric ANOVA

V.2.2 Transitional and IgM memory B cells suppress proliferation of CD56+CD3- NK cells

IL-10 producing B regulatory cells have been shown to sharply suppress T cell proliferation (Khoder et al., 2014). To assess whether IL-10 enriched transitional and IgM memory B cell subsets exert a similar inhibitory effect on NK cell proliferation, I cultured magnetically purified allogeneic CFSE labelled NK cells alone or with sort purified B cell subsets in the presence of exogenous IL-2 (500U/ml) for 8 days (n=12) at a ratio of 1:1 as described in II.9.1. Transitional and IgM memory B cells suppressed NK cell proliferation [median percentages of proliferating NK cells, 40.5% (31.6-62.7%) and 48.6% (38.5-58.5%), respectively], when compared with NK cells cultured alone [median percentage of proliferating NK cells, 79.2% (69.8-89.1%)], or cultured with naïve nor switched memory B subsets **(figure V-6)**. I further compared the suppressive capacity of IgM memory and transitional B cells on NK proliferation with that of Tregs. At a 1:1 ratio, the inhibition of NK-cell proliferation by transitional IgM memory B cells was comparable to that of Tregs (**figure V-6**)**.** Together, these results show that transitional and IgM memory B cells share with Tregs a robust capacity to suppress proliferation of NK cells. Transitional and IgM memory B cells suppressed NK cell proliferation in a dose-dependent manner, with near complete abrogation of NK cell proliferation at a ratio of 10 B cells: 1 NK cells (**figure V-7**)**.**

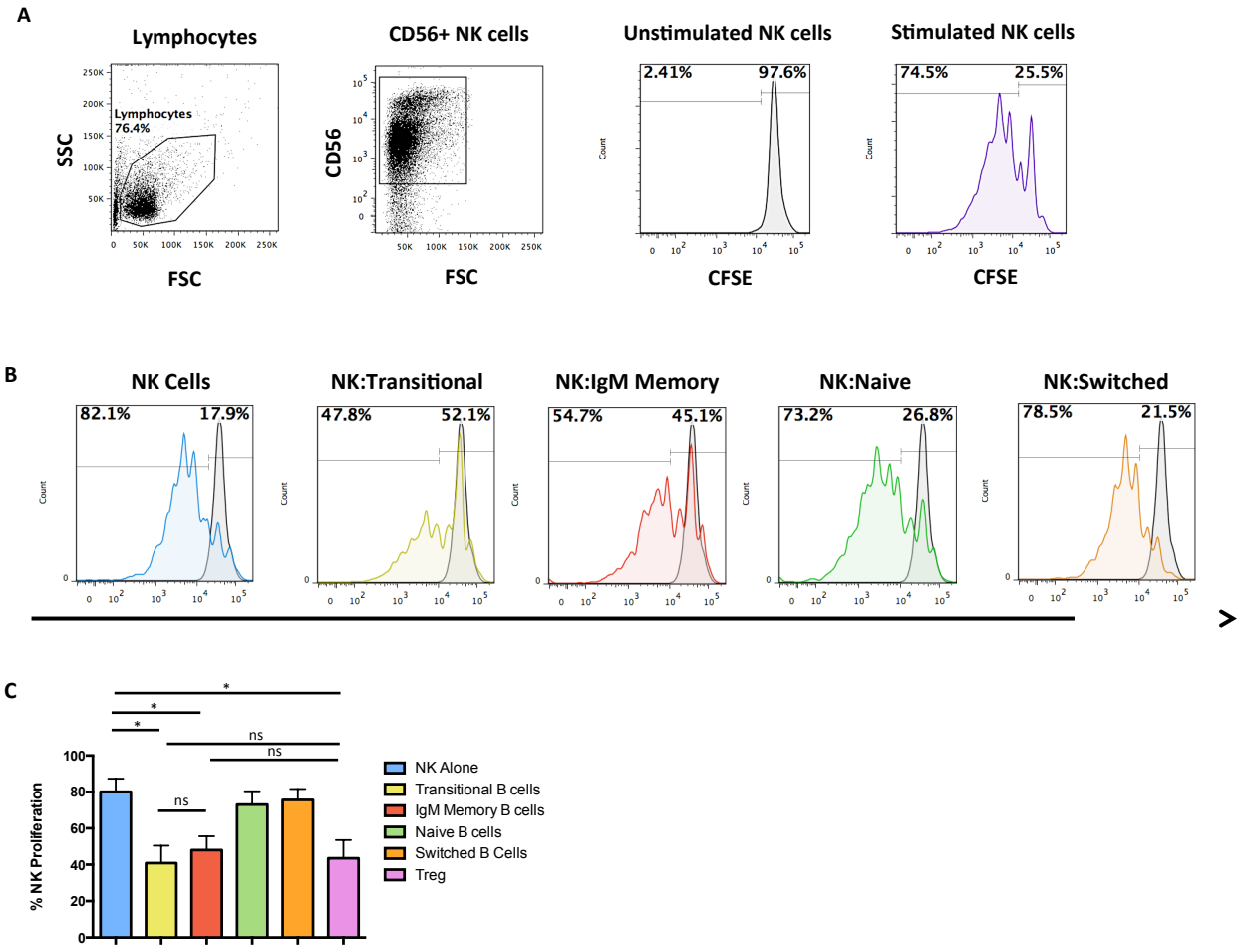


Figure V-6: Transitional and IgM memory B cells suppress NK proliferation
Sort purified B cell subsets were co-cultured with magnetically selected and CFSE stained NK cells at a 1:1 ratio in the presence of 500IU/mL of exogenous IL-2 for 8 days. **A**. Gating strategy illustrating i. lymphocyte gated population ii. CD56+ NK cells iii unstimulated CFSE-stained NK cells (negative control) iv. IL-2 stimulated NK cells (positive control) **B**. Representative histograms illustrating the proliferating CD56+ NK cells labeled with CFSE (eBioscience) plated either alone (blue, proliferation index 1.85) or at a 1:1 ratio with transitional (yellow, proliferation index 1.47), IgM memory (red, proliferation index 1.51), Naïve (green, proliferation index 1.97) or Switched memory B cells (orange, proliferation index 1.84). **C.** Cumulative data summarizing the suppressive effect of Breg subsets and autologous Treg on NK proliferation (n=8). Bars represent median values and upper whisker of error bars represents the range. *P < 0.05 by nonparametric ANOVA; ns, not significant

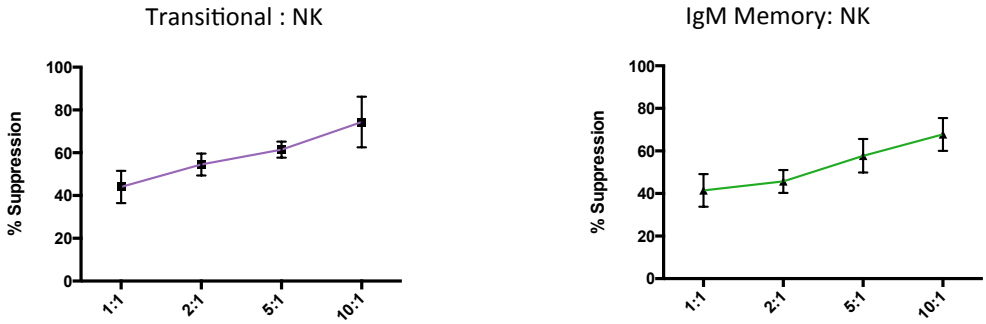


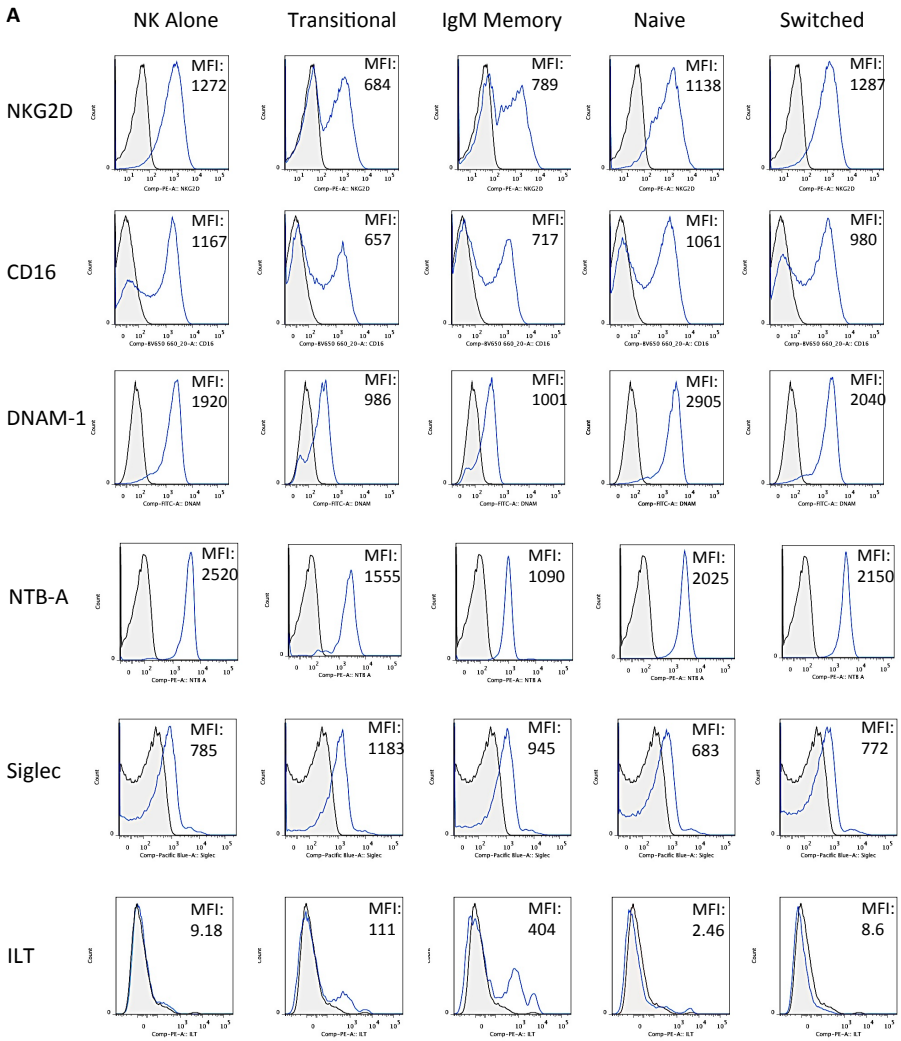
Figure V-7: The suppressive effect of transitional and IgM memory B cells on NK proliferation is cell dose dependent (n=4)
The figure reports mean values and error bars represent range.

V.2.3 Human IL-10 producing transitional and IgM memory B cell subsets modulate expression of NK co receptors and suppress ADCC

To investigate the underlying mechanisms through which transitional and IgM memory B cells suppress NK cell function, I next examined whether co-culture with these regulatory B cell subsets modulated the phenotypic expression of 11 inhibitory and activating receptors, as depicted in Table II-2, on the surface of NK cells (section II-9.3). Co-culture of healthy donor NK cells with transitional and IgM memory B cells, but not naïve or switched memory B cells, resulted in downregulation of NKG2D, CD16, DNAM-1, and NTB-A expression on the surface of NK cells (**figure V-8**). The expression of other activating molecules did not vary following co-culture with B cell subsets. In contrast, 2 inhibitory receptors, ILT and Siglec-7, were upregulated on the surface of NK cells co-cultured with transitional and IgM memory B cells but not naïve or switched memory B cell subsets. I did not find a difference in NKG2A or KIR expression. Taken together, these results indicate that defects in NK cell phenotype modulated via Bregs may contribute to the development and persistence of the disease in patients with AML.

CD16 is transmembrane protein that induces a potent series of signals resulting in cytokine production and cytotoxic effector activity via ADCC.

These results suggest that Breg subsets may suppress NK mediated ADCC. To determine whether transitional and IgM memory B cells suppress IFN-y, TNF-a and CD107a production induced by CD16 activation, magnetically selected CD56+CD3- human NK cells were activated with plate bound anti-CD16 mAB for 5 hours alone or with sort purified B cell subsets at a 1:1 (B cell: NK cell) ratio as described in section II.9.4. Breg-NK co-culture resulted in significant suppression in IFN-y and TNF-a expression, and CD107a degranulation by NK cells following CD16 activation, when compared to the corresponding positive control (**figure V-9).** These results highlight the role of transitional and IgM memory B cell subsets as potent suppressor cells of NK activity and ADCC**.**



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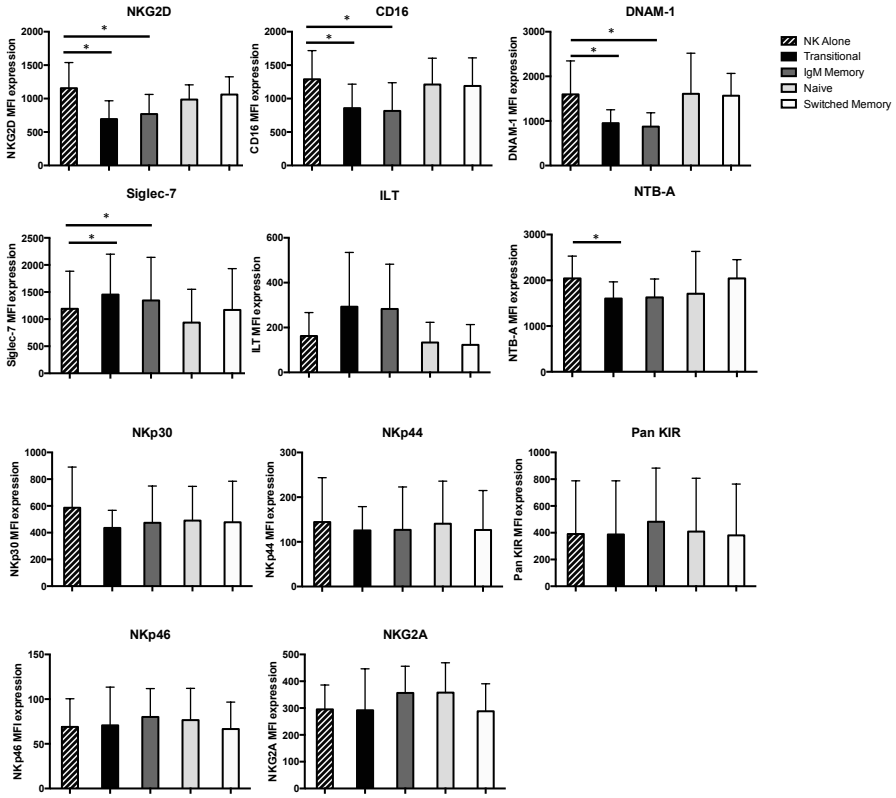

Figure V-8: Transitional and IgM memory B cells modulate NK phenotype
A. Representative histograms were generated using FlowJo and gates for each marker (blue) was determined by corresponding FMO (shaded grey) with calculated MFI to determine the effect of NK: B co-culture **B**. cumulative data (n=8) illustrating the changes in NK phenotype when cultured alone or with B cell subsets. Bars represent median values and upper whisker of error bars represents the range. *$P <$ 0.05 by nonparametric ANOVA

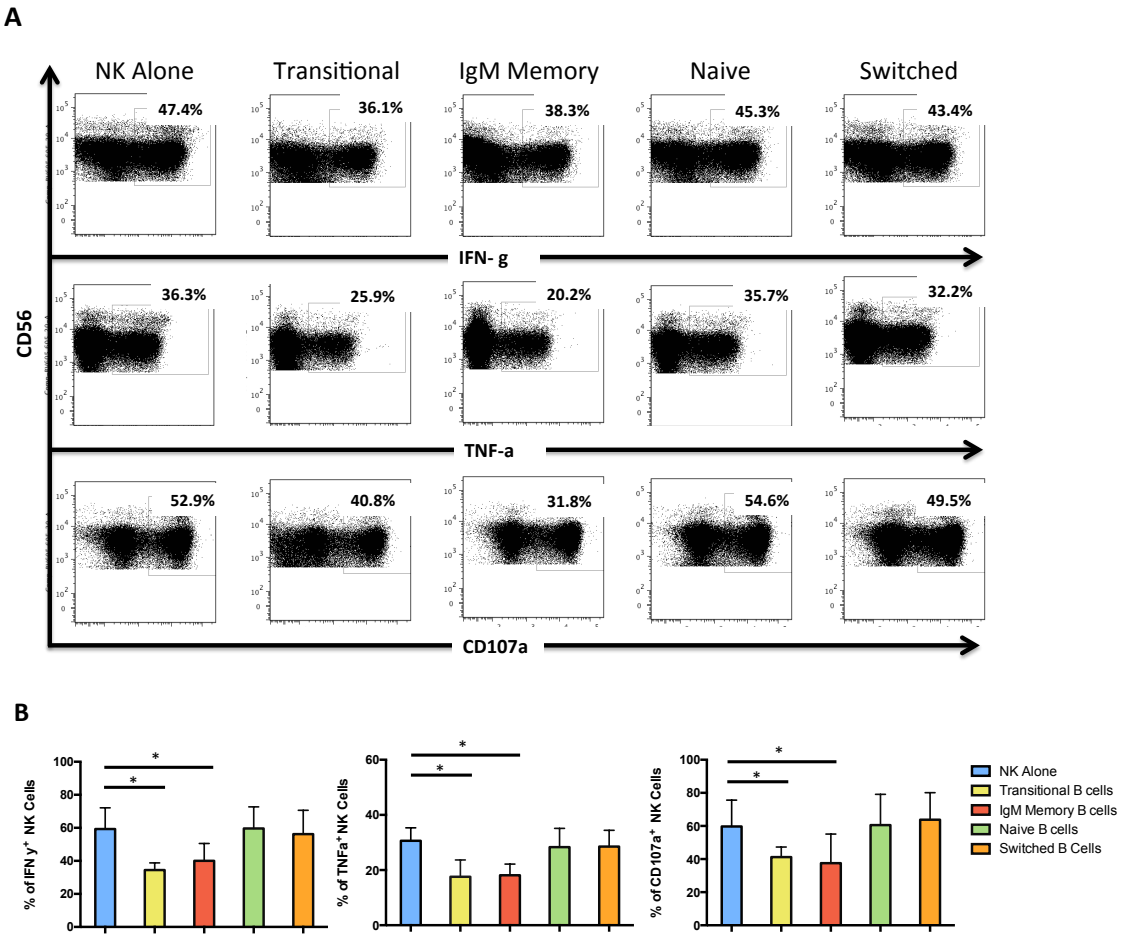

Figure V-9: Transitional and IgM memory B cells suppress NK mediated ADCC. NK cells were activated with plate bound anti CD16 mAB either alone or with sort purified B cell subsets. After culture, cells were collected and stained with corresponding antibodies to assess the frequencies of TNFa, IFN-g and CD107a. **A.** Dot plots were generated by gating on lymphocyte population then live cells, then CD56+CD3- NK cells (n=3) **B**. Cumulative data illustrating Transitional and IgM Memory B cells suppress NK mediated ADCC. (n=3). Bars represent median values and upper whisker of error bars represents the range. *$P < 0.05$ by nonparametric ANOVA

V.2.4 IL-10-producing transitional and IgM Memory B cells suppress NK cell function via both IL-10 dependent and cell-cell contact-mediated mechanisms

Given the reported ability of transitional and IgM memory B cells to suppress T cell immune effector responses through IL-10 (Blair et al., 2010, Mauri et al., 2013, Khoder et al., 2014) I next examined whether IL-10 produced by these B cell subsets with regulatory capacity was responsible for the suppression of NK cytotoxicity and proliferation. To determine if IgM memory and transitional

B cells suppress NK cell proliferation and cytotoxicity though IL-10, magnetically isolated NK cells were cultured with sort purified IgM memory or transitional B cells at a 1:1 ratio in the presence or absence of mAbs against IL-10 and IL-10 receptor (IL-10R) as described in section II.9.5. IL-10 blockade only partially restored NK effector when co-cultured with transitional or IgM memory B cell subsets as observed through intracellular cytokine staining and ELISA by harvesting the supernatant after B cell and NK cell culture (**figure V-10A-C).**

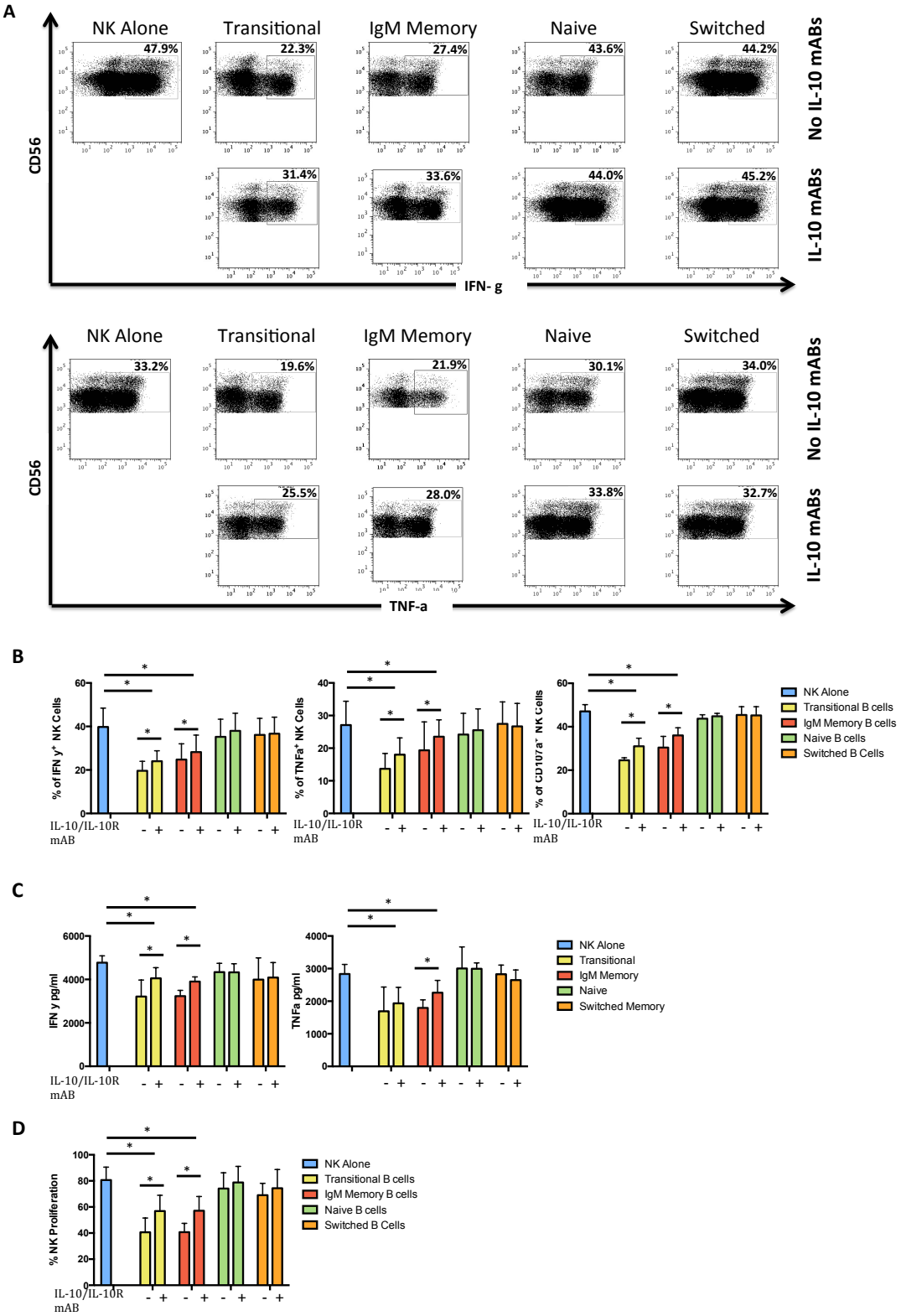

Figure V-10: The suppressive ability of B cells on NK function is partially reversed upon IL-10mAB blockade

A. Dot plots of IFNγ and TNFa expression by CD56+ NK cells cultured alone (positive control) or in the presence of indicated B cell subsets with and without IL10 blocking antibodies. **B**. Bar charts compare between frequencies of IFNγ, TNFa and $CD107a^{+}$ CD56+ NK cells. The results were compared with paired t test analysis, $^{*}p < 0.05$. (n=3) **C**. Bar charts compare representing IFNγ and TNFa production by NK cells with or without B cell co-culture and IL-10 blockade through ELISA (n=3) **D**.IL-10 blocking partially reverses the inhibitory effect of Breg subsets on NK proliferation. (n=4). In **B-D**, bars represent median values and upper whisker of error bars represents the range. $^{*}P < 0.05$ by nonparametric ANOVA

Further, IL-10 blockade did not fully reverse the suppressive capacity of either transitional or IgM memory B cell subsets on NK cell proliferation [median percentages of proliferating NK cells, 52.7% (47.4-70.5%) and 60.4% (50.5-66.4%), respectively; (n=4) compared with 81.9% (71.2-88.1%) for the positive control] **(figure V-10D)**. Addition of exogenous IL-10 to NK cells cultured alone induced marginal suppression of NK cell cytolytic function (**figure V-11**), but this effect was substantially less than that seen when NK cells are cultured with transitional or IgM memory B cells. Taken together, these data suggest that the immunoregulatory properties of transitional and IgM memory B cells on NK cell function are likely mediated through mechanisms other than IL-10.

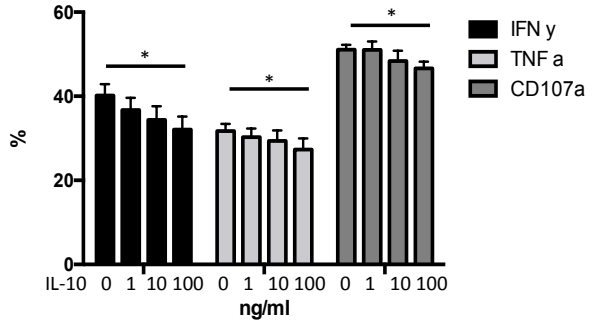

Figure V-11: Addition of exogenous IL-10 to NK cells induced marginal suppression of NK effector function when assessed by intracellular staining for of IFNγ, TNFa and CD107a^{+}
Bars represent median values and upper whisker of error bars represents the range. *P < 0.05 by nonparametric ANOVA and comparisons were made between each concentration of IL-10 ng/ml.

To pursue the notion that TGF-β might mediate at least part of the immunoregulatory effects of Bregs on NK cells, we performed additional blocking experiments using TGF-β-specific mAbs. TGF-β blockade had no significant impact on the suppression of NK effector function by either transitional or IgM memory B cells (p=0.46 and p=0.86, respectively; n=3),

indicating that TGF-β lacks any significant role in human Breg-mediated inhibition of NK cell effector function (**figure V-12**).

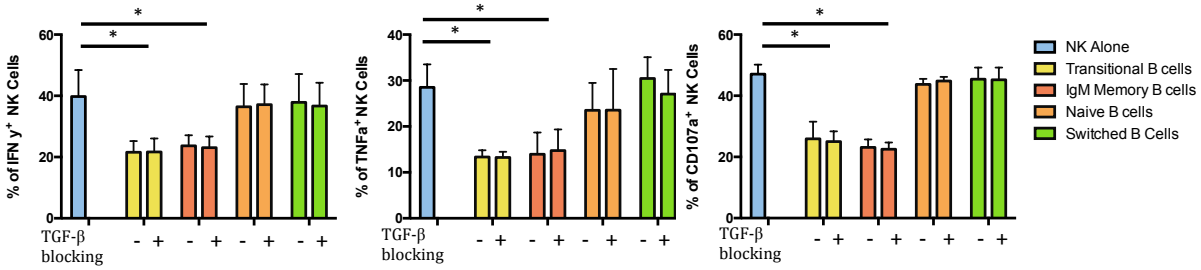

Figure V-12: The suppressive capacity of transitional and IgM memory regulatory B cell subsets is independent of TGF-β
Bars represent median values and upper whiskers of error bars indicate the ranges (n=3). *P < 0.05 by nonparametric ANOVA

A dynamic, bi-directional interaction exists between NK cells and CD19+ B cells (Yuan et al., 2010, Gao et al., 2006). However, to date, no studies have assessed the interaction between Bregs and NK cells. To examine if the suppressive effect of Bregs on NK cells requires direct cell-to-cell interaction, I cultured purified CD56+CD3- NK cells either alone or at a 1:1 ratio with sorted IgM memory or transitional B cells either in direct contact or separated in a transwell by a permeable membrane as described in section II.9.6. The proliferation of K562-stimulated and CFSE-stained CD56+CD3- NK cells was measured 8 days after the culture was initiated. Separation of transitional and IgM memory B cells from K562-activated CD56+ NK cells by a transwell membrane reversed their suppressive effect on NK effector function nearly completely when compared with NK cells co-cultured in direct contact with these B cell subsets when assessed by both intracellular staining through flow cytometry and ELISA assay performed on supernatants harvested from NK/B cell co-cultures (**figure V-13A-B**). Concurring with my findings, abrogation of

cell-to-cell contact by a transwell assay also significantly reversed the suppressive effect of transitional and IgM memory B cells on NK cell proliferation (**figure V-13C).** These results indicate that the suppressive effect of transitional and IgM memory B cells on NK cell proliferation and effector function is mediated mainly by direct cell-to-cell contact.

I next determined if a combination of IL-10 blockade and abrogation of direct cell-cell contact could completely reverse the suppressive effect of candidate regulatory B cells on CD56+ NK-cell proliferation and cytolytic function. Whereas IL-10 blockade alone did not fully reverse the suppressive effect of either sort-purified transitional or IgM memory B cells on the proliferation or cytotoxic function of CD56+CD3- NK cells, the addition of IL-10 and IL-10R Abs in a transwell setting completely abolished the suppressive effect of these B cell subsets on NK-cell proliferation and cytotoxic function (**figure V-14)**. These results suggest that both IL-10 and cell-to-cell contact are pre-requisites for regulatory B cells to achieve their full suppressive potential.

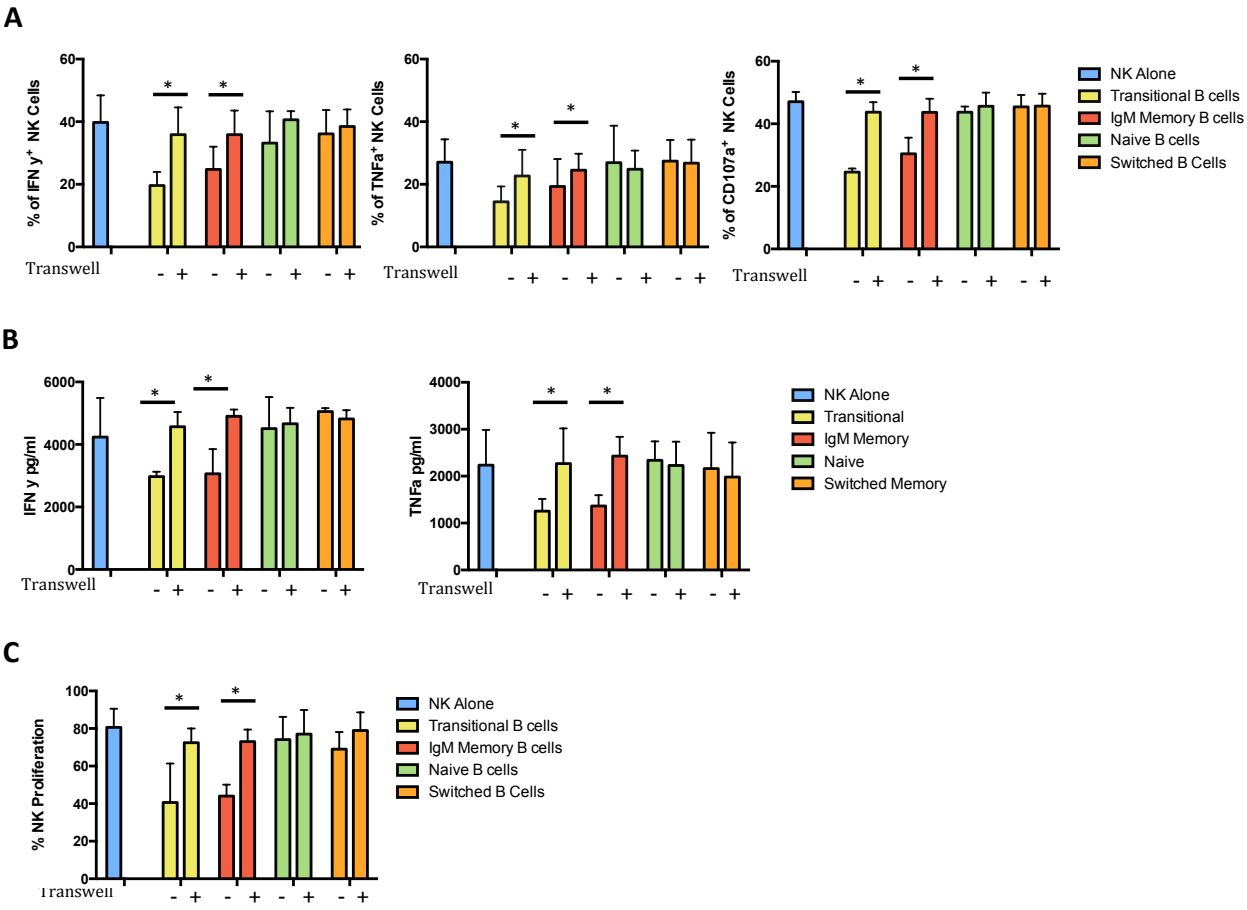

Figure V-13: Cell to Cell contact is required for the inhibitory effect of Bregs on NK cell function

A. Bar graphs illustrate the suppressive ability of B cells on NK effector function was reversed upon abrogation of cell-to-cell contact by a permeable membrane in a transwell assay. Sort purified B cell subsets were co-cultured with NK cells at a ratio of 1:1. Regulatory B cell subsets appeared to suppress frequencies of NK IFNy, TNFa and CD107a production at cell-cell contact but not in the absence of contact. (n=4) **B**. Bar graphs depicting results from supernatants that were collected from NK-B co-cultures in a transwell setting and measured by ELISA assay for TNFa and IFNy (pg/ml) production after 48 hours of co-culture. Data is representative of 3 experiments **C.** Negatively selected CFSE stained CD56+CD3- NK cells were co-cultured with sort purified with B cell subsets either directly or in the presence of a permeable membrane in a transwell assay for 48 hours. In the absence of cellular contact B cell inhibitory effect on NK proliferation was reversed significantly. (n=4) In **A-C,** bars represent median values and upper whiskers of error bars indicate the ranges. *$P < 0.05$ by nonparametric ANOVA

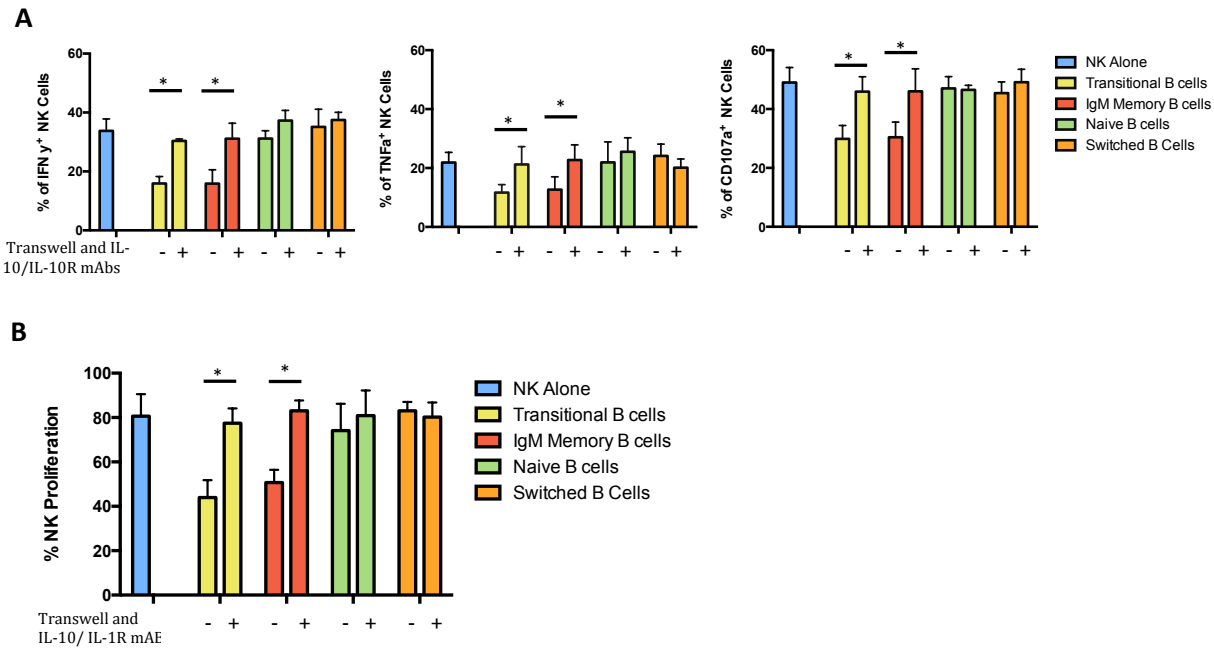

Figure V-14: Both IL-10 and Cell to Cell contact are required for the full potential of Breg suppression on NK cell function
A. Bar graphs illustrate the suppressive ability of B cells on NK effector function was significantly reversed upon abrogation of cell-to-cell contact and IL-10 suggesting that both IL-10 and cell-to-cell contact are contributors to the full potential of Breg suppression (n=3) **B**. Bar graphs depicting the absence of IL-10 and cell-to-cell contact inhibited the suppressive function of Breg on NK proliferation (n=3). In **A and B**, bars represent median values and upper whiskers of error bars indicate the ranges. *$P < 0.05$ by nonparametric ANOVA

V.2.5 Breg-NK cell cross-talk is mediated through the 2B4-CD48 axis

Previous studies have shown that 2B4, a co-receptor on NK cells, interacts with CD48, widely expressed on hematopoietic cells, to generate bidirectional signals and cross-talk (Yuan et al., 2010, Gao et al., 2006, Messmer et al., 2006, Mathew et al., 2005, Assarsson et al., 2004). Recent murine and human studies suggest that 2B4 can also function as an inhibitory rather than an activating co-stimulatory receptor when engaged by CD48-expressing tumor targets (Lee et al., 2006). I therefore next investigated whether CD48 on transitional and IgM memory B cells can act as an inhibitory receptor upon interaction with 2B4 on NK cells.

To test the contribution of 2B4 and CD48 co-stimulatory signaling to the suppressive capacity of sort-purified transitional or IgM memory B cell subsets, blocking antibodies against CD48 and 2B4 molecules were added to co-cultures of B cell subsets and NK cells as described in section II.9.5 to study the interaction of Breg-NK interaction through the 2B4 and CD48 axis. Addition of CD48 and 2B4 molecules to co-cultures of sort purified B cell subsets and magnetically selected NK cells nearly completely reversed the suppressive effect of transitional and IgM memory B cells on NK cell proliferation and cytotoxic function (**figure V-15**). Thus, the suppressive effect of human Breg cells on PB-NK function is mediated primarily by 2B4/CD48 co-stimulatory signaling. Further, whereas IL-10 blockade alone did not fully reverse the suppressive effect of either sort-purified transitional or IgM memory B cells on the proliferation or cytotoxic function of CD56+CD3- NK cells (V.2.4), the addition of IL-10 and IL-10R to CD48 and 2B4 blocking completely abolished the suppressive effect of these B cell subsets on NK-cell proliferation and cytotoxic function (**figure V-16)**, which in accord with my transwell with IL-10 blocking assays as described previously.

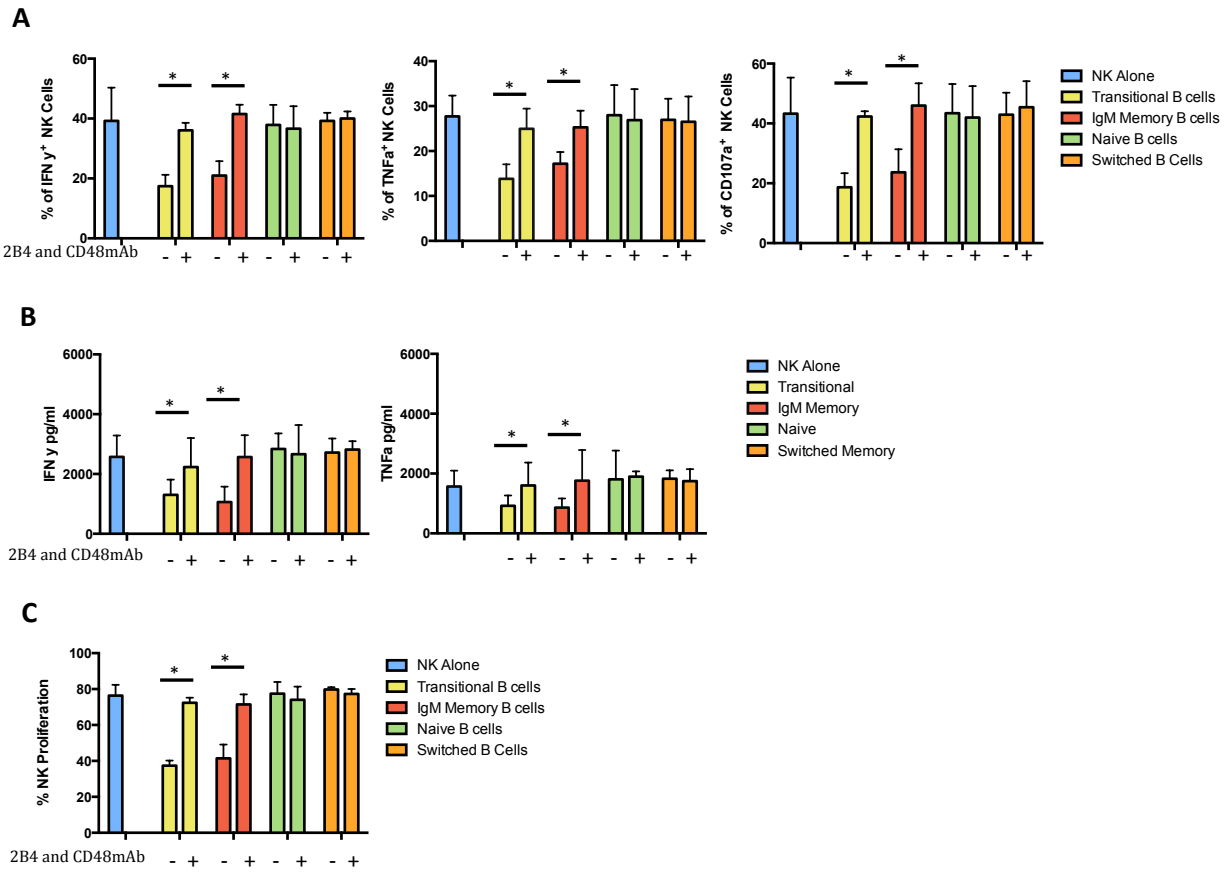

Figure V-15: The suppressive ability of B cells on NK effector function is mediated by 2B4 and CD48 interaction

A. Blocking antibodies against CD48 and 2B4 were added to B cell and NK cell co-cultures at a 1:1 ratio to prevent the interaction between 2B4 and CD48. Frequencies of CD107a+, TNFa+ and IFNy+ CD56+CD3- NK cells was assessed. (n=4). **B.** Supernatants were collected from NK-B co-cultures to measure the presence of TNFa and IFNy with the blocking of 2B4 and CD48 (n=4). **C.** Blocking antibodies against CD48 and 2B4 were added to B cell and NK cell co-cultures at a 1:1 ratio to prevent the interaction between 2B4 and CD48. Frequencies CFSE+ CD56+CD3- NK cells was assessed. (n=3). In **A-C**, bars represent median values and upper whiskers of errors bars indicate the ranges. *P < 0.05 by nonparametric ANOVA

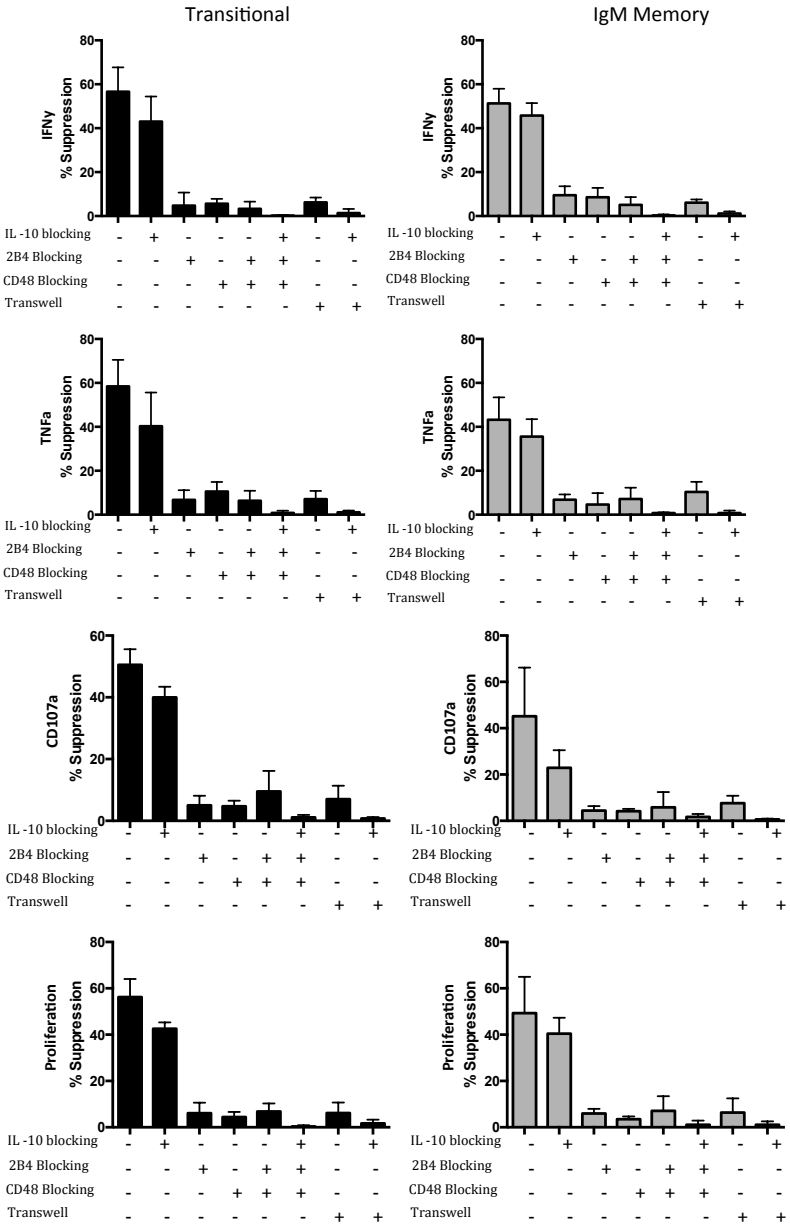

Figure V-16: Cell-to Cell contact and IL-10 are both required for the full capacity of suppression by Transitional and IgM Memory B cells on NK cytotoxic function and proliferation

Percentage suppression was calculated for NK+IFNy+ cells, NK+TNFa+ cells, NK+CD107a+ and CFSE+NK+ cells in the presence of IL-10 blocking, 2B4 blocking, CD48 blocking and transwell. Data shows that a combination of IL-10 blocking and abrogation of cell to cell contact reversed the suppressive effect of transitional and IgM memory B cell subsets. Bars represent median values and upper whiskers of error bars indicate the ranges from 4 independent experiments.

V.2.6 Reduced expression of SAP and upregulation of pSHP-1 mediates B regulatory cell suppression of NK cells through CD48 and 2B4 interaction

Triggering of 2B4 (CD244) on the surface of NK cells can induce NK–cell activation, co-stimulation, or even inhibition (Morandi et al., 2005, Eissman et al., 2005, Endt et al., 2007). The signaling lymphocyte activation molecule–associated protein (SAP) can bind to all 4 immuno-receptor tyrosine-based switch motifs (ITSMs) of 2B4. The phosphorylated ITSM can additionally recruit the phosphatases SHP-1, SHP-2 and SHIP. SAP can also act as an inhibitor of interactions between 2B4 and these inhibitory molecules.

To test the contribution of 2B4 and CD48 co-stimulatory signaling to the suppressive capacity of sort-purified transitional or IgM memory B cell subsets, I co-cultured NK cells with sort purified B cells subsets (naïve, switched memory, IgM memory and transitional) at a 1:1 ratio for 48 hours as described in section II.10.2, to determine the inhibitory effect of transitional and IgM memory B regulatory subsets on the expression of SAP+NK+ cells through CD48 and 2B4 signalling. Co-culture of NK cells with both transitional and IgM Memory B cells results in downregulation of the 2B4-activated SAP in NK cells [median percentages of NK+SAP+ cells, 52.7% (47.4-70.5%) and 60.4% (50.5-66.4%), respectively; (n=4)] compared to 97.5% (94.9-98.8%)] in NK cells cultured alone (**figure V-17).** These results highlight an important mechanism of Breg suppression and indicate that CD48 on the surface of Bregs block 2B4-mediated NK cell activation by reducing expression of the 2B4-associated SAP molecule, which in turn contributes to inhibition of NK function.

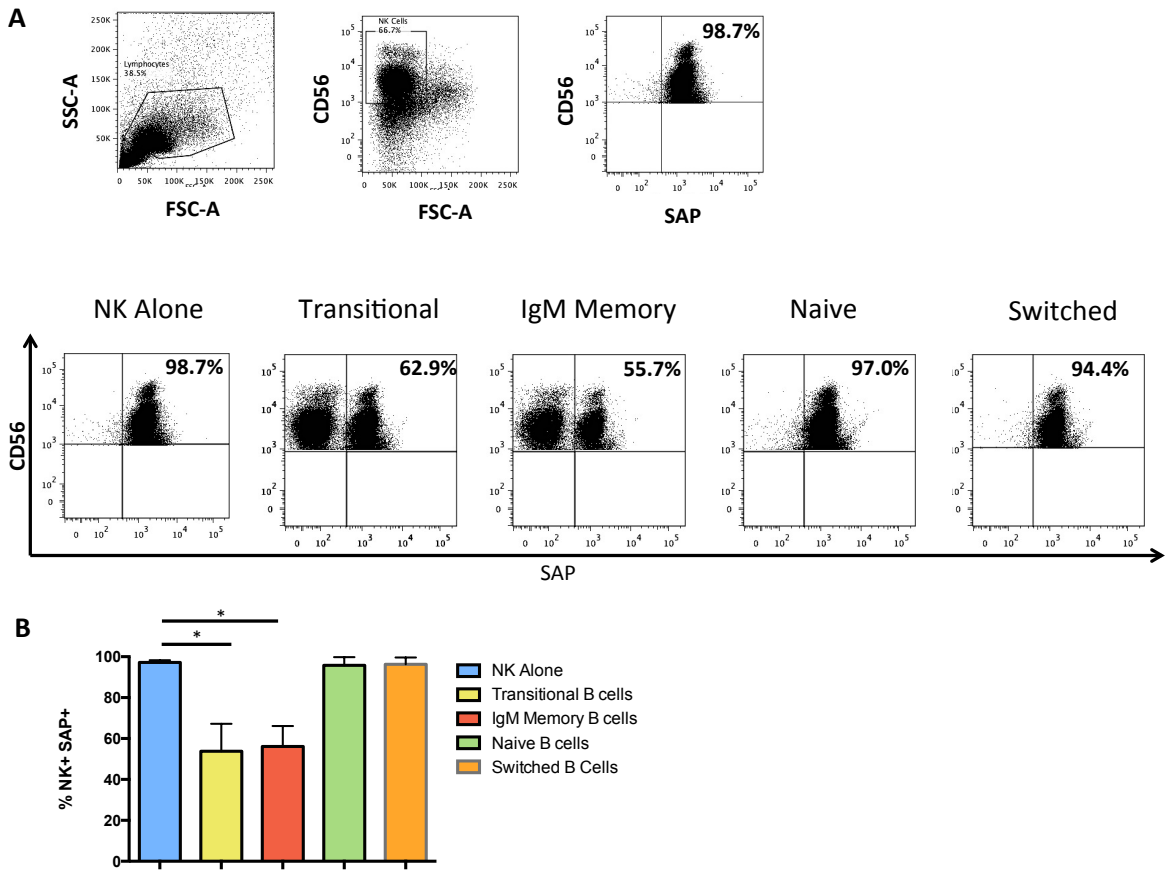

Figure V-17: Co-culture of NK cells with both transitional and IgM Memory B cells results in downregulation of the 2B4-activated SAP in NK cells
A. Gating strategy illustrating CD56bright+SAP+ NK cells. Magnetically selected CD3-CD56+ NK cells were cultured either alone or with sort purified B cell subsets: naïve, switched, transitional and IgM memory for 48 hours. Following incubation, cells were collected and stained for CD56+SAP+ NK cells. Cells were gated on lymphocytes, CD56+bright NK cells and CD56bright+SAP+ NK cells. **B.** Transitional and IgM Memory B cells downregulated the expression of SAP+NK+ cells when co-cultured with NK cells at 1:1 cell ratio. Data is representative of 3 independent experiments. Bars represent median values and upper whiskers of error bars indicate the ranges. *$P < 0.05$ by nonparametric ANOVA

To further dissect how Bregs may be modulating the expression of NK+SAP+ NK cells I first determined the impact of IL-10 on SAP expression, as both transitional and IgM memory B cells are enriched in IL-10 producing cells. NK cells were cultured with IgM memory or transitional B cells at a 1:1 ratio in the presence or absence of mAbs against IL-10 and IL-10 receptor (IL-10R). IL-10 blockade had minimal effect on restoring SAP expression on NK cells when co-cultured with transitional or IgM memory B cell subsets (**figure V-**

18A). Addition of exogenous IL-10 to NK cells cultured alone induced marginal suppression of SAP expression on NK cells (**figure V-18B**). These data suggest that IL-10 by Bregs does not play a significant role modulating SAP expression in NK cells.

To determine if cell-cell contact between Breg subsets and NK cells is required for downregulation of SAP expression by NK cells, NK-B cell interaction was prevented either by a transwell system through a permeable membrane or by the addition of blocking antibodies against CD48 and 2B4 molecules, either separately or together, as described in section II.10.2. Prevention of NK-B cell interaction by a transwell membrane or by addition of CD48 and/or 2B4 molecules to co-cultures completely reversed NK cell SAP downregulation by transitional and IgM memory B cells (**figure 19**). My results highlight an important role for 2B4-CD48 interaction in inducing downregulation of the activating molecule SAP, thereby facilitating interactions of negative regulatory molecules, such as SHP-1 with 2B4. Hence, the suppressive effect of transitional and IgM memory B cell subsets may be mediated by through 2B4-CD48 interaction, which induces inhibitory signalling through 2B4 by down regulation of SAP.

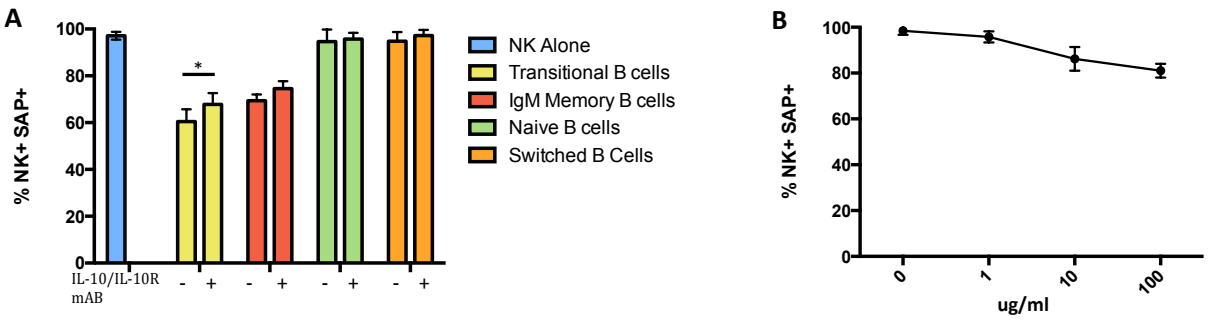

Figure V-18: IL-10 had minimal contribution to SAP expression on NK cells when co-cultured with transitional or IgM memory B cell subsets
A cumulative data representing the effect of IL-10 blockade on restoring suppressed SAP expression by transitional and IgM memory B cell subsets (n=3). Bars represent median values and upper whiskers of error bars indicate the ranges. *$P < 0.05$ by nonparametric ANOVA **B**. Addition of exogenous IL-10 had minimal impact on SAP expression in CD56+ NK cells (n=3). The figure represents median values with range. No statistically significant difference was found between different individual groups by nonparametric ANOVA.

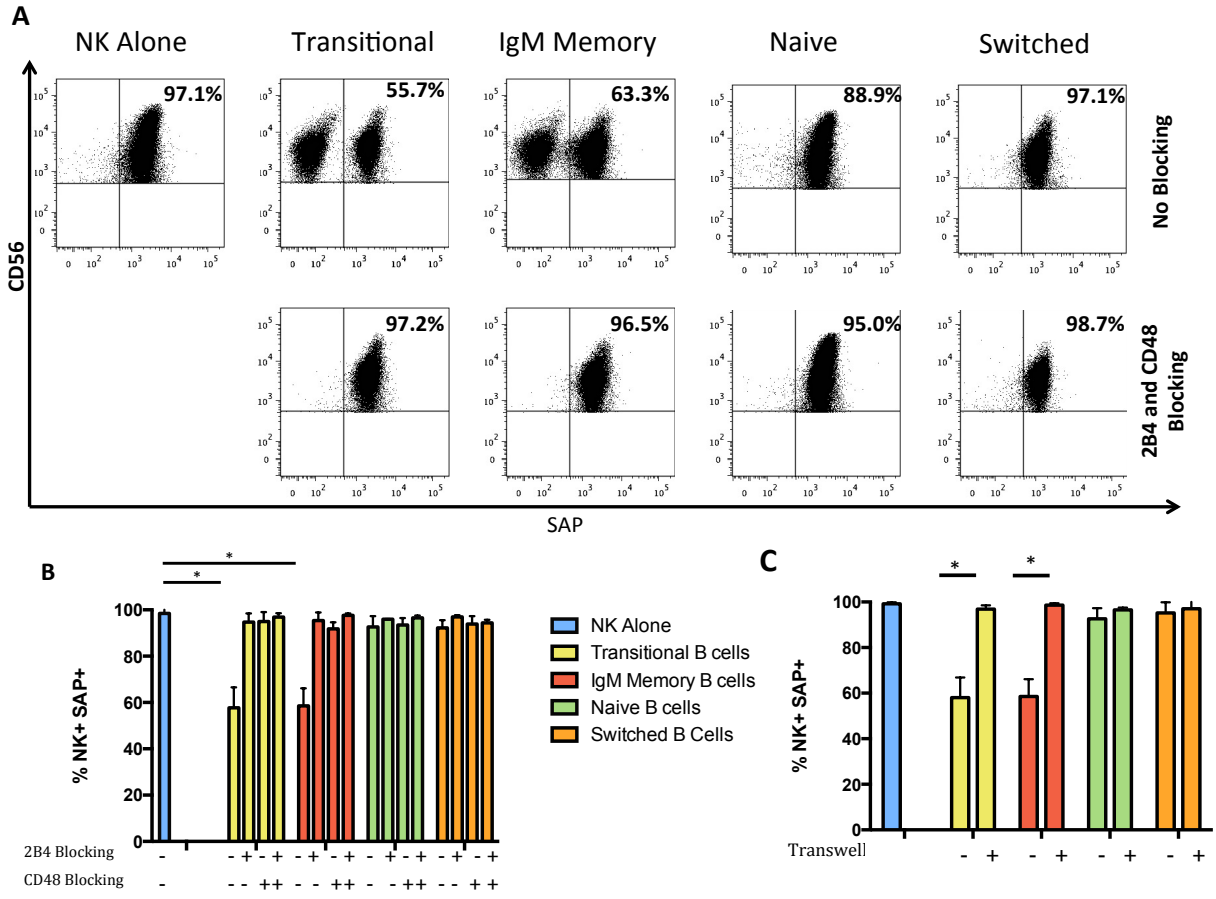

Figure V-19: Prevention of NK-B cell cell-to-cell interaction completely reversed NK cell SAP downregulation by transitional and IgM memory B cells
A. Dot plots representing frequencies of NK+SAP+ expression with 2B4 and CD48 blocking which reverses the downregulation SAP by transitional and IgM memory B cells when compared with NK cells cultured with naïve or switched memory B cell subsets or NK cells cultured alone (positive control) **B** Bar graphs representing negatively selected CD3-CD56+ NK cells were cultured at a 1:1 ratio with sort purified B cell subsets for 48

hours in the presence of 2B4 and CD48 mAbs. In the absence of 2B4 and CD48 interaction, the inhibitory activity of Bregs was reversed (n=3) **C**. In the absence of NK-B cellular contact by a transwell membrane, inhibitory effect of transitional and IgM Memory B cells on SAP expression on NK cells was reversed. N=4. In **B and C**, bars represent median values and upper whiskers of error bars indicate the ranges. *$P < 0.05$ by nonparametric ANOVA

To test if 2B4-CD48 interaction induces expression of the inhibitory molecule pSHP-1 in NK cells, sort purified transitional and IgM memory B cell subsets were co-cultured with NK cells for 48 hours and expression of pSHP-1 in NK cells was examined using the phosphoflow assay as described in section II.10.3. NK cells cultured either alone or with naïve or switched memory B cell subsets did not express pSHP-1, however NK cells cultured with transitional and IgM memory B cells upregulated pSHP-1 expression [**figure V-20].** These results support my hypothesis that Breg mediated suppression of NK cells is dependent on 2B4-CD48 interaction by inducing downregulation of the activating molecule SAP, and thereby facilitating interactions of negative regulatory molecules, such as SHP-1 with 2B4.

To test the contribution of IL-10 by Bregs to upregulation of pSHP-1 in NK cells, IgM memory or transitional B cells were cultured with NK cells at a 1:1 ratio in the presence or absence of mAbs against IL-10 and IL-10 receptor (IL-10R). IL-10 blockade had minimal effect on pSHP-1 expression in NK cells (**figure V-21A).** Addition of exogenous IL-10 to NK cells cultured alone induced marginal expression of pSHP-1 on NK cells (**figure V-21B**).

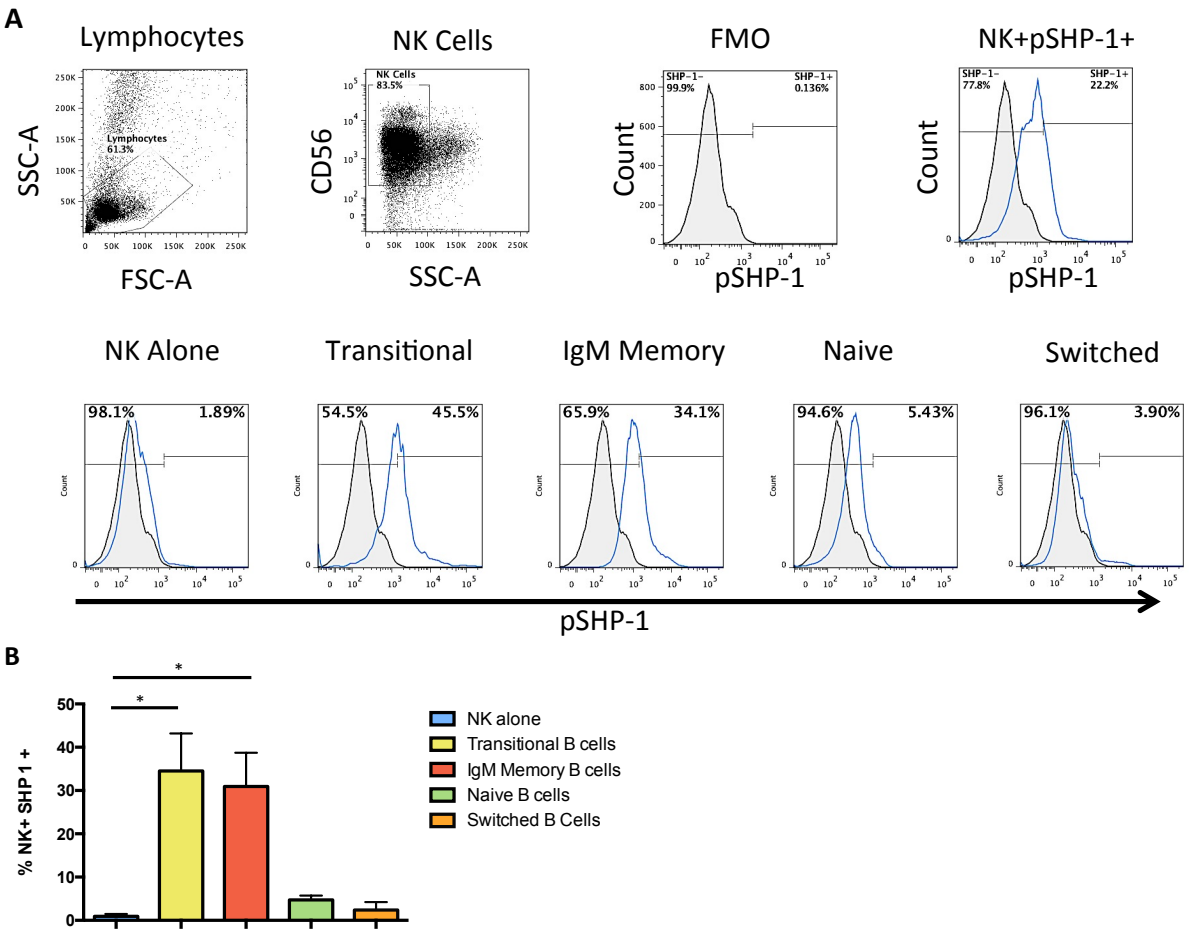

Figure V-20: NK cells cultured with transitional and IgM memory B cells upregulated pSHP-1 expression

A. Sort purified transitional and IgM Memory B cells upregulate the expression of pSHP-1. Magnetically selected CD56+CD3- NK cells were co-cultured with sort purified B cell subsets at a 1:1 ratio for 48 hours after which cells were collected and tested for the presence of pSHP-1 by phosflow assay kit (Beckman Coulter). Cells were gated on lymphocytes population and then CD56+NK cells. CD56+SHP-1+ cells (blue) were determined by SHP-1 Alexa fl 400 FMO (shaded grey). Transitional and IgM memory B cells up regulated the expression of SHP-1. **B** Bar graphs illustrate NK co-cultured with transitional or IgM memory B cells upregulated the expression of pSHP-1, inhibitory downstream protein of 2B4 signalling pathway (n=6). Bars represent median values and upper whiskers of error bars indicate the ranges. *$P < 0.05$ by nonparametric ANOVA

Next, in order to determine if CD48-2B4 interaction induces pSHP-1 expression in NK cells, NK-B cell interaction was prevented by transwell or with the addition of blocking antibodies against CD48 and 2B4 molecules. Addition of CD48 and 2B4 molecules to co-cultures or prevention of cell-cell contact using a transwell device completely reversed upregulation of pSHP-1 in NK cells by transitional and IgM memory B cells (**figure V-22**).

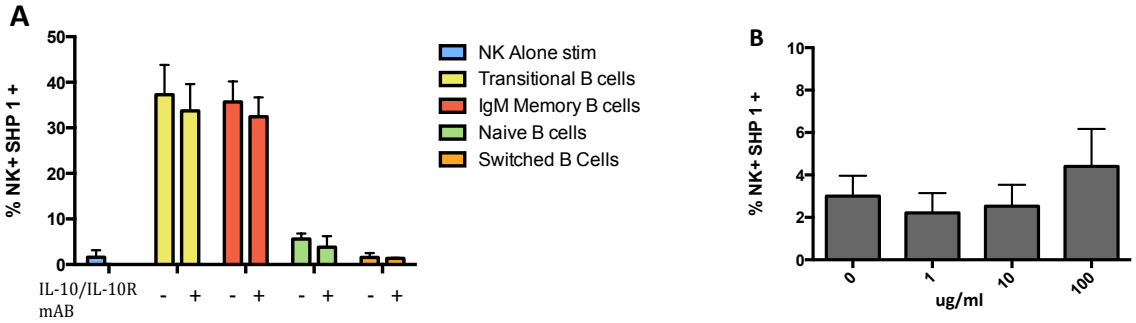

Figure V-21: IL-10 had minimal contribution to pSHP-1 expression on NK cells when co-cultured with transitional or IgM memory B cell subsets
A. Bar graphs illustrate IL-10 blocking alone had minimal effect on the expression of pSHP-1 in NK co-cultures with transitional and IgM memory B cells (n=3) **B** The effect of varying concentrations of adding exogenous IL-10 had no significant effect on the expression of pSHP-1 on CD56+CD3- NK cells (n=3). Both **A and B**, the bars represent median values and upper whisker of error bars indicate the range. No significant differences were found by nonparametric ANOVA.

In order to determine the relation between pSHP-1 and SAP expression I studied a time course and found that as regulatory B cell subsets down-regulates SAP expression, pSHP-1 expression is augmented in a time dependent manner (**figure V-23**). Thus, the suppressive effect of human Breg cells involves not only the release of IL-10 and cell-cell contact, but also mediation of these factors by 2B4/CD48 co-stimulatory signaling.

Taken together, these data support my hypothesis that Breg subsets inhibit NK cell activation primarily through direct cell-cell contacted mediated via CD48-2B4 interactions, by downregulating expression of the activating molecule SAP and upregulating expression of the inhibitory molecule pSHP-1.

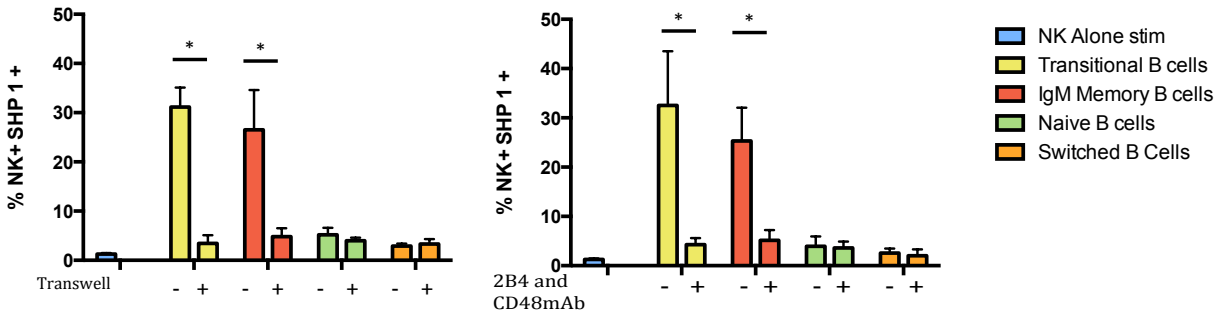

Figure V-22: In the absence of NK-B cellular contact via 2B4 and CD48 blocking (right) and transwell (left) assays inhibitory effect of Bregs on SHP-1 expression was reversed

Data is representative of 3 independent experiments and bars represent median values and upper whiskers of error bars indicate the ranges. *P < 0.05 by nonparametric ANOVA

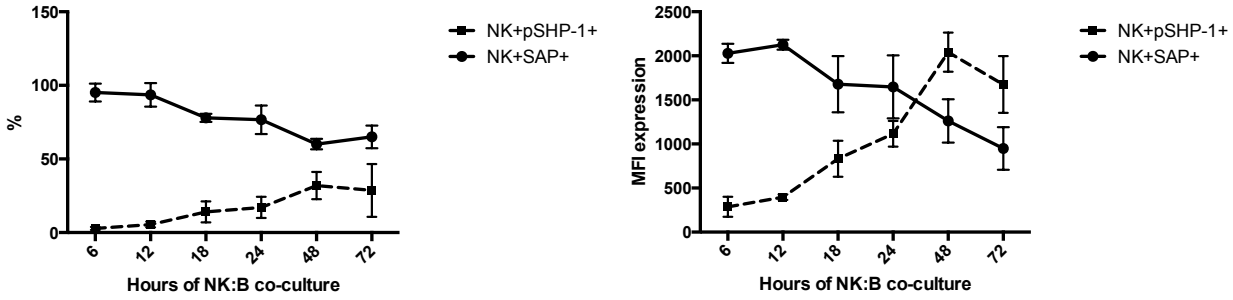

Figure V-23: Time kinetics of SAP and pSHP-1 expression on NK cells highlighting the unique relationship between both the frequency (left) and mean fluorescence intensity (MFI) (right) of negative regulatory molecule pSHP-1 expression and downregulation of activating 2B4-associated SAP molecule (n=4). The figures represent mean values and error bars represent range.

V.2.7 2B4 and CD48 mediated NK-B cell interaction activates JAK/STAT3 pathway in transitional and IgM Memory regulatory B cell subsets

Next I investigated the bi-directional cross-talk between NK and regulatory B cell subsets. I hypothesized that Breg mediated suppression of NK cells may be a consequence of 2B4 and CD48 mediated NK-B cell interaction, which activates JAK/STAT3 pathway in transitional and IgM Memory regulatory B cell subsets, activating the regulatory function of the B cell subsets. Greater production of IL-10 was observed in NK-B cell co-culture than resting B cells alone (**figure V-24).** This suggests that cell-to-cell contact between NK cells and B cells may perhaps activate B cells to regulate their IL-10 mediated suppressive function. We next examined if this phenomenon was mediated through phosphorylation of the STAT3 pathway (p-STAT3) in Breg subsets.

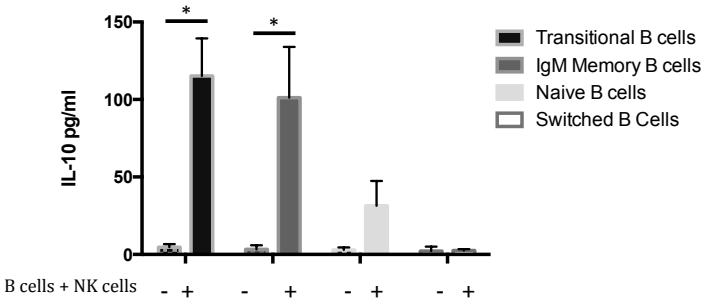

Figure V-24: B cells co-cultured with NK cells produced greater IL-10 than resting B cells cultured alone.
Supernatants were harvested after cultured NK-B cell co-culture and measured for IL-10 production through ELISA assay (n=4). Bars represent median values and upper whisker of error bars represents the range. *$P < 0.05$ by nonparametric ANOVA

Coculture of NK cells with transitional and IgM memory B cells from healthy controls resulted in upregulation of p-STAT3 in transitional and IgM memory and to a much lesser extent in naïve and switched B cell subsets **(figure V-25A-B)**. These findings indicate that NK cell interaction with B cells results in phosphorylation of the STAT3 pathway in transitional and IgM memory B cells and suggest that proximal signals induced by NK cells may induce regulatory activity in transitional and IgM memory B cells. I next explored whether preventing NK-B cell interaction, either by culturing the cells the presence of a transwell membrane or by the addition of blocking antibodies against CD48 and 2B4 molecules could reverse NK-induced STAT3 phosphorylation in B cell subsets. Inhibition of cell-cell contact completely NK cell-induced STAT3 phosphorylation in transitional and IgM memory B cell subsets [**Figure V-25C**].

Taken together, these data indicate that 2B4 on NK cells interacts with CD48 on transitional and IgM memory B cells to induce regulatory function by generating bidirectional signals and cross-talk.

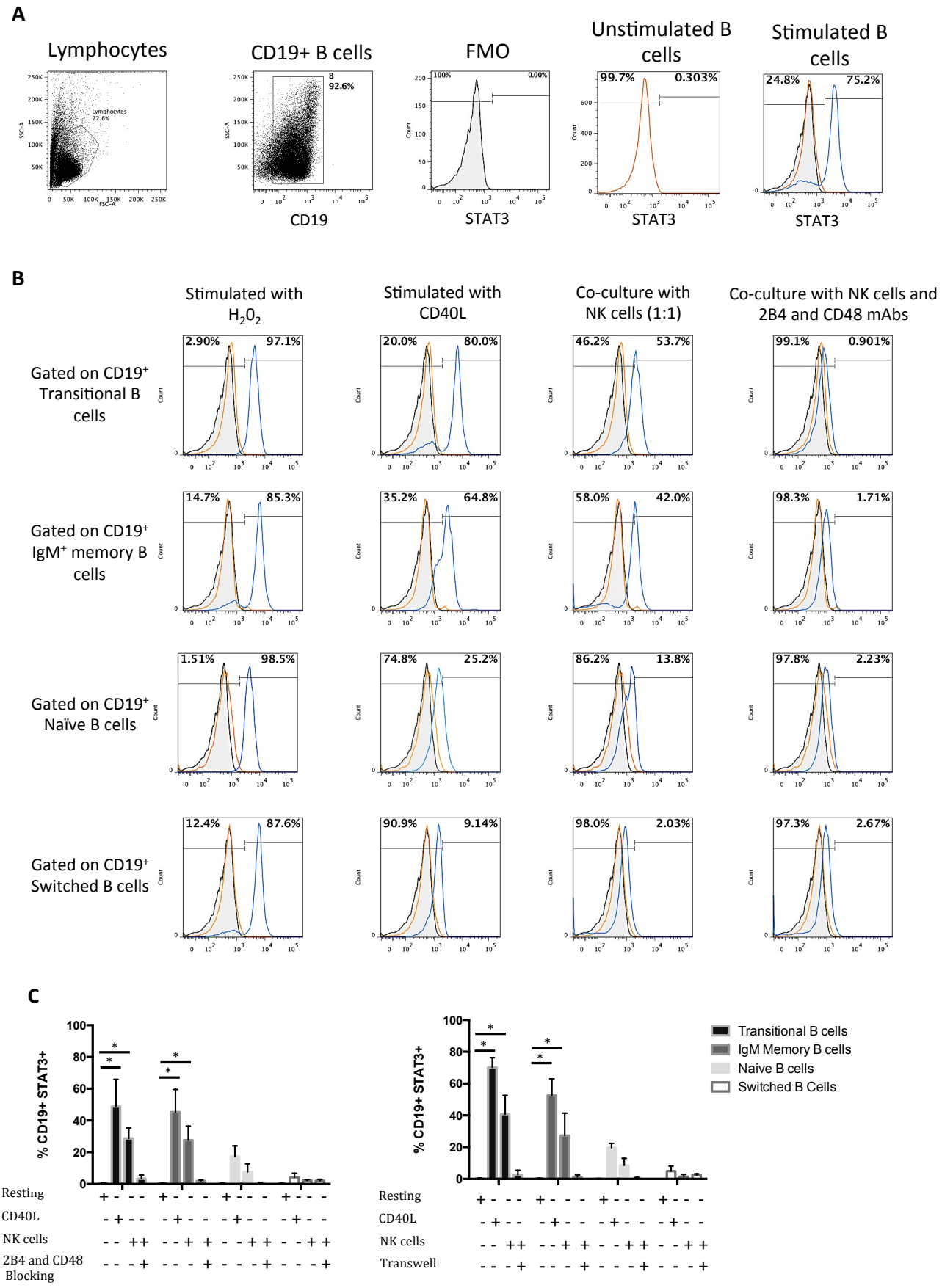

Figure V-25: Activated B cells upregulate JAK/STAT3 pathway.
A. Sort purified CD19+B cell subsets were cultured with NK cells or fibroblasts expressing CD40L to determine the frequency of STAT3 expression. B cells were derived from the lymphocyte population then CD19+ gate. CD19+JAK/STAT3+ cells (blue) were determined by PE FMO as indicated (shaded grey). **B.** Histograms representing expression of STAT3 in B

cell subsets after stimulation with CD40L, NK cells, NK cells with CD48/2B4 blocking antibodies or with H202 (positive control). CD19+STAT3+ cells (blue) were determined by PE FMO as indicated (shaded grey) and unstimulated B cells (orange) **C**. Bar graphs represent STAT3 expression in transitional and IgM memory B cell subsets. Addition of CD48 and 2B4 mAbs inhibited the upreglation of the JAK/STAT3 pathway in NK-B co-cultures. Bars represent median values and upper whisker of error bars represents the range. *P < 0.05 by nonparametric ANOVA

V.2.8 Patients with AML have increased frequencies of IL-10 producing B cells

The microenviroment of acute myelogenous leukemia (AML) is suppressive for immune effector cells (Stringaris et al., 2014). To determine if patients with AML have higher frequencies of B cell subsets with regulatory capacity, I measured transitional and IgM memory B cells in the PB of 13 patients with AML (**Table V-1**) at diagnosis and13 healthy controls. In comparison with healthy controls, AML patients had significantly higher frequencies and absolute numbers of transitional and IgM memory B cells [**figure V-26**].

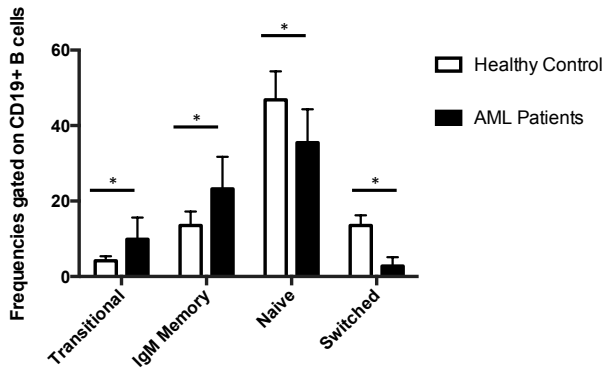

Figure V-26: Patients with AML have greater frequencies of transitional and IgM memory B cells than healthy controls
Frequencies of B cell subsets: naïve, switched, transitional and IgM memory B cells were significantly different in AML patients than healthy controls. AML patients expressed expanded population of B regulatory subsets transitional and IgM memory B cells (n=13). Bars represent mean values and upper whisker of error bars represents the range. *P < 0.05 by unpaired t-test.

Table V-1: Clinical Characteristics of Patients with AML

	All N=13
Age in years Median (range)	68 (20-80)
Sex	
Female, n (%)	7 (53.8%)
Male, n (%)	6 (46.2%)
Race	
White	8 (63.5%)
Black	1 (7.7%)
Hispanic	4 (30.8%)
Diagnosis, n (%)	
Primary AML	9 (69.2%)
Secondary AML	4 (30.8%)
Cytogenetics, n (%)	
Poor Risk	12 (92.3%)
Day 30 ALC (k/μL)	
Median, range	1.98 (0.19 – 8.18)
Mean, S.D.	2.4 (4.1)
Disease status	
Remission	2 (15.4%)
Died	11 (84.6%)
WBC (k/μL)	
Median, range	15.5 (0.8-65.9)
Mean, S.D.	22.97 (21.4)
PB Blasts (%)	
Median, range	34 (11-97)
Mean, S.D.	48 (29.4)
% CD19+ B Cells	
Median, range	15.2 (4.46 - 35.4)
Mean, S.D.	17.9 (9.4)
% CD19+ IL-10+ Cells	
Median, range	11.5 (2.12 - 27.5)
Mean, S.D.	12.8 (8.8)

To determine if the expanded B cell populations in AML had the capacity to produce IL-10, I measured IL-10+CD19+ B cells following stimulation with L cells as described in section II.6.5. AML patients had significantly higher frequencies and absolute numbers of IL-10-producing B cells compared to healthy controls [**figure V-27**]. I next determined whether this functional difference noted in the overall population of IL-10+CD19+ B cells may contribute to an altered ratio of regulatory-to-effector cells in AML patients. Patients with AML had a significantly higher IL-10+CD19+ B-cell/ CD56+CD3- NK-cell ratio, whether in terms of relative frequency or absolute counts, compared with that for healthy control group [**figure V-28**]. These results suggest that patients with AML present an expanded population of potential IL-10 producing B regulatory cells, which may contribute to suppressed NK frequencies, absolute counts and function, thus, promoting cancer progression through an imbalance in immunoregulatory and effector subsets. For this reason, I next investigated if the expanded population of IL-10 producing candidate regulatory CD19+ B cells derived from AML patients could suppress NK cell function *in vitro* in a similar fashion to healthy PB transitional and IgM memory B cells as described previously.

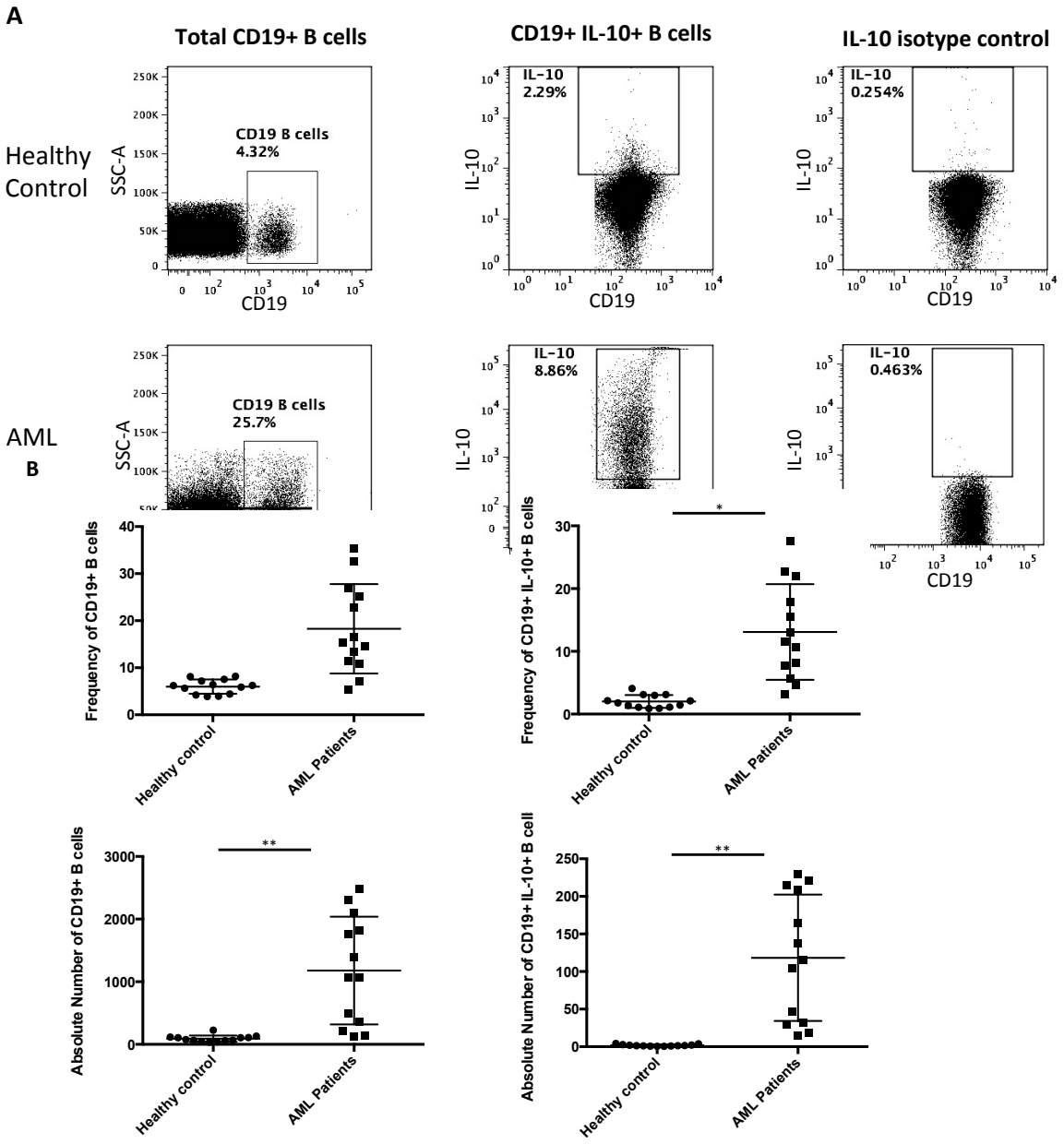

Figure V-27: Patients with AML have abnormally higher frequencies and absolute numbers of IL-10-producing B cells at diagnosis compared to healthy controls
A. Magnetically selected CD19+ B cells from AML patients and healthy controls were incubated with L cells alone for 48 hours. PMA, Ionomycin and BFA were added for the last 6 hours of culture and cells were surface stained for the expression of CD19 and intracellular staining for IL10. Representative FACS plot of IL10 IC staining in gated CD19+ B cells AML patients and Healthy controls. Frequencies of IL10+ CD19+ B cells were assessed relative to isotype controls n=13 **B.** B cells from AML patients had higher frequencies and absolute numbers of total CD19+ B cells and produced higher frequencies and absolute numbers of IL10+ B cells when stimulated with L cells compared with healthy controls (n=13). Figure represents mean value and whiskers of error bars represent the ranges. *$P < 0.05$ and **p<0.01 by unpaired t-test.

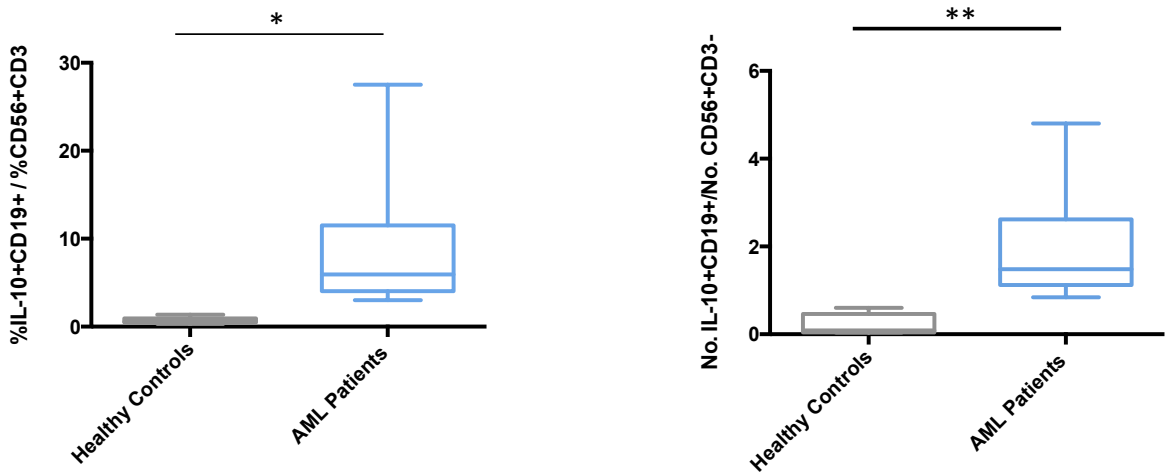

Figure V-28: Patients with AML had a significantly higher IL-10+CD19+ B-cell/ CD56+CD3- NK-cell ratio, whether in terms of relative frequency or absolute counts, compared with that for healthy control group
The box and whiskers plot extends from the 25th to 75th percentiles and were computed by Graphpad Prism software from 13 independent experiments. The line in the middle of the box is plotted at the median and the whiskers represent the range. *P < 0.05 and **p<0.01 by unpaired t-test.

Purified CD19+ B cells from the PB of AML patients and healthy controls were co-cultured at a 1:1 ratio with magnetically purified healthy control NK cells for 48 hours as described in section II.9.2. NK cell effector function was then assessed against K562 target cells by measuring the frequencies of degranulated (CD107a+) CD56+ NK cells and TNF-α and IFN-γ producing CD56+ cells by flow cytometry. AML CD19+ B cells induced significant suppression of CD107a degranulation, and expression of IFN-γ and TNF-α by NK cells in response to K562 targets when compared to B cells from healthy controls [**figure V-29A-B**]. The potent suppressive effect of AML CD19+ B cells on NK cytotoxicity was further confirmed using chromium (51Cr) release assay [**figure V-29C**]. These results support my hypothesis and suggest that expanded population of IL-10 producing CD19+ B cells in AML patients may act directly on suppressing NK cells and thus contributing to progression of AML.

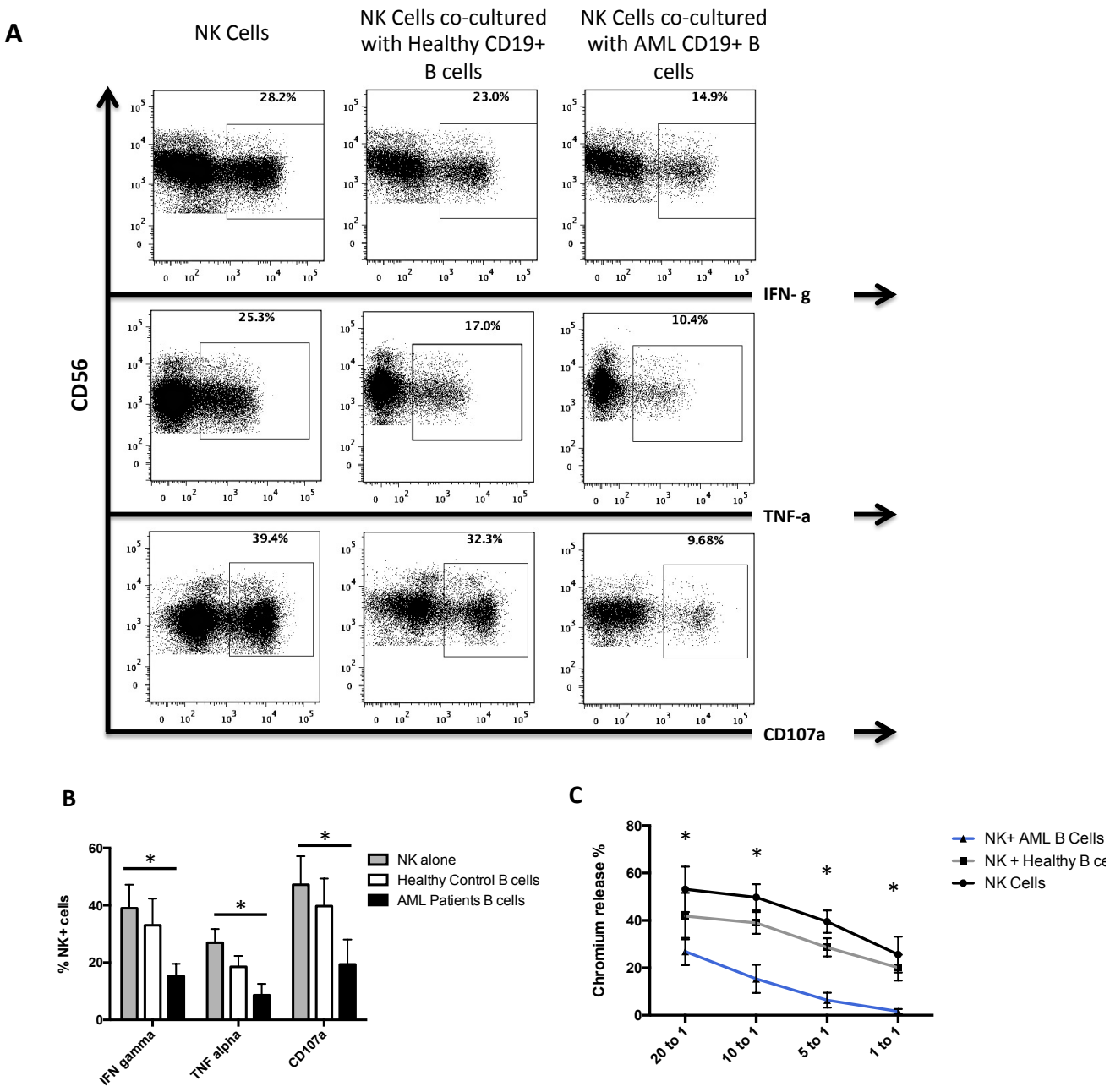

Figure V-29: Total CD19+B cells from AML patients exhibited greater suppressive function on NK effector function than total CD19+ B cells from healthy control

A. Magnetically selected total CD19+ B cells from AML patients and Health controls were co-cultured with selected healthy NK cells at a 1:1 ratio for 48 hours. Representative dot plots illustrate gated frequencies of CD56+IFN-y+ NK cells, CD56+TNF-a NK cells and CD56+CD107a+ NK cells cultured alone (positive control), with total CD19+ B cells from AML patients or total CD19+ healthy B cells **B.** Bar graph represents total CD19+B cells from AML patients exhibited greater suppressive function on NK effector function than total CD19+ B cells from healthy control (n=9). Bars represent median values and upper whiskers of error bars represent the range. *$P < 0.05$ by nonparametric ANOVA **C.**Total CD19+B cells from AML patients exhibited greater suppressive function on NK cytotoxicity than total CD19+ B cells from healthy control when compared with healthy NK cells alone (positive control) (n=6). The figure represents mean values and error bars represent range. *$P < 0.05$ by nonparametric ANOVA

I also investigated if coculture of purified AML CD19+ B cells with healthy donor NK cells induced changes in the expression of activating and inhibitory receptors on NK cells [**figure V-30**]. Co-culture resulted in downregulation of the activating NK co-receptors NKG2D, DNAM-1, NTB-A and natural cytotoxicity receptors, NKp46 and NKp30 and upregulation of the inhibitory receptors NKG2A, Siglec-7 and ILT.

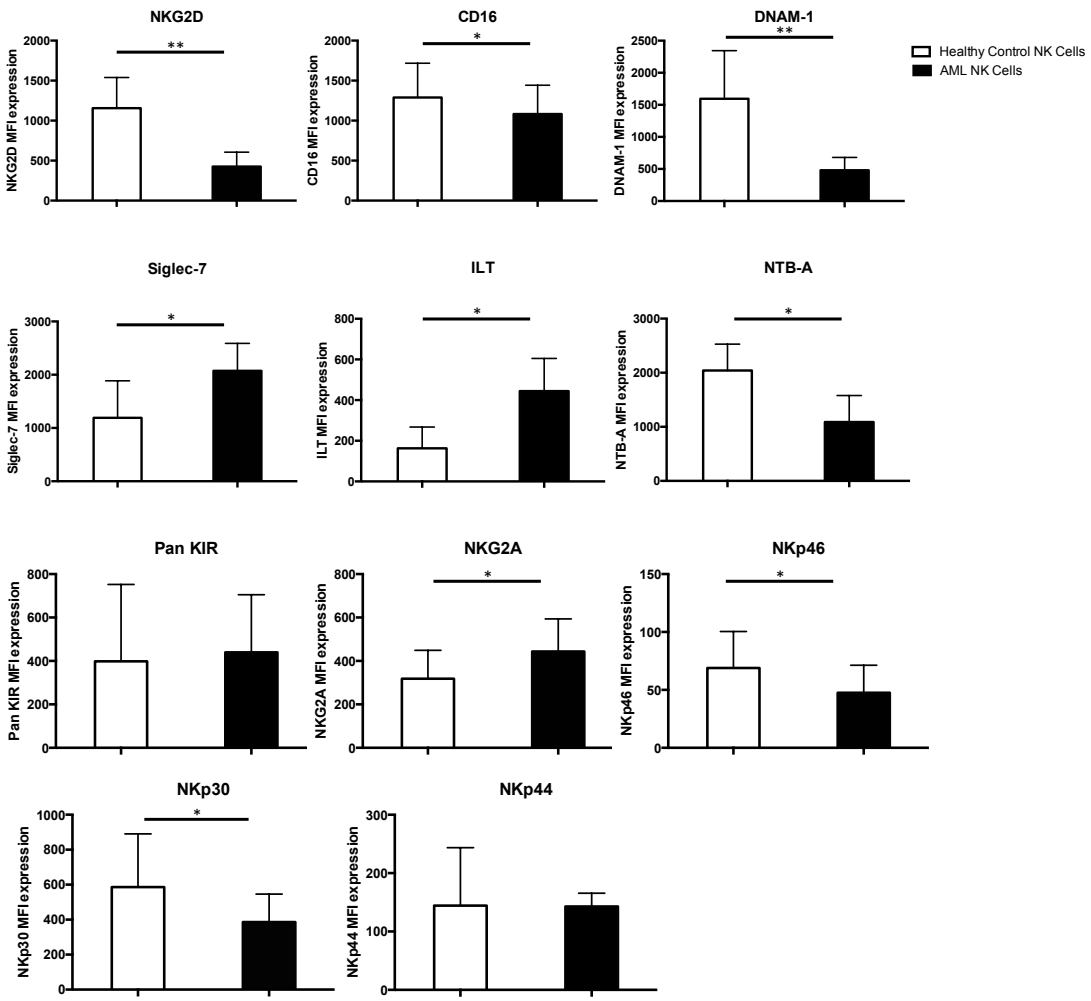

Figure V-30: NK cells from AML patients at diagnosis were shown to have dysfunctional expression of receptors when compared to health control NK cells

Frequencies of NKG2D, CD16, DNAM-1, NKp46, and NKp30 was significantly lower in AML NK cells whereas, expression of Siglec-7, ILT and NKG2A was significantly elevated when compared to the expression of these receptors in healthy controls (n=9). No differences of receptor expression between the 2 groups was found for Pan KIR and NKp44. Bars represent mean values and error bars represent range. *p<0.05 and **p<0.01. Statistical analysis was performed using a non-paired t test.

Given my earlier findings that IgM memory and transitional B cells suppress NK cell function via 2B4-CD48 interaction, resulting in down regulation of the activating molecule SAP and upregulation of the inhibitory regulator pSHP-1, I further evaluated whether AML B cells suppressed NK cell function through a similar mechanism. NK cells from AML patients had significantly lower expression of SAP and higher expression of pSHP-1 than healthy control NK cells [**figure V-31**]. Further, AML CD19+ total B cell significantly suppressed the expression of SAP and consequently upregulated the expression of pSHP-1 in healthy PB NK cells, when compared with total CD19+ B cells from healthy controls [**figure V-32**].

Collectively, my findings demonstrate transitional and IgM memory B regulatory cells inhibit NK cell activity suggesting that B regulatory cells can directly act on NK cells, a unique phenomenon which may recognize Bregs as contributing factors that may be recruited and exploited by leukemic cells to evade immunesurveillance.

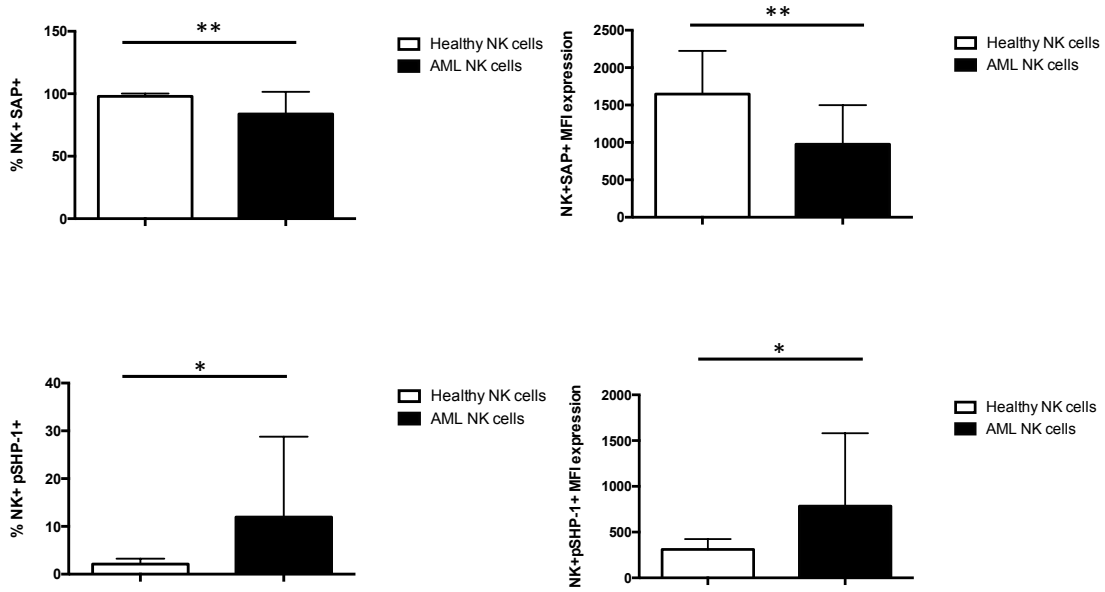

Figure V-31: Dysfunctional NK cells from AML patients had reduced expression of SAP and increased expression of negative regulator pSHP-1 than healthy controls

Magnetically selected CD3-CD56+ NK cells from AML patients and healthy controls were stained for SAP and SHP-1 expression. Data representative of n=12 and highlight both the frequency and MFI of SAP and pSHP-1 expression. Bars represent mean values and errors bars represent range. *p<0.05 and **p<0.01 by a non-paired t test.

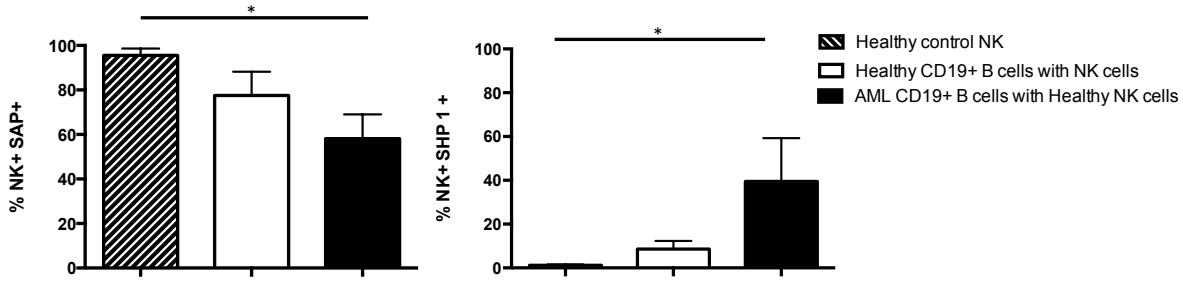

Figure V-32: Dysfunctional B cells from AML patients significantly reduced expression of SAP and increased expression of negative regulator pSHP-1

Magnetically selected CD19+ B cells from AML patients were co-cultured with healthy NK cells at a 1:1 ratio for 48 hours. After the culture period NK cells were stained for SAP and SHP-1 expression (n=8). Bars represent mean values and errors bars represent range. *p<0.05 by a non-paired t test.

V.3 Discussion

In recent years, a number of studies in mice and humans have identified a distinct subpopulation of B cells with significant regulatory function. Despite increasing evidence showing an important role for Bregs in autoimmunity (Mauri et al., 2013, Yang., 2013), lack of a clear phenotype or the paucity of specific markers have hampered their in depth characterization. In this study, I demonstrate the capacity of human CD19+CD24hiCD38hi transitional and CD19+IgM+CD27+ memory B cells to significantly inhibit the effector function and proliferation of NK cells in a contact-dependent manner.

Previous studies in mice and humans have shown that the Breg-mediated suppression of cell proliferation and effector function is mediated through both IL-10 and cell-cell contact-mediated mechanisms (Yang et al., 2013, Blair et al., 2010, Iwata et al., 2011, Khoder et al.,2014). In addition, TGF-β has also been shown to be an important mediator of Breg suppression in experimental models of diabetes (Tian et al., 2001). In contrast, I show here that IL-10 secretion is not a major mechanism for regulatory B cell-mediated suppression of NK cell proliferation and function. Instead, transitional and IgM memory B cells require direct cell-cell contact to suppress NK cells.

There is evidence for bi-directional crosstalk between NK cells and B cells, mediated through interactions involving CD48-2B4 and CD40-CD40L (Yuan et al., 2010, Gao et al., 2006, Blanca et al., 2001, Sinha et al., 2010). I did not detect any soluble CD40L in my NK-B cell co-culture experiments (data not shown) and thus did not pursue this interaction further. Instead, I found an

important role for CD48-2B4 in mediating suppression of NK cells by Breg subsets. CD48 is expressed widely on hematopoietic cells including B, T and NK cells and binds to the 2B4 co-receptor on the surface of NK cells (Yuan et al., 2010, Gao et al., 2006, Sinha et al., 2010, Assarsson et al., 2005, Lee et al., 2006). B cells can activate NK cells (Gao et al., 2006, Sinha et al., 2010). However, in my study I show an important role for CD48-2B4 interaction in mediating the suppressive effect of Breg subsets on NK cell proliferation and effector function. Blocking either CD48 or 2B4 resulted in near complete abrogation of the suppressive effect of CD19+CD24hiCD38hi transitional and CD19+IgM+CD27+ memory Breg subsets on NK cells and resulted in restoration of NK cell proliferation and effector function. My findings are supported by murine studies reporting that CD48 on tumor cells can interact with 2B4 on NK cells and inhibit their function (Lee et al, 2006), supporting the notion that CD48-2B4 interaction can induce both positive and negative signals.

To understand this phenomenon more mechanistically, I studied the 2B4 signaling pathway in NK cells. In humans, NK cell activation through 2B4 is accompanied by the phosphorylation of immunoreceptor tyrosine-based switch motifs (ITSMs) in its cytoplasmic tail and the recruitment of SLAM-associated protein (SAP), a signaling adaptor protein (Stark et al., 2006,Eissman et al., 2005). SAP is crucial for 2B4 to deliver activating signals to NK cells. In the absence of SAP, the phosphorylated ITSM of the 2B4 receptor recruits inhibitory signaling molecules such as SHP-2, SHP-1, SHIP (Assarsson et al., 2005, Eissmann et al., 2005), resulting in dephosphorylation

of downstream molecules involved in NK cell activation (Hallet et al., 2006, Moretto et al 2003). The importance of SAP in immune regulation was first highlighted by SAP mutation found in cases of X-linked lymphoproliferative (XLP) disease, an immunodeficiency disorder characterized by lymphoproliferation and abnormal responses to Epstein-Barr virus (EBV) infection. Patients with XLP have also been found to exhibit impaired NK cell cytotoxicity (Eissmann et al., 2005, Hallet et al., 2006, Lanier et al., 2008).

Here, I showed that the interaction between CD48, on transitional and IgM memory Breg subsets, but not naïve or switched memory B cells, and 2B4 on NK cells results in downregulation of, SAP and recruitment of pSHP-1. The effect of Breg subsets on SAP and SHP was completely reversed upon disrupting the interaction between 2B4 and CD48 using blocking antibodies, suggesting a crucial role for this axis in the mechanism of Breg-mediating NK cell suppression. It is quite possible that additional signaling pathways also play a role in Breg-mediated suppression of NK function. Indeed, Assarsson et al (2005) have noted that 2B4 and CD48 both reside within glycolipid-rich microdomains, which contain many other molecules that may be involved in signaling, and additional studies of 2B4-CD48 signaling pathways involved in Breg-mediated suppression of NK cells are underway.

Blair et al (2010) highlighted that CD19+CD24^{hl}CD38^{hl} transitional B cells require the activation of the CD40/STAT3 signaling pathway to exert their immunoregulatory function on CD4+ T cells. Here I show that following coculture with B cells subsets, NK cells trigger phosphorylation of STAT3 in

both IgM memory as well as transitional B cell subsets (but to a much lesser extent in naïve and switched memory B cells). Taken together, these data support the existence of bidirectional 2B4-CD48-mediated cross-talk between NK cells and B cells and suggest that NK cells can deliver signals to regulatory B cell subsets, and the latter in turn deliver inhibitory signals to NK cells by inducing downregulation of SAP and recruitment of pSHP.

Recent reports in murine models indicate that Bregs can potently inhibit antitumor immune responses (Balkwill et al., 2013, He et al., 2014) and clinically, IL-10-producing B cells have been shown to play an important role in resistance to anti-CD20 mAb therapy in patients with B-cell lymphoma (Horikawa, et al., 2011). My report provides for the first time functional evidence for a significant increase in Bregs with potent suppressive activity against NK cells in the peripheral blood of patients with AML.

Coculture of AML CD19+ B cells with healthy donor NK cells resulted in significant suppression of SAP, recruitment of pSHP-1, and significant suppression of healthy donor NK cell proliferation and function. It is noteworthy that ex vivo-purified NK cells from AML patients expressed significantly less SAP and, higher pSHP-1 than their normal counterpart, further supporting the notion that in AML, engagement of 2B4 on NK cells by CD48 on B cells will deliver inhibitory signals to NK cells, resulting in suppression of their effector function. Although earlier studies have demonstrated that CD48 on hematopoietic cells interacts with 2B4 on NK cells to generate bidirectional activating signals and cross-talk (Gao et al., 2005, Yuan et al., 2010, Messmer et al., 2006, Mathew et al., 2005, Assarsson et

al., 2004), this is the first study to provide mechanistic insights into inhibitory cross-talk between NK cells and Breg subsets and to demonstrate the relevance of this inhibitory axis in acute myeloid leukemia. However, The AML patients were derived from a very heterogeneous group and factors such as immunosuppressive AML blast cells may also have affected NK properties in AML samples and require further consideration.

The immune microenvironment in AML displays a spectrum of abnormalities, including aberrant expression of immunomodulatory cytokines (Stringaris et al., 2014, Ferrara and Schiffer., 2013, Lion et al., 2012) and numerical and functional alterations in immunoregulatory subsets such as Tregs and myeloid-derive suppressor cells (Ustan et al., 2011, Szczepanski et al, 2009). Our findings point to the existence of a broader network of immunoregulatory cells, also involving Bregs, that can be recruited and exploited by leukemic cells to evade immunesurveillance. Breg-mediated suppression of NK cell function may have important implications not only on cancer immune surveillance and defense against pathogens but also in determining response to therapy. For instance, Bregs may negatively impact the effectiveness of immunotherapies in AML, such as antibody-dependent cellular cytotoxicity (ADCC) of tumor-targeted monoclonal antibodies (*e.g.*, anti-CD33 mAbs). This data will have implications for other cancers and studies to explore the role of Bregs in other types of cancer and the consequences of their depletion on NK cell antitumor activity *in vivo* are currently under way. Finally, I propose that depletion of Breg subsets may be a novel strategy to enhance cancer immunity in humans.

Chapter V1. General Discussion and Future plans

VI.1 General Discussion

In recent years, a distinct newly described subpopulation of IL-10-producing B regulatory (Breg) cells, that have been shown to exert significant immunoregulatory functions both *in vitro* and *in vivo*, has been the focus of intense immunological research (Mauri and Bosma., 2012). Breg cells have been shown to exert suppressive functions through the production of IL-10 in various diseases, including inflammation, cancer, autoimmunity and more recently cGVHD (DiLillo et al., 2010, Sarantopoulos et al., 2015, Khoder et al., 2014). Bregs suppress a variety of immune cells through differing mechanisms (Rosser et al., 2014). Further, the understanding of human Breg suppressive function and interaction with other effector cells may facilitate the understanding of imbalance between regulatory and effector subsets that leads to loss of tolerance and induction of allo-reactivity in GVHD.

Despite the extensive body of evidence reinforcing the notion that B cells can exert immunoregulatory function, controversy over the paucity of markers that can unequivocally identify Bregs particularly in humans still exists. Blair and colleagues have elegantly described human Bregs as $CD19^{+}CD24^{hi}CD38^{hi}$, a phenotype that typically delineates human transitional B cells (Blair et al., 2010). Conversely, Iwata *et al* (2011) described human IL-10 producing B cells to be contained mainly within the $CD24^{hi}CD27^{+}$ memory B cell compartment (de Masson et al., 2015, Iwata et al., 2011). In addition to these studies, Khoder *et al* syndicates previous studies and demonstrates that IL-10-secreting CD19+IgM+CD27+ memory B cells coexist with IL10+$CD24^{hi}CD38^{hi}$ transitional B cells in healthy human donors and

significantly suppresses the proliferation and cytokine production of autologous CD4+ T cells through both IL-10-dependant and cell-to-cell contact mediated mechanisms. Further, considerable evidence from murine studies supports the presence of more than one Breg phenotype. Thus, discrepancies in the cell surface antigens studied and a lack of consensual definitions of the Breg subset phenotypes hampers their in depth characterization.

In my PhD thesis, I studied the phenotypic and functional characterization of Breg subsets in peripheral blood and cord blood and their potential interaction with other immune effector cells (CD4+ T cells and CD56+ NK cells). This understanding may aid the development of Breg cells as novel therapeutics for the treatment of immune-mediated diseases including GVHD and cancer.

For the phenotypic characterization of B cells, I designed an extended panel of surface antibodies, using a constellation of markers reported in a number of B-cell classification schemes. The panel was validated using freshly isolated healthy control samples and their frozen counterparts. I correlated the different B cell subsets namely, transitional, naïve, IgM+ memory and non-switched memory B cells according to different B cell classifying schemes, each of which had limitations as a result of limited surface markers used. I therefore, extensively phenotyped CD19+ B cell subsets based on CD24 and CD38 axis, with use of additional markers including IgM, CD27, CD21 and CD10 to characterize B cell subsets and demonstrated that CD38/24 combination with IgM could be used as a sorting panel.

The intracellular detection of IL-10 combined with flow cytometric phenotyping demonstrated the presence of IL-10–producing B cells that were enriched within the $CD19^{+}CD24^{hi}CD38^{hi}IgM^{hi}CD27^{-}$ transitional and $CD19^{+}CD38^{-/lo}$ $CD24^{hi}$ $CD27^{+}IgM^{+}$ memory B cell population and relatively fewer in the $CD19^{+}CD38^{+}CD24^{+}CD27^{-}$ naïve and $CD19^{+}CD24^{hi}CD38^{-/lo}CD27^{+}IgM^{-}$ switched memory B cell subsets. The functional assessment of sort purified transitional and IgM memory B cells supports the immunoregulatory functions of transitional B cells and identify IgM+ memory B cells as a new candidate Breg subset in healthy individuals. This discovery broadens the proportion of PB-derived regulatory B cell subset within circulating total CD19+ B cells, suggesting a prominent role for Bregs in the maintenance of immune tolerance. Both IL-10 enriched transitional and IgM memory B cells exerted suppressive function on proliferation and cytokine production of CD4+ T cells in a manner that was comparable with that of Tregs. Although I also found the presence of IL-10 producing B cells within naïve and switched memory B cell subsets, albeit at much lower frequencies, they lacked any suppressive effect on CD4+ T cell function. This is in agreement with other studies that have also shown lack of suppressive activity in naïve B cells (CD27- IgM+) (Blair et al., 2010, Iwata et al., 2011). Moreover, the regulatory capacity of these human Bregs on CD4+ T cell proliferation and cytokine production was dependent on both cell-cell contact and IL-10 production. These finding are consistent with Blair et al's report where the suppressive effect of CD19+CD24hiCD38hi transitional B cells was also partially mediated by IL-10 (Blair et al., 2010). On the other hand, although Tedder et al's study also demonstrated a subset of IL-10 producing CD24hiCD27+CD148+ memory B cells, this subset failed to

suppress CD4+T cells and instead was shown to suppress both IFNγ and TNFα production of CD14+ monocytes (Iwata et al., 2011). Thus contrary to these results, in this study, IL-10+ B cells were defined as $CD27^{+}IgM^{+}$ $CD38^{-/lo}$ $CD24^{hi}$ memory B cells and were found to suppress CD4+ T cells. Conclusively, for clinical purposes, sort-purification based on a phenotype of transitional and IgM memory B cells is likely to yield an enriched population of IL-10+Breg. Further, in the majority of clinical trials, systemic administration of recombinant IL-10 was not associated with clinical improvement with the exception of psoriasis (Sanz et al., 2008). Similarly in my experiments, the addition of exogenous IL-10 to co-cultures of naïve or switched memory B cells failed to suppress CD4+ T cells to the same extent as seen with transitional and IgM memory B cell subsets, suggesting that cellular contact may be required for IL-10 to deliver immune regulation. Indeed, transwell experiments and CD80/CD86 blockade confirmed that cell-to-cell contact was needed for IgM memory and transitional B cells to exert their full suppressive activity on CD4+ T cell function. These findings may provide support for future investigations of regulatory B cell–based therapy to tip the scales in favor of immune regulation for the treatment of GVHD.

As I found that human Bregs are enriched within both the transitional and IgM memory B cell subsets and since CD19+CD24hiCD38hi transitional B cells are abundant in cord blood (CB) (near 50% of B cells), I next studied Bregs in CB and their potential regulatory function. My findings support the notion that CB may offer an invaluable source of off-the-shelf regulatory B cells for the treatment of GVHD and other autoimmune conditions.

Allogeneic hematopoietic SCT (HSCT) is a potentially curative option for many patients with high-risk hematological malignancies, however the high onset rate of acute and chronic graft-versus-host disease (GVHD) remains a major obstacle its success (Daikeler et al, 2009, Barrett and Battiwalla, 2010). Human CB is widely used as a source of HSC for many patients and a lower incidence of chronic extensive GVHD has been reported after CBT compared with other stem cell sources, despite broader HLA disparity (Beaudette-Zlatanova et al., 2013, Komanduri et al., 2007). Alloreactive reactions between donor-derived CD4+ and CD8+ T lymphocytes have typically been considered to be the chief effector cells arbitrating GVHD pathogenesis (Shimabukuro-Vornhagen et al., 2009, Rezvani et al., 2006). Our understanding of the function of Bregs in this disease is limited. However, recent evidence has postulated a role for B cell dysregulation in the development of GVHD (Shimabukuro-Vornhagen et al., 2009, Sarantopoulos et al., 2015). In keeping with these studies, previous research has also highlighted protective role for IL-10 producing regulatory B cells in the regulation of GVHD (Rowe et al., 2006, Lee et al., 2012, Huu et al., 2013, Weber et al., 2014, Khoder et al., 2014, de Masson et al., 2015). However, further insights into the mechanistic role of B cells in this debilitating disease is required to highlight the therapeutic benefits of targeting B cells in GVHD.

I therefore next determined whether cord blood-derived B cells possess regulatory function as seen with peripheral blood-derived Bregs, which may contribute to the lower rates of chronic GVHD seen in CBT recipients.

Extensive phenotypic characterization of CB-derived B cells revealed the presence of two distinct B cell populations: $CD19^{+}CD38^{hi}CD24^{hi}$ transitional B cells (a population that includes immature B cells) and $CD19^{+}CD38^{int}CD24^{int}$ naïve B cells (primarily mature B cells). In contrast to peripheral blood B cells, $CD24^{hi}CD38^{-}$ $CD27^{+}$ memory B-cells are almost absent in CB and only become detectable in the first year of life (Cuss et al., 2006, Ha et al., 2008). The phenotype panel was validated on both freshly isolated CB-derived B cells and their frozen counterparts before applying it to frozen samples from post-CBT patients.

I next demonstrated that IL-10+CD19+ B cells are enriched within both CB-derived transitional and naïve subsets and exert suppressive function on allogeneic CD4+ T cell function. I propose that within the CB-CD19+ B cell pool there exist 2 distinct subsets; $CD19^{+}CD38^{hi}CD24^{hi}$ transitional B cells and $CD19^{+}CD38^{int}CD24^{int}$ naïve B cells, both of which are functionally regulatory.

Despite several phenotypic and functional similarities to PB-B cells, this study revealed a number of key differences between human PB- and CB-derived Bregs. In contrast to PB-derived CD24hiCD27+ and CD27+IgM+ memory cells that have been ascribed with regulatory capacity by previous studies, memory B-cells were absent in CB (Iwata t al., 2011, Khoder et al., 2014). Further, unlike PB-naïve B cells that failed to exert suppressive activity on CD4+ T cells, our discovery presents a novel suppressive role for naïve B cells in CB and broadens the proportion of Breg in CB, suggesting a prominent role for these functional CB-derived B cells in the maintenance of immune tolerance.

Functional assessment of CB-derived total CD19+B cells as well as sort purified transitional and naïve B cell subsets revealed that these populations suppressed both the proliferation and effector cytokine production of allogeneic peripheral blood-derived CD4+T cells in a manner that was comparable to CB-derived Tregs. This suppressive capacity was further augmented in the presence of pre-activated B cells co-cultured with CD4+T cells suggesting that in human PB, Breg designation may not be limited to the IgM memory and transitional B cell subsets described previously and it is likely that discrete subsets of naïve and switched memory B cells could also be induced to exert regulatory function in response to CD40 ligand signaling provided by activated T cells, analogous to reports of inducible Tregs during inflammation (Feuerer et al., 2009). Similar to previous reports of PB-Bregs, the mechanism by which CB-derived naïve and transitional B cells suppress CD4+T cell function was mediated synergistically through IL-10 production and cell-to-cell contact involving CD80/CD86 and CTLA-4 (Blair et al., 2010, Khoder et al., 2014). These findings are in agreement with previous studies with human PB Bregs that describe the involvement of CD80 and CD86 as an important feature of their suppressive capacity (Blair et al., 2010, Khoder et al., 2014) and with murine studies of intestinal inflammation where CD86 was noted to facilitate B cell suppression (Mann et al., 2007, Mizoguchi et al., 2000). Interestingly, I found involvement of CTLA-4 in the suppressive mechanism of CB-Bregs; this is in contrast to data with PB Bregs where CTLA-4 was not found to play a major role in their suppressive function (Khoder et al. 2014). However, a similar mechanism is employed by CD80 expressed on DCs, which acts preferentially as a ligand for CTLA-4 and

mediates Treg cell suppression (Zheng et al., 2004). This study provides support that CD80 and CD86 on Bregs may also function as ligands for CTLA-4 and could play a role in Breg suppressive function.

The role of Bregs in cord blood transplantation and GVHD has not been previously studied. I next investigated whether CB-Bregs account for lower rates of GVHD after CBT. Several studies have reported that immune reconstitution after CBT is characterized by an expansion in B cells during the first year post transplant (Komanduri et al., 2007, Nakatani et al., 2014, Beaudette-Zlatanova et al., 2013, Saliba et al., 2015, Lucchini et al., 2015). In accord with these published work, I found a robust expansion in both the frequencies and absolute numbers of CD19+ B cells and IL-10 producing CD19+B cells during the first 3-9 months post CBT, after which the B cell population progressively decreased and by 1-year post CBT there were no significant differences in the frequencies and numbers of circulating B cells and IL-10+B cells in CBT recipients and healthy donors. The recovering B cells at 6-9 months post-CBT were found to be more suppressive on a cell per cell basis when compared to B cells derived from cord blood. These results suggest a role for the expanded population of IL-10 producing B-cell population in CBT patients in mediating T-cell suppression and thus reducing the severity of GVHD. A previous study by Stiff et al also postulated that robust B cell recovery might attenuate T cells responses post CBT (Beaudette-Zlatanova et al., 2013). I further demonstrated that CD19+IL-10+ B cell recovery was impaired in patients with GVHD compared to those without GVHD after CBT, and that B cells isolated from CBT recipients with

GVHD were refractory to stimulation and were unable to produce IL-10. These results are in accord with similar studies that have also found low frequencies of IL-10 producing B cells in cGVHD patients following AHSCT than in AHSCT recipients without cGVHD (Rowe et al., 2006, Khoder et al., 2014, Weber et al., 2014), suggesting that the early recovery of B cells post CBT may define a protective role for Bregs in GVHD setting.

This study defines a novel potential role of CB derived donor Bregs in reducing the risk of GVHD, emphasizing the potential of novel B-cell directed therapies for the prevention or treatment of GVHD.

Further, although there is no unique marker to purify exclusively suppressor B cells that express IL-10, my studies illustrate that at IL-10+B cells are enriched in selected B cell compartments in both peripheral and cord blood. This serves as an advantage in using Breg based therapies compared to Treg for use in the treatment of GVHD, since there is currently no defined Treg phenotype to accurately identify human Treg as a result of discrepancies between surface markers and cell plasticity. Additionally, recent in vitro human data, indicating that activated B cells directly suppress allogeneic CD4+ T-cell proliferation through inducing the expansion of alloantigen-specific suppressor Tregs further supports a significant advantage in the development of Breg cell based immunotherapy and suggests the use of inducing B-cell expanded Treg cells for treatment of GVHD (Chen et al., 2009).

Moreover, the use of anti-CD20 B-cell depleting agent, Rituximab in cases where B cells are considered the main arbitrators for disease pathology may

need to be avoided as all described regulatory B cells express CD20. Hence, strategies to selectively target B effector cells whilst preferentially sparing regulatory B cells are required. Thus, to tip the scales in favor of immune regulation and induce protection against GVHD, so that GVHD is either prevented or attenuated, infusion of donor-derived Bregs early in the patient's post-transplant regime may be essential. Therefore, the potential of in-vitro expanded CB-derived IL-10 producing Bregs as an invaluable source of off-the-shelf treatment of human GVHD also merits further investigation.

To date, recent evidence has firmly identified the suppressive capacity of Bregs on T cell function in murine models and humans, which maintains immune tolerance and is critical in host suppression of autoimmune diseases (Iwata et al., 2011, Mauri and Bosma., 2012, Rosser and Mauri., 2015). However, recent studies have also highlighted a role for Bregs in cancer progression (Inoue et al., 2006, DiLillo et al., 2013, Horikawa et al., 2011, Shao et al. 2014). Despite mounting evidence supporting a role for Breg cells in autoimmunity and promoting tumor growth through inhibition of T cell proliferation and effector function, there is limited data on the interaction of Bregs with other immune effectors important in tumor-immune surveillance such as natural killer (NK) cells. NK cells are important components of the innate immune system and play an important role in tumor immune surveillance (O'Hanlon, 2004, Ljunggren and Malmberg, 2007, Waldhauer and Steinle, 2008). In cancer patients, NK cell activation can be hampered by downregulation of major histocompatibility complex (MHC) class I molecules on target cells (missing self theory) (Smyth *et al*, 2002, Ljunggren and Karre,

1990) and/or upregulation of proteins such as NKG2D ligands on 'distressed' cells (induced self theory) (Vivier *et al*, 2008), but Giringhelli *et al* suggested that other mechanisms may also play a role in blunting NK cell responses against cancer. Indeed, increased frequencies of regulatory T cells (Tregs) have been shown to correlate with cancer progression and hamper NK cell function (Ghirenghelli *et al*, 2005, Pedrozo-Pacheo *et al*, 2013). Although recent studies have shed light on the existence of NK-B cell bidirectional cross-talk, through the interaction of CD48 on B cells with, 2B4, on NK cells (Yuan *et al*, 2010., Gao *et al*, 2006., Gao *et al*, 2005, Lee *et al*, 2006), no studies to date have explored the role of Breg and NK interaction.

I therefore next determined the effect of Breg mediated suppression of NK function. Both PB-derived and IL-10 enriched transitional and IgM memory B cells suppressed the proliferation, effector function and cytotoxicity of CD56+CD3-NK cells in a manner that was comparable with that of PB-Tregs. Previous studies in mice and humans have shown that Breg-mediated suppression of T cells is mediated through both IL-10 and cell-cell contact-mediated mechanisms (Yang et al., 2013, Blair et al., 2010, Iwata et al., 2011, Khoder et al.,2014). In contrast, I found that IL-10 secretion is not a major mechanism for regulatory B cell-mediated suppression of NK cell proliferation and function. Instead, transitional and IgM memory B cells require direct cell-cell contact to suppress NK cells. Although, there is evidence for bi-directional crosstalk between NK cells and B cells, mediated through interactions involving both CD48-2B4 and CD40-CD40L (Yuan et al., 2010, Gao et al., 2006, Blanca et al., 2001, Sinha et al., 2010), I did not detect any soluble CD40L in my NK-B cell co-culture experiments and thus did not pursue this

interaction further. Instead, I found an important role for CD48-2B4 interaction in mediating suppression of NK cells by Breg subsets. Although, previously published work has shown that B cells can activate NK cells through B cell-NK cell cross-talk (Gao et al., 2006, Sinha et al., 2010), in my study I show an important role for CD48-2B4 signalling pathway in mediating suppression of NK function through induction of inhibitory signalling via 2B4 by Bregs. Although 2B4 is commonly associated with delivering activating signals to NK cells through SAP (a signalling adapter molecule), my findings are in accord with murine studies reporting that CD48 on tumor cells can interact with 2B4 on NK cells and inhibit their function (Stark and Watzl., 2006, Lee et al, 2006). The mechanistic suppression of NK cells by Breg through CD48-2B4 signalling was mediated through downregulation of SAP, which is crucial for 2B4 to deliver activating signals to NK cells and recruitment of pSHP-1, an inhibitory signalling molecule recruited in the absence of SAP. These findings are supported by cases of X-linked lymphoproliferative (XLP) disease, where patients have also been found to exhibit impaired NK cell cytotoxicity through 2B4/SAP dysfunction (Eissmann et al., 2005, Hallet and Murphy., 2006, Lanier., 2008). Furthermore, it is quite possible that additional signaling pathways also play a role in Breg-mediated suppression of NK function. Indeed, Assarsson et al have noted that 2B4 and CD48 both reside within glycolipid-rich microdomains, which contain many other molecules that may be involved in signaling, and additional studies of 2B4-CD48 signaling pathways involved in Breg-mediated suppression of NK cells are underway (Assarsson et al., 2005).

Since the interaction between CD48, on transitional and IgM memory Breg subsets, but not naïve or switched memory B cells induces an inhibitory effect on NK function via 2B4, I studied the effect of NK cells on Breg subsets. Blair et al highlighted that CD19+CD24^{hl}CD38^{hl} transitional B cells require the activation of the CD40/STAT3 signaling pathway to exert their immunoregulatory function on CD4+ T cells (Blair et al., 2010). In keeping with these findings, I found that NK cells trigger phosphorylation of STAT3 in both IgM memory as well as transitional B cell subsets (but to a much lesser extent in naïve and switched memory B cells). Collectively, these data support the existence of bidirectional 2B4-CD48-mediated cross-talk between NK cells and B cells and suggest that NK cells can deliver signals to regulatory B cell subsets, and the latter in turn deliver inhibitory signals to NK cells by inducing downregulation of SAP and recruitment of pSHP. To further unravel whether Bregs may exert potent suppressive activity promoting cancer progression and NK effector dysfunction, I functionally characterized CD19+ B cells in the peripheral blood of patients with AML. The immune microenvironment in AML displays a spectrum of abnormalities, including aberrant expression of immunomodulatory cytokines (Stringaris et al., 2014, Ferrara and Schiffer., 2013) and numerical and functional alterations in immunoregulatory subsets such as Tregs and myeloid-derive suppressor cells (Ustan et al., 2011, Szczepanski et al, 2009).

AML patients had increased frequencies and absolute numbers of both CD19+ B cells and IL-10-producing B cells in comparison to healthy controls. Further, patients with AML had a significantly higher IL-10+CD19+ B-cell/ CD56+CD3- NK-cell ratio, compared with that for healthy control group

suggesting that an altered ratio of regulatory-to-effector cells in AML may suppress NK cell number and function, thus, promoting cancer progression. Coculture of AML CD19+ B cells with healthy donor NK cells resulted in significant suppression of SAP, recruitment of pSHP-1, and significant suppression of healthy donor NK cell function. It is noteworthy that ex vivo-purified NK cells from AML patients expressed significantly less SAP and, higher pSHP-1 than their normal counterpart, further supporting the notion that in AML, engagement of 2B4 on NK cells by CD48 on B cells will deliver inhibitory signals to NK cells, resulting in suppression of their effector function. Conclusively, this is the first study to provide mechanistic insights into inhibitory cross-talk between NK cells and Breg subsets and to demonstrate the relevance of this inhibitory axis in acute myeloid leukemia.

These findings point to the existence of a broader network of immunoregulatory cells, also involving Bregs that can be recruited and exploited by leukemic cells to evade immunesurveillance. Breg-mediated suppression of NK cell function may have important implications not only on cancer immune surveillance and defense against pathogens but also in determining response to therapy. For instance, Bregs may negatively impact the effectiveness of immunotherapies in AML, such as antibody-dependent cellular cytotoxicity (ADCC) of tumor-targeted monoclonal antibodies (*e.g.*, anti-CD33 mAbs). These data may have implications for other cancers and studies to explore the role of Bregs in other types of cancer and the consequences of their depletion on NK cell antitumor activity *in vivo* are currently under way. Finally, I propose that depletion of Breg subsets may be a novel strategy to enhance cancer immunity in humans.

VI.2 Conclusion

Based on these data, I propose that an imbalance between regulatory B cells and immune effector subsets leads to loss of tolerance and perpetuation of an inflammatory process that may induce allo-reactivity in GVHD or suppress anti-tumor activity through inhibition of immune effector responses. These findings have important clinical implications and suggest that Bregs may be exploited to treat immune-mediated diseases. Adoptive transfer of donor-derived Bregs early in the patient's post-transplant course may tip the scales in favor of immune regulation, so that GVHD is either prevented or attenuated, an may offer a potentially effective immunomodulatory therapy for the treatment of GVHD. Conversely, strategies to deplete Bregs for optimal anti-cancer immunotherapy may benefit antitumor activity in AML and other cancers.

VI.3 Future Plans

In my thesis, I have proposed that an imbalance between regulatory and proinflammatory networks leads to loss of tolerance and induction of GVHD. I have shown a robust expansion in both frequencies and absolute numbers of CD19+ B cells and IL-10 producing CD19+B cells in the first 3-9 months after CBT, which were also found to exert greater suppressive function on healthy PB-CD4+T cells when compared to CD19+ B cells from cord blood units. These results postulate that attenuated T cell responses (Komanduri et al., 2007, Beaudette-Zlatanova et al., 2013) and the lower risk of cGVHD after CBT may be a result of robust regulatory B cell recovery. To further assess whether robust expansion of B cells attenuates T cell responses post CBT leading to protection against GVHD, I propose to phenotypically and functionally characterize T cells subsets and determine effector T cell/regulatory B cell ratio in terms of frequency and absolute counts in CBT recipients with and without GVHD development post transplant. These results will highlight whether a reduced regulatory network may cause an increased proinflammatory effector function leading to inflammation and GVHD pathology post CBT.

I have further shown that AML patients had increased frequencies and absolute numbers of both CD19+ B cells and IL-10-producing B cells in comparison to healthy controls. Further, patients with AML had a significantly higher IL-10+CD19+ B-cell/ CD56+CD3- NK-cell ratio, compared with that for healthy control group suggesting that an altered ratio of regulatory-to-effector cells in AML may suppress NK cell number and function, thus, promoting

cancer progression. I further plan to determine if this phenomena is still present in AML patients after remission to better understand whether NK dysfunction in relation to effector function and expression of SAP and an altered Breg/NK ratio may be a prognostic marker for survival outcome in patients with cancer.

I have demonstrated that IL-10-producing regulatory B cells are enriched within PB-derived transitional and IgM memory B cell subsets and within CB-derived transitional and naïve subsets. Both CB-derived and PB-derived Breg have been shown to exert suppressive capacity on CD4+ T cell effector function, which has implications in a cGVHD setting post transplant as described previously. Subsequently, as CD8+T cells possess a pathogenic role in aGVHD, I propose to explore the suppressive function of Breg on this effector subset, that may facilitate a broader understanding of the therapeutic benefits of Breg therapy in GVHD and their potential in aGVHD setting.

In my study, I found that PB-derived Breg subsets, transitional and IgM memory B cells share functional similarities. I propose to study the gene expression profiling of these subsets with the aim of identifying a unique signature for this population of regulatory cells, by using IL-10 cytokine capture beads to purify IL-10+B cells. The identification of a specific marker for regulatory B cell subsets may aid strategies to target the selected depletion of these cells for treatment of AML and other cancers. Finally to confirm that Bregs can attenuate or suppress cGVHD, I propose to infuse these CB-derived and PB-derived regulatory B cells in minor and major

mismatched mouse models of cGVHD to evaluate the effect of Breg infusion as a potential therapy.

In particular, murine studies of B-cell depletion have reported a preferential expansion of the Breg cell subset in the reconstituting B-cell population has (Palanichamy et al, 2009, Blair et al., 2010, Yang et al., 2013). Hence, it will be noteworthy to evaluate the effect of adoptive transfer of PB-Bregs and CB-Bregs either alone or with *in vivo* B-cell depletion as a treatment option for GVHD and other autoimmune conditions.

Further, whereas the direct administration of IL-10 has had limited beneficial effect due its short half life, the adoptive transfer of Bregs is likely to be more effective as they are able to continually secrete IL-10 (Yang et al., 2013).

I therefore, propose to develop strategies to trigger the expansion of Breg population to maintain or induce immune tolerance. To study this, I will stimulate B cells with appropriate stimulations including CD40, BCR ligation and/or CpG with growth factors and other cytokines *in vitro* for the generation of Bregs for adoptive transfer, which could play a pivotal role in immunosuppression to achieve tolerance in transplantation.

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In preparation

Sarvaria, A et al. Control of NK functions by Human IL-10 producing $CD19^{+}CD24^{hi}CD38^{hi}$ Transitional and $CD19^{+}CD27^{+}IgM^{+}$ memory cells in Acute Myeloid Leukemia

Sarvaria, A et al. IL-10 producing regulatory B cells are enriched in cord and may play a role in protection against GVHD after cord blood transplantation

Abstract Presentations and Awards

Sarvaria, A, Khoder, A, Alsuliman, A, Chew, C, Sekine, T, Cooper, N…& Rezvani, K. (2013). Immunoregulatory B Cells Are Enriched within Transitional and IgM Memory B Cell Subsets in Healthy Donors but Are Reduced and Functionally Impaired in Patients with Chronic Graft-Versus-Host Disease. American Society of Haematology (ASH) annual meeting & exposition **Poster Presentation and Abstract achievement award**

Sarvaria, A, Khoder, A, Alsuliman, A, Chew, C, Sekine, T, Cooper, N…& Rezvani, K. (2014). 'B Cells With Regulatory Function Are Enriched Within Transitional and IgM Memory B Cell Subsets In Healthy Donors But Are Reduced and Functionally Impaired In Patients With Chronic Graft-Versus-Host Disease' American Society of Bone Marrow Transplantation (BMT) Tandem Meeting. **Oral Presentation**