The role of B cells in the pathogenesis of chronic graft-versus-host disease

A thesis submitted in fulfilment of the requirements

for the degree of Doctor of Philosophy

By

Ahmad Khoder

Centre of Haematology

Division of Experimental Medicine,

Faculty of Medicine

Imperial College London

2014

Declaration of Originality

I herein, Ahmad Khoder, 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

'The copyright of this thesis rests with the author and is made available under a Creative Commons Attribution Non-Commercial No Derivatives licence. Researchers are free to copy, distribute or transmit the thesis on the condition that they attribute it, that they do not use it for commercial purposes and that they do not alter, transform or build upon it. For any reuse or redistribution, researchers must make clear to others the licence terms of this work'

Dedication

*This thesis is dedicated to my father "**Mahmoud**" and mother "**Halima**" to whom I owe my life.*

*I would also like to dedicate this work to my beloved wife "**Nahed**"; your love and care has been the secret ingredient for the success of this work. To my daughter "**Hala**" and my son "**Mahmoud**"; the joy you brought into my life has made it all worth it!*

*To my sisters and brother; **Nada, Mona, Huda and Mohammad**; thank you for everything you mean to me.*

*To **Syria**, I never thought I loved you so much!*

ACKNOWLEDGEMENTS

My utmost gratitude is to almighty ALLAH for giving me the strength and courage to finish this work.

I would like to thank my PhD supervisors; Professor Katy Rezvani, Professor Jane Apperley and Dr. Donald MacDonald, for generously supporting me during my PhD. Katy is the finest advisor and one of the smartest people I know. The unconditional support and trust she had in me has allowed the completion of this work. Katy has given me the freedom to pursue various projects and set the perfect example of the successful academic scholar. I am also sincerely grateful to Jane who has been very supportive and caring throughout the years of my PhD. I am indebted to her rich experience and genuine kindness which I was privileged to have whilst she mentored me during clinical training.

Very special thanks go to my dear friends and colleagues; Abdullah Alsuliman, Asad Luqmani, Talat Malik, Mustafa Daghistani and Basim Alshammari, who made this journey pleasant and knowledgeable.

I would also like to thank my colleagues in Prof. Rezvani's lab for their joyful friendship, practical solutions and willingness to share their expertise in various laboratory techniques: Hugues de Lavallade, Takuya Sekine, Kate Stringaris, Paula Garland, Ian Gabriel, Sarvaria Anushruthi and Muharram Muftuoglu.

Finally, I wish to express gratitude to the staff, nurses and doctors of the Hammersmith Hospital, London for providing samples even during busy times.

Abstract

Chronic graft-versus-host disease (cGVHD) remains a major complication of allogeneic haematopoietic stem cell transplantation. Traditionally, GVHD has been approached as a T-cell-mediated process and current therapies reflect this understanding. The successful treatment of a proportion of refractory cGVHD patients with a CD20-depleting-agent supports a pathological role for B cells. To study the role of B cells in cGVHD, I designed a comprehensive multi-parameter panel of surface antibodies to distinguish different B cell subsets. The panel was validated on a number of healthy volunteers and applied to peripheral blood samples from cGVHD patients. I showed that patients with active chronic GVHD have an expanded population of $CD21^-$ with an 'exhausted' phenotype, based on upregulation of inhibitory receptors, altered expression of chemokine receptors and poor proliferative capacity. A similar subset of $CD21^-$ exhausted B cells was described in patients with chronic HIV viraemia. Interestingly, there was a significant correlation between frequencies of $CD21^-$ B cells and the severity of cGVHD. Further work to identify the potential antigens that drive the expansion of these cells is currently underway. I also explored the role of IL10 producing regulatory B cells (Breg); powerful regulators of immune responses. A number of B cell subsets have been described in murine models of autoimmunity and some human autoimmune diseases. I investigated the presence of IL10 producing B cells in the peripheral blood of healthy donors. Using intracellular cytokine staining for IL10 and surface phenotyping, I showed that IL10 producing B cells are enriched within the $CD27^+$ IgM^{hi} (IgM memory) B-cell subset. The purified populations of IgM memory B cells had regulatory properties and are capable of suppressing $CD4^+$ T cell proliferation and IFNγ in vitro. Finally, I showed that B cells from cGVHD patients produce significantly less IL10 compared with both HC and "no-GVHD" patients. I thereby propose that B cells play a significant role in the pathogenesis of cGVHD. Ex vivo expanded and adoptively transferred IgM memory B-cells may offer a potentially effective immunomodulatory therapy for the treatment of refractory cGVHD.

Table of Contents

DECLARATION OF ORIGINALITY 2

COPYRIGHT DECLARATION 3

DEDICATION 4

ACKNOWLEDGEMENTS 5

ABSTRACT 6

TABLE OF CONTENT 7

LIST OF FIGURES 14

LIST OF TABLES 17

LIST OF ABBREVIATIONS 18

CHAPTER I. INTRODUCTION 23

I.1 Allogeneic Haematopoietic Stem cell Transplantation (AHSCT) 23

I.2 Graft-versus Host Disease (GVHD) and Graft-versus Leukaemia (GVL) 24

I.3 B cell development: 26

I.4 Peripheral blood B cell subsets: 29

I.4.1 Immature and transitional B cells: 29

I.4.2 Mature B cells; naïve and memory B cells: 30

I.4.2.1 Naïve B cells: 30

I.4.2.2 Memory B cells 32

I.5 IgM^+ IgD^+ $CD27^+$ memory B cells; "IgM memory B cells": 35

I.5.1 IgM memory B cell characteristics and phenotype: 35

I.5.2 IgM memory B cells in pathological conditions: 36

I.5.3 IgM memory B cells; source and maintenance: 37

I.5.4 IgM memory B cell development: 38

I.5.5 IgM memory B cell ontogeny: 39

I.5.6 IgM memory B cell function: 41

I.6 CD21 ⁻B cells: 45

I.6.1 CD21 (CR2) molecule: 45

I.6.2 CD21 expression by peripheral blood B cells: 46

I.6.3 Loss of CD21 expression: 46

I.6.4 Low CD21 expression and B cell exhaustion: 48

I.7 B cell and Graft versus host disease: 51

I.7.1 Stem cell transplantation (SCT): 51

I.7.2 GVHD- clinical manifestations and classification: 52

I.7.3 Pathophysiology of GVHD: 54

I.7.4 B cell in cGVHD 56

I.7.4.1 B cell reconstitution following SCT 56

I.7.4.2 B cells disturbances in human GVHD: 58

I.7.4.2.1 The role of B cells in GVHD- mouse models: 58

I.7.4.2.2 The role of B cells in human aGVHD: 59

I.7.4.2.3 The role of B cells in human cGVHD: 59

I.7.4.2.4 Chronic GVHD effects on B cells 62

I.8 Regulatory B cells 64

I.8.1 Evidence for regulatory B cells: 64

I.8.2 B cells and immune regulation; the regulatory B cell: 65

I.8.3 Regulatory B cell functions: 67

I.8.4 Regulatory B cells: polyclonal versus antigen specific 68

I.8.5 Regulatory B cells in mouse models: 70

I.8.5.1 Induction of regulatory phenotype in B cells: 70

I.8.5.2 Regulatory IL10^{+} B cell phenotype(s): 71

I.8.5.3 IL10 independent Breg cells: 72

I.8.6 Human regulatory B cells: 73

I.8.6.1 Human IL10^{+} regulatory B cells: subset(s) and phenotype(s) 73

I.8.6.2 Human regulatory B cells in disease conditions: 75

HYPOTHESIS AND AIMS 78

CHAPTER II. MATERIAL AND METHODS 79

II.1 Patients: 79

II.2 Sample processing: 79

II.2.1 Isolation of peripheral blood mononuclear cells: 79

II.2.2 Plasma collection: 80

II.2.3 Cell freezing: 80

II.2.4 Cell thawing: 80

II.3 Cell lines- L cells and control cells: 80

II.4 B cell culture and activation protocol: 83

II.4.1 ELISA experiments: 83

II.4.2 IL10 quantitative ELISA: 83

II.5 Cell selection 84

II.5.1 B cell selection: 84

II.5.2 T cell selection: 86

II.5.2.1 Total CD4^{+} T cell selection: 86

II.5.2.2 $CD4^{+}CD127^{lo}CD25^{-}$ and Treg magnetic selection "Treg depletion": 87

II.6 Cell sorting: 89

II.6.1 Sample preparation and sorting: 89

II.7 Proliferation Assay: 93

II.8 Phenotypic and functional characterization of B cells using flow cytometry: 96

II.8.1 Intracellular cytokine assay for detection of $IL10^{+}$ B cells and phenotyping: 96

II.8.2 Phenotypic analysis of IL10 producing B cells: 97

II.8.3 Phenotypic characterization of B cells: 98

II.8.3.1 Antibody titration: 99

II.8.3.2 Cell staining and FACS acquisition: 102

II.9 In vitro suppression Assay: 106

II.9.1 Proliferation Suppression: 106

II.9.2 Suppression of cytokine production: 107

II.9.3 IL10 blocking: 108

CHAPTER III. B CELL SUBSET AND CHARACTERIZATION OF $CD21^{-}$ B CELLS IN HEALTHY CONTROLS 109

III.1 Introduction 109

III.2 Results 112

III.2.1 Titration of monoclonal antibodies and flow cytometry settings 112

III.2.2 Characterization of human B cell subsets and panel validation 114

III.2.2.1 Transitional B cells 114

III.2.2.2 Naïve B cells 117

III.2.2.3 Memory B cells 119

III.2.2.4 Plasmablasts ____ 121

III.2.2.5 CD21$^-$ B cells ____ 122

III.2.3 Peripheral blood B cell subset frequencies ____ 124

III.2.3.1 B cell subsets ____ 124

III.2.3.2 CD21$^-$ B cells ____ 126

III.2.3.3 Gender has no effect of B cell subsets ____ 127

III.2.3.4 Effect of Cryopreservation of the frequencies of B cell subsets ____ 127

III.3 Discussion ____ 130

CHAPTER IV. IL10 PRODUCING B CELLS ____ 134

IV.1 Introduction ____ 134

IV.2 Results ____ 136

IV.2.1 In vitro production of IL10 by B cell ____ 136

IV.2.1.1 IL10 production by B cells from fresh PBMC: ____ 136

IV.2.1.2 Effect of cryo-preservation on in vitro IL10 detection by ELISA: ____ 138

IV.2.2 Intracellular staining for interleukin 10 in human B cells: ____ 138

IV.2.2.1 Optimizing negative controls for IL10 intracellular staining ____ 139

IV.2.2.2 Optimization of IL10 intracellular staining technique ____ 140

IV.2.3 Kinetics of inducible IL10 production using intracellular staining ____ 142

IV.2.3.1 Anti-CD3/ anti-CD28 stimulated CD4 T cells upregulated surface expression of CD154 ____ 142

IV.2.3.2 Stimulation with activated T cells ____ 142

IV.2.3.3 Stimulation with L cells with and without anti-BCR ____ 143

IV.2.4 Comprehensive phenotyping of human IL10$^+$ B cell ____ 144

IV.2.5 Effect of cryopreservation on IL10 IC staining ____ 148

IV.2.6 Functional assays ____ 150

IV.2.6.1 Sort purification of B cell subsets ____ 150

IV.2.6.2 Suppression of $CD4^+$ T cell proliferation ____ 153

IV.2.6.3 The suppressive property of IgM memory B cells is cell dose dependent ____ 156

IV.2.6.4 IgM memory B cells suppressed IFNγ but not TNFα production by $CD4^+$ T cells ____ 157

IV.2.6.4.1 IgM memory B cells Suppress $IFN\gamma^+$ $CD4^+$ T cells ____ 158

IV.2.6.4.2 IgM memory B cells do not suppress $TNF\alpha^+$ $CD4^+$ T cells ____ 159

IV.2.6.4.3 IgM memory B cell induced suppression is comparable to Treg ____ 160

IV.2.6.5 Direct $CD4^+$ T cells suppression by IgM memory B cells is not dependent on the presence of Tregs in the culture system ____ 163

IV.2.7 Suppressive effects is IL10 dependent and cell specific ____ 165

IV.2.7.1 IL10 blocking experiments: ____ 165

IV.2.7.1.1 Effect of IL10 blocking on IFNγ production by $CD4^+$ T cells ____ 165

IV.2.7.1.2 Effect of IL10 blocking on $TNF\alpha^+$ $CD4^+$ T cell frequencies ____ 168

IV.3 Discussion ____ 169

CHAPTER V. IL10 PRODUCING B CELLS IN CGVHD ____ 173

V.1 Introduction ____ 173

V.2 Results ____ 175

V.2.1 Patients characteristics ____ 175

V.2.2 Chronic GVHD is associated with reduced $IL10^+$ B cell population ____ 176

V.2.2.1 B cells from cGVHD patients have impaired in vitro IL10 responses to CD154 stimulation in comparison with “no GVHD” and HC groups ____ 176

V.2.2.2 B cells from cGVHD patients produce Less IL10 in response to stimulation with CD40L ____ 179

V.2.3 Chronic GVHD patients have comparable relative IgM memory B cell population ____ 180

V.2.4 $IFN\gamma^+$ $CD4^+$ T cells are increased in post-transplant patients ____ 181

V.2.5 Active cGVHD is characterised by increased $IFN\gamma^+$ $CD4^+$ T cell / $IL10^+$ B cell ratio ____ 183

V.3 Discussion 185

CHAPTER VI. GVHD AND B CELLS 189

VI.1 Introduction: 189

VI.2 Results: 191

VI.2.1 Patient Characteristics: 191

VI.2.2 B cells subsets in cGVHD: 195

VI.2.2.1 Chronic GVHD patients have lower number of circulating B cells: 195

VI.2.2.2 Chronic GVHD patients have lower number of naive B cells: 196

VI.2.2.3 Chronic GVHD patients have higher percentage of $CD27^+$ B cells 197

VI.2.2.4 Chronic GVHD patients have higher frequencies of $CD21^-$ B cells: 199

VI.2.2.5 Phenotypic characterization of $CD21^-$ B cells in cGVHD patients: 202

VI.2.2.6 B cells from cGVHD patients have impaired proliferative potential: 206

VI.2.2.7 The frequency of $CD21^-$ B cells correlates with cGVHD severity: 209

VI.2.3 Discussion: 211

CHAPTER VII. GENERAL DISCUSSION AND FUTURE PLANS 216

VII.1 General Discussion 216

VII.2 Conclusion 227

VII.3 Future Plans 228

REFERENCES: 230

LIST OF PUBLICATIONS 259

List of Figures

Figure I-1: B cell development 28

Figure I-2 : Regulatory mechanisms of IL10-producing B cells 69

Figure I-3: Phenotypic profiles of IL10-producing Breg in mice 73

Figure II-1: CD154 expressing L cells and their controls 82

Figure II-2: Example of standard curves for IL 10 detection 84

Figure II-3: Purity checks of B cell selection 85

Figure II-4: Purity checks of T cell selection 87

Figure II-5: Purity check of Treg selection 89

Figure II-6: Gating strategies of B cell sorting 91

Figure II-7: Purity checks of B cell sorting 92

Figure II-8 : CD4 and CD19 proliferation assay 95

Figure II-9: Intracellular staining of IL10 98

Figure II-10 : stain index 101

Figure II-11: Antibody titration: CD21 APC 102

Figure II-12 : Gating strategies of B cell surface phenotyping 104

Figure III-1: $CD19^+$ B cell classifications 111

Figure III-2: Stain index and MFI: CD21 APC 113

Figure III-3: Surface expression of B cell markers 115

Figure III-4: Transitional B cell subsets: T1, T2 and T3 116

Figure III-5: B cell subsets correlation with different B cell classifications 118

Figure III-6: Memory B cells 121

Figure III-7: IgM/CD27 B cell classification scheme 122

Figure III-8: CD21^{-} CD19^{+} B cells inter-individual variation _____ 123

Figure III-9: B cell subsets in healthy controls _____ 125

Figure III-10: Cryopreservation effect on B cell subset phenotypes _____ 129

Figure IV-1: IL 10 ELISA_____ 137

Figure IV-2: Comparison of intracellular staining negative controls _____ 140

Figure IV-3: IL10 IC staining of CD19^{+} B cells _____ 141

Figure IV-4: Inducible expression of CD154 by activated CD4^{+}T cells _____ 143

Figure IV-5: Determination of optimal time point for IL10^{+} B cell study_____ 145

Figure IV-6: Phenotypic characterization of IL10^{+}CD19^{+} B cells _____ 147

Figure IV-7: IL 10 IC staining in frozen samples _____ 149

Figure IV-8: Gating strategy of B cell sorting _____ 152

Figure IV-9: Proliferation suppression assay (CD4^{+} T cells) _____ 155

Figure IV-10: IFNγ suppression assay (CD4^{+} T cells)_____ 159

Figure IV-11: TNFα suppression assay (CD4^{+} T cells) _____ 160

Figure IV-12: Breg suppression capacity is comparable to Treg _____ 162

Figure IV-13: Proliferation suppression assay (Treg)_____ 164

Figure IV-14: IgM^{+}CD27^{+} CD19^{+} B cell suppression is dependent on IL10. _____ 167

Figure V-1: cGVHD is associated with lower IL10 production _____ 178

Figure V-2: B cells from cGVHD patients produce Less IL10 in response to stimulation with CD40L_____ 180

Figure V-3: IFNγ^{+} CD4^{+} T cells post SCT _____ 182

Figure V-4: IFNγ^{+}CD4^{+}/IL10^{+} CD19^{+} ratio in patients with and without cGVHD _____ 183

Figure VI-1: lymphocyte and $CD19^{+}$ B cell counts among patients with and without cGVHD and HC _____ 195

Figure VI-2: B cell subsets in “no GVHD”, cGVHD and a HC _____ 197

Figure VI-3: Comparison of B cell subset frequencies in “no GVHD” patients, cGVHD patients and healthy controls _____ 198

Figure VI-4: CD21 expression examination in cGVHD, no GVHD and HC _____ 201

Figure VI-5: Inhibitory and chemokine receptor profile of naïve, classical memory and $CD21^{-}$ B cells _____ 205

Figure VI-6: Proliferation assessment of B cells in no GVHD, GVHD and HC _____ 208

Figure VI-7: The frequency of exhausted B cells per cGVHD grades _____ 210

Figure VII-1: Schematic flow chart summarizes the proposed mechanisms involved in B cell exhaustion in chronic GVHD. _____ 227

List of tables

Table I-1: NIH consensus criteria for GVHD diagnosis _____ 53

Table II-1: antibodies used for B cell characterization: _____ 105

Table II-2: experimental conditions for CFSE suppression assay_____ 107

Table II-3: Experimental conditions for cytokine production suppression assay ____ 108

Table III-1: titrated antibodies' volumes in the final master mix _____ 113

Table VI-1: Patient characteristics _____ 193

Table VI-2: chronic GVHD characteristics _____ 194

Table VI-3: Absolute counts of cell subsets in "no GVHD" patients, cGVHD patients and healthy controls _____ 199

Table VI-4: NIH-defined cGVHD grade and exhausted B cells percentage, based on a cut off of 5%. _____ 210

List of Abbreviations

ABCB1	ATP-binding cassette B1
Ag	antigen
AHSCT	Allogeneic Hematopoietic Stem Cell Transplantation
AID	activation induced deaminase
ANOVA	analysis of variance
APC	antigen presenting cells or allophycocyanin
BAFF	B cell activating factor
BCR	B cell receptor
BD	Becton Dickinson
BFA	Brefeldin A
BM	Bone marrow
Breg	Regulatory B cells
CCR	Chemokine receptor
CD	cluster of differentiation
CFSE	carboxyfluorescein diacetate succinimidyl ester
CIA	collagen induced arthritis
Cntl	control cells
CpG	cytosine-p-guanine dinucleotide
CR	complement receptor
CsA	cyclosporine A
CVID	common variable immune deficiency

CXCR	CXC chemokine receptor
DC	dendritic cells
DLI	donor lymphocyte infusion
DMSO	dimethylsulphoxide
EAE	experimental autoimmune encephalomyelitis
ECP	extracorporeal photopheresis
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FCR-L4	Fc receptor–like 4
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FSC	forward side scatter
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

IBD	inflammatory bowel disease
ICOS	inducible co-stimulator
IFNγ	Interferon-gamma
Ig	immunoglobulin
IL	interleukin
Iono	Ionomycin
ITP	idiopathic thrombocytopaenic purpura
KO	knockout
L_D	live dead marker
LN	Lymph node
LPS	Lipopolysaccharides
mAb	monoclonal antibodies
MFI	median fluorescence intensity
MHC	major histocompatibility antigens
mHA	minor histocompatibility antigens
µl	microlitre
MS	multiple sclerosis
MSC	mesenchymal stem cells
mTOR	mammalian target of rapamycin
MZ	marginal zone
MZB	marginal zone B cells
MZP	marginal zone precursors

NF-κB	nuclear factor kappa- B
NK	natural killer
NKT	natural killer T cells
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBSCT	Peripheral blood stem cell transplantation
PC	positive control
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
Pg	picogram
PI	propidium iodide
PMA	phorbol 12-myristate 13-acetate
PNH	paroxysmal nocturnal haemoglobinuria
RF	relative frequency
RIC	reduced intensity conditioning
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
SHM	somatic hypermutation
siRNA	small interfering RNA
SLE	systemic lupus erythematosus
SSC	side scatter

Tr	Transitional B cells
TD	T cell dependent
TGFβ	transforming growth factor beta
Th	T helper
TI	T cell independent
TLR	Toll-like receptor
TNFα	tumour necrosis factor alpha
Treg	regulatory T cells

Chapter I. Introduction

I.1 Allogeneic Haematopoietic Stem cell Transplantation (AHSCT)

Allogeneic hematopoietic stem cell transplantation (AHSCT) is currently the only curative option for numerous haematological diseases. Since the first successful allogeneic bone marrow transplantation in humans in 1968(Bach et al., 1968, Gatti et al., 1968), the indications for AHSCT have been continuously growing to include immune deficiency syndromes, bone marrow failure syndromes, metabolic disorders, enzymatic deficiencies, autoimmune conditions, solid tumours and malignant and non-malignant haematological diseases (Tan et al., 2007). The introduction of reduced intensity conditioning protocols, growing cumulative experience of transplantation centres, and well-established supportive care practices have extended its use to older patients and a new spectrum of medical conditions (Resnick et al., 2005). In Europe alone, over 24,000 stem cell transplants are currently performed annually (ESH-EBMT, 2008, Tan et al., 2007).

Despite the high curative potential of AHSCT, its wide application in clinical practice is offset by the associated high rate of complications (Pallera and Schwartzberg, 2004) and mortality (Gratwohl et al., 2005). Careful consideration of the multiple interacting factors is therefore crucial before a decision is made to proceed to transplant (Ringden et al., 2003).

The therapeutic effect of AHSCT is attained through the myeloablative and immunosuppressive qualities of the conditioning regimen on the recipient haematopoietic system (Georges and Storb, 2003). Furthermore, the immunological

disparity between the donor and recipient gives rise to the graft-versus-leukaemia effect (Slavin, 2003, Alousi and de Lima, 2007), albeit at the risk of developing graft versus host disease (Resnick et al., 2005). Achieving GVL without the detrimental risk of GVHD is the ultimate goal in allogeneic stem cell transplantation.

I.2 Graft-versus Host Disease (GVHD) and Graft-versus Leukaemia (GVL)

The beneficial effect of the alloimmune GVL effect was first observed in transplant murine models in the 1950s when it was shown that irradiated mice receiving bone marrow from a different strain of mice died of a wasting diarrhoeal syndrome, now known as GVHD, but without any signs of residual leukaemia(Barnes and Loutit, 1957). Later studies in human leukocyte antigen (HLA)-matched AHSCT provided evidence that both GVL and GVHD effects result from the cellular allo- immune responses induced by disparities of minor histocompatibility antigens (mHA) between recipient and donor (Mutis et al., 2010). This observation was first harnessed clinically in the 1990s by demonstrating the curative potential of donor lymphocyte infusion (DLI) in relapsed leukaemia following AHSCT (Kolb et al., 1995, Deol and Lum, 2010, Helg et al., 1998).

A number of leukaemia-specific antigens and mHA have been described and are believed to be the targets for the cytotoxic T lymphocytes (CTL) responses in GVL (Mutis and Goulmy, 2002). However, in the majority of cases the beneficial GVL effect is associated with the harmful effects of GVHD.

Graft versus host disease continues to be a major cause of morbidity and mortality following AHSCT (Gratwohl et al., 2005). Similar to the pathophysiology of GVL, GVHD involves activation of donor T-cells directed against mismatched mHA presented on primed recipient antigen presenting cells (APC) early after AHSCT (Shlomchik, 2007). Much work has been centred around the role of T cells in the pathogenesis of GVHD and clinical practice reflects this paradigm; for instance, T cell depletion of the graft and T cell-directed immunosuppression form the cornerstone of the prevention and treatment of GVHD (Ferrara et al., 2009); although, in some cases without significant success (Khaled et al., 2009).

Recent data support the involvement of B cells in the pathogenesis of GVHD (Shimabukuro-Vornhagen et al., 2009). However, more insight into their role is required to help tailor more specific treatments with the aim of improving the overall outcome of AHSCT.

I.3 B cell development:

Differentiation of irreversibly committed PAX5 expressing B cell precursors, that derive from the common lymphoid progenitors, is guided by several micro-environmental components and cellular interaction in the bone marrow niches (Nagasawa, 2006). After the rearrangement of the D_H (diversity) and J_H (junction) segments of Ig heavy chain gene, $CD34^+CD19^+$ pro B cells undergo further rearrangement of the V_H segment, yielding a pre B cell. Upon coupling of cytoplasmic μH (rearranged DJV segments) with surrogate light chain Vpre B and λ 5 components, Pre B cells express Pre B cell receptor (pre-BCR) at a later stage of their differentiation; **figure (I-1)** (Cambier et al., 2007). Signalling from the pre-BCR prevents further recombination and promotes positive selection and proliferation. In contrast, a failure of pre-BCR signalling will cause developmental arrest, apoptosis or receptor editing (Edry and Melamed, 2004). This stage of development is Ag independent and takes place in the bone marrow stromal environment with a central role for IL7 (Maddaly et al., 2010). Hardy and colleagues illustrated that the stepwise transition is marked by differential expression of surface markers (Hardy et al., 1991). Pre B cells then initiate preferentially κ light chain rearrangement resulting, when successful, in surface IgM and later IgD co-expression; namely the BCR (Marie-Cardine et al., 2008). The release of immature B cells to the peripheral blood is strictly governed by negative selection checkpoints where auto-reactive B cells are removed from the overall B cell repertoire (Sims et al., 2005, Hartley et al., 1991, Duty et al., 2009). In this process, the vast majority of the newly produced immature B cells, i.e. transitional B cells, die rapidly while the surviving B cells make their way to the peripheral blood (Forster and Rajewsky, 1990, Allende et al., 2010,

Wardemann et al., 2003). Transitional B cells mature mainly in the splenic micro-environment into follicular or marginal zone B cells depending on the strength of BCR signalling and Ag specificity (Allman and Pillai, 2008, Carsetti et al., 2004). Ag activated naïve B cells can receive T cell help to form germinal centres and undergo isotype switch and somatic hyper-mutation. This process produces memory B cells, short and long-lived, and plasma cells and forms the basis for the adaptive humoral immune response (Good-Jacobson and Shlomchik, 2010). Marginal zone B cells on the other hand, are T cell independent with regards to Ag response and bear lower levels of somatic mutation (Weill et al., 2009). A better understanding of B cell development is essential to improve the outcome of therapy for a number of non-malignant and malignant medical conditions involving B cell disorders (Vale and Schroeder, 2010).

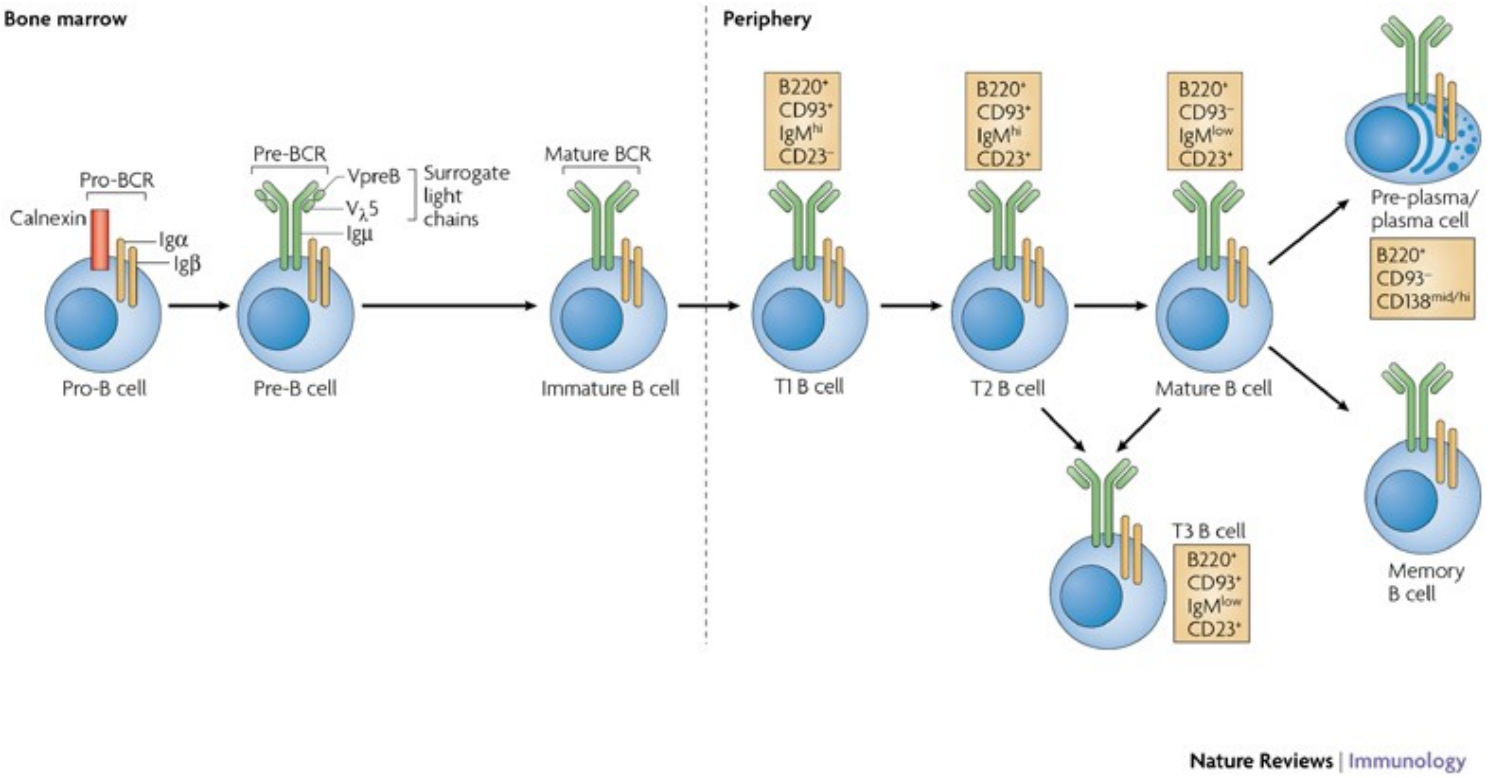


Figure I-1: B cell development
Schematic figure shows the stages of B cell development in both bone marrow and the periphery. Adopted from Cambier et al (Cambier et al., 2007).

I.4 Peripheral blood B cell subsets:

I.4.1 Immature and transitional B cells:

Transitional B cells, the early emigrants from the bone marrow, are functionally immature i.e. short lived and undergo apoptosis rather than proliferation upon BCR engagement (Sims et al., 2005, Palanichamy et al., 2009). The transition from a less to more mature stage within the transitional B cell populations is associated with considerable cell loss (Allman et al., 2001). Transitional B cells play an important role in repopulating the B cell ontogeny following bone marrow transplantation (Marie-Cardine et al., 2008) and B cell depletion therapy (Anolik et al., 2007). In mice spleen, two distinct transitional B cell populations, T1 and T2 cells, were characterised based on their high expression of both heat-stable antigen (HSA, CD24) and IgM and the differential higher expression of CD21, CD23 and IgD in T2 (Loder et al., 1999). Allman et al identified 3 subsets based on the expression of the B-lineage precursor marker CD93/AA4, CD23 and IgM (Allman et al., 2001). Furthermore, Meyer-Bahlburg et al, using CD21 expression, divided transitional B cells into two phenotypically and functionally distinguished $CD21^{lo}$ T1 and $CD21^{int}$ T2 populations (Meyer-Bahlburg et al., 2008).

Similarly in humans, two subsets of transitional B cells that phenotypically resemble murine T1 and T2 were described in the peripheral blood (Sims et al., 2005). A third subset (T3) defined by lack of the ATP-binding cassette Transporter (ABCB1) was later characterised in the peripheral blood following rituximab therapy and considered a developmental intermediate between T2 and mature B cells (Palanichamy et al., 2009). Since the majority of transitional B cells are self-reactive, they are subject to rigorous

early negative and later positive selection processes which take place principally in the bone marrow (Hartley et al., 1991) and spleen (Meyer-Bahlburg et al., 2008).

Transitional B cells are found in the bone marrow, mostly (T1), spleen (T2), peripheral blood and cord blood mainly (T3) (Palanichamy et al., 2009) and in small numbers in lymph nodes (Carsetti et al., 2004). Maturation of transitional B cells to follicular naive B cells or marginal zone B cells occurs in the spleen, depending on the strength of BCR signalling (Carsetti et al., 2004, Weill et al., 2009). Moreover, there is some evidence to suggest that transitional B cells could mature to IgM producing memory cells *in vitro* upon TLR activation (Capolunghi et al., 2008).

Transitional B cells constitute 2.2 to 7 % of circulating human B cells (Sims et al., 2005, Palanichamy et al., 2009, Marie-Cardine et al., 2008). They could be identified in the peripheral blood by their high expression of two developmentally regulated markers, CD24 and CD38(Carsetti et al., 2004, Palanichamy et al., 2009), or $CD38^{hi}$ IgD^{+} (Sims et al., 2005) within the $CD19^{+}$ population. Transitional B cells are negative for CD27 and express increasingly CD44, CD21, CD23, CD22 and decreasing levels of IgM, CD5, and CD10 in accord with their maturation (Palanichamy et al., 2009).

I.4.2 Mature B cells; naïve and memory B cells:

I.4.2.1 Naïve B cells:

Further maturation of transitional B cells to naive B cells bearing functional BCR in germline configuration occurs in the spleen (Pascual et al., 1994). The functional maturity is reflected by acquisition of surface IgD expression, longer life and proliferation upon BCR engagement (Allman et al., 2001). Naive B cells account for 40 to

60% of total B cells and express $CD24^{int}$, $CD38^{int}$ and CD21 and are negative for CD27, CD10 and CD5 (Carsetti et al., 2004, Palanichamy et al., 2009). Naive B cells can be discriminated from $CD27^{-}$ memory B cells by their expression of the ABCB1 (Wirths and Lanzavecchia, 2005).

Mature B cells attain the capacity to circulate in the periphery and home to and survive in the follicular niches of the secondary lymphoid tissue (BAFF dependent) and around the vascular sinusoids in the bone marrow (BAFF- independent) (Allman and Pillai, 2008). Having escaped the elimination checkpoints, naïve B cells have high affinity to specific non-self-antigens. However, only a minority of auto-reactive anergic naive B cells expressing IgD can normally be found in the periphery (Duty et al., 2009). Activation of the follicular B cells requires BCR engagement and CD40 signalling (T cell dependent TD) (Allman and Pillai, 2008). In the TD germinal centre, Ag-specific B cells undergo numerous rounds of division, somatic hyper-mutation (SHM), class-switch recombination, and affinity selection. Several hypotheses have been proposed to explain why some activated B cells differentiate into memory or plasma cells; however the determining factors remain unclear (Good-Jacobson and Shlomchik, 2010).

Lower Ag affinity and weaker BCR signalling direct T2 B cells to differentiate towards marginal zone B cells (Carsetti et al., 2004, Weill et al., 2009). Recently, Srivastava et al proposed that marginal zone B cells are derived from $CD21^{hi}CD23^{+}$ intermediate precursors enriched within the T2 population (Srivastava et al., 2005). Marginal zone B cells (MZB), located to the marginal zone in the spleen, are innate-like cells and mediate a T cell independent response to blood borne lipid antigens, differentiating into short-

lived plasma cells (Allman and Pillai, 2008). Marginal zone B cells share a similar phenotype to the so-called IgM^+IgD^+ memory B cells and are $CD27^+$ (Weill et al., 2009).

I.4.2.2 Memory B cells

Memory B cells are large Ag experienced cells, that secrete Ig following stimulation, persist in the absence of the immunising agents and respond rapidly during a secondary response (Good-Jacobson and Shlomchik, 2010, Weill et al., 2009). They are characterised by the acquired somatic mutation in their rearranged Ig V region genes. A direct link between somatic hyper-mutation and surface CD27 expression was established by Klein and co-workers (Klein et al., 1998, Tangye et al., 1998). CD27 expression has, therefore, been used as a universal marker for memory B cells. However, CD27 is expressed briefly at early stages of B cell development in the bone marrow; pro‐/pre‐B cells. These cells do not normally circulate in the peripheral blood and can be identified by flow cytometry based on the expression of CD34 and the lack of BCR expression (Vaskova et al., 2008, Scheeren et al., 2008).

Therefore, the classification of memory B cells on the basis of a single marker expression is far too simplistic (Sanz et al., 2008). About a third of the circulating memory B cells carrying mutated BCRs are $CD27^-$ (Fecteau et al., 2006). The mutation rate of this subgroup of memory cells is somewhat lower than in $CD27^+$ memory B cells, possibly as a result of incomplete GC reaction. The origin and function of this category of cells is yet to be understood (Sanz et al., 2008). $CD27^-$ memory B cells are larger than naive B cells, are isotype switched i.e. IgG^+ and express higher levels of CD80 and CD86 (Tangye and Good, 2007, Fecteau et al., 2006).

Furthermore, Shlomchik and colleagues have identified, using a transgenic Ag specific BCR knock-in mouse model, that Ag specific memory B cells contained both mutated as well as un-mutated memory B cells with the latter being enrich in the $CD35^{+}CD80^{+}$ fraction (Anderson et al., 2007).

In the peripheral blood of healthy people, $CD27^{+}$ memory B cells can be divided into two major groups, $IgM^{-}IgD^{-}$, isotype switched, and $IgM^{+}IgD^{+}$ memory B cells. Isotype switched memory B cells are the final product of a successful T-cell dependent (TD) germinal centre reaction (Good-Jacobson and Shlomchik, 2010). On the other hand, $IgM^{+}IgD^{+}$ memory B cells develop in the marginal zone of the spleen (Weller et al., 2004). Contrary to the isotype switched memory B cells, $IgM^{+}IgD^{+}$ memory B cells show a higher clonal diversity, comparable to naive B cells, while harbouring somatic mutations. Upon activation with a polysaccharide antigen, this pre-diversified subset of memory cells produces IgM, IgG and IgA, independent of T cell help (Weill et al., 2009, Weller et al., 2004).

Two smaller subsets of $CD27^{+}$ B cells exist in the peripheral blood; namely, IgM only and IgD only memory B cells. IgM only memory B cells, occasionally confused with $IgM^{+}IgD^{+}$ memory B cells , are thought to be GC derived and the direct precursor of the isotype switched cells (Weill et al., 2009). Similarly, a small population of germinal centre memory cells have class-switched to IgD at the genetic level referred to as Cδ-CS; these cells are enriched in auto-reactivity (Koelsch et al., 2007).

When defined by CD27 expression, memory B cells represent 30-60% of total B cells with equal shares of each major subset (10-15%) (Sanz et al., 2008, Weill et al., 2009). IgD only and IgM only memory cells account for a small percentage in healthy subjects and

may be expanded in some autoimmune conditions e.g. SLE (Weill et al., 2009). The $CD27^-$ memory B cells can be recognised by negativity for CD27, IgD and IgM and account for 5% of total B cells i.e. around a third of total IgG^+ and IgA^+ isotype switched memory B cells (Sanz et al., 2008, Weill et al., 2009, Wei et al., 2007). Memory B cells express $CD24^{int}$, $CD38^{int}$, however, the level of expression of these markers varies relative to CD27 expression (Sanz et al., 2008).

Germinal centre derived memory B cells differentiate to early (T cell dependent) and late (BAFF dependent) Ig secreting plasmablasts upon re-exposure to a specific antigen. This process is associated with early up-regulation of CD27, followed by acquisition of division-linked CD38 in the late phase (Avery et al., 2005). Plasmablasts represent a minor subset in the peripheral blood accounting for around 1% of total B cells (Piatosa et al., 2010).

Memory B cells survive in secondary lymphoid tissues and mediate secondary responses upon re-challenge with specific antigens. Plasma cells, on the other hand, are terminally differentiated cells that home to spleen and bone marrow and are the main providers for Ig. However, it is unlikely that long-lived plasma cells will maintain a persistent supply of specific antibody over the human lifetime. Lazavache and colleagues proposed that constant polyclonal activation of memory B cells via bystander T cells and CpG DNA provides a means to preserve serological memory for a human lifetime (Bernasconi et al., 2002).

I.5 IgM$^+$ IgD$^+$ CD27$^+$ memory B cells; "IgM memory B cells":

I.5.1 IgM memory B cell characteristics and phenotype:

IgM memory B cells possess somatically hypermutated variable region genes but have not undergone class switching (Tangye and Good, 2007, Moens et al., 2008). IgM memory B cells are large cells with abundant cytoplasm (Shi et al., 2003, Takizawa et al., 2006) and constitute 40 to 50% of CD27$^+$ B cells in the peripheral blood (Lucas, 2004, Weller et al., 2005). The number of IgM memory B cells in the peripheral blood is low after birth (Agematsu et al., 1997) and increases gradually during the first year of life (Kruetzmann et al., 2003, Weller et al., 2003). Some investigators detected IgM memory B cells in neonatal cord blood, foetal liver, LNs and BM at 14 and 16 weeks of gestation, however, others could not detect IgM memory B cells in cord blood (Kruetzmann et al., 2003). The number peaks at the age of 30 to 40 years and declines remarkably after 70 years of age (Shi et al., 2003, Shi et al., 2005).

IgM memory B cells are IgM^{hi}, IgD$^+$, CD1c$^+$, CD23$^-$, CD21^{hi}, CD27$^+$ and CD5$^-$ (Weill et al., 2009, Weller et al., 2004, Won and Kearney, 2002). The higher expression level of CD21 (complement receptor 2) compared to follicular B cells and recirculating mature naive B cells (Pillai and Cariappa, 2009, Capolunghi et al., 2008) is necessary to recognise bacterial polysaccharides, coupled with complement component C3d (Zandvoort et al., 2001). Membrane-bound IgM i.e. B cell receptor on the surface of IgM memory B cells, is in a monomer or dimer configuration in contrast to the pentamer structure of the secreted IgM (Ehrenstein and Notley, 2010, Pierce and Liu, 2010).

Compared with switched memory B cells, IgM memory B cells have higher surface expression of CD72 (but lower than in naive B cells), lower CD80 and absent CD86 and CD95 expression (Shi et al., 2003). IgM memory B cells express chemokine receptors: CXCR4, CXCR5 and CCR7 (Tangye and Tarlinton, 2009). In contrast to switched memory B cells which produce IgM, IgA and IgG upon in-vitro stimulation, IgM memory B cells do not switch isotype and predominantly produce IgM (Shi et al., 2005). This subset of B cells cannot be induced to differentiate into switched memory B cells under different in-vitro experimental conditions. Upon stimulation, IgM memory B cells mature to $CD20^-$ IgM producing plasmablasts with higher CD27 and CD38 expression (Capolunghi et al., 2008, Shi et al., 2003).

Some authors define IgM memory B cells as $CD27^+$, IgM expressing B cells with no or little expression of IgD (Liu et al., 1988, Klein et al., 1997) or $CD27^+$, IgD expressing B cells with or without co-expression of IgM (Shi et al., 2003), whilst others consider IgM only B cells as a distinct subset of B cells (Klein et al., 1997). In this thesis, I will refer to the non-switched $IgM^+IgD^+CD27^+$ memory B cells as IgM memory B cells.

I.5.2 IgM memory B cells in pathological conditions:

IgM memory B cells are reduced in SLE (Odendahl et al., 2000, Wehr et al., 2004), PNH (Richards et al., 2000), CVID (Hart et al., 2007, Carsetti et al., 2005), IBD (Di Sabatino et al., 2005, Di Sabatino et al., 2004) and HIV, suggesting a possible immunoregulatory role for this subset (Nagase et al., 2001, Hart et al., 2007). In patients with Crohn's disease, anti-TNFα treatment is associated with the recovery of IgM memory B cell number and function (Di Sabatino et al., 2008). In HIV positive patients, there is a direct correlation

between IgM memory B cells and the total CD4 count i.e. lower CD4 count is associated with lower IgM memory B cell frequencies (D'Orsogna et al., 2007). A similar association is also observed with the switched memory B cell compartment, supporting a role for $CD4^+$ helper T cells in B cell memory maintenance (Hart et al., 2007). There is some evidence that EBV may preferentially infect IgD^+CD27^+ B cells (Ito et al., 2011). Conversely, expanded clonal IgM memory B cells may have a pathogenic role in anti-myelin-associated glycoprotein (MAG) neuropathy (Maurer et al., 2012) and HCV positive patients with mixed cryoglobulinaemia (Terrier et al., 2011, Charles et al., 2008). These data suggest that IgM memory B cells may play a role in immune pathologies.

I.5.3 IgM memory B cells; source and maintenance:

IgM memory B cells are considered to be the circulating counterpart of marginal zone B cells (MZB), as they share the same gene-expression profile and phenotype. MZB cells reside mainly in the marginal zone (MZ) of the spleen (Weller et al., 2004). Therefore, the spleen has been implicated as the source for IgM memory B cell maintenance (Kruetzmann et al., 2003). A number of observations support this notion; congenitally asplenic individuals have undetectable levels of IgM memory B cells in the peripheral blood (Kruetzmann et al., 2003) and the progressive appearance of IgM memory B cells in the circulation during the first two years of life correlates with the development of the splenic MZ (Lucas, 2004, Kruetzmann et al., 2003). Conversely, the decline in spleen function with ageing is associated with a reduction in IgM memory B cell frequencies in the periphery (Shi et al., 2005, Birjandi et al., 2011, Zago, 1989, Markus and Toghill, 1991). MZB cells also reside in the MZ of other lymphoid organs such as the tonsils

(Takizawa et al., 2006) and lymph nodes (Tierens et al., 1999). A large registry study reported that splenectomised patients lose their IgM memory B cells by 6 months post splenectomy. However, the loss is not complete which supports the existence of other sources of IgM memory B cell maintenance (Cameron et al., 2011). Likewise, older splectomised patients have lower IgM response to pneumococcal vaccine compared to younger patients splenectomised for hereditary spherocytosis, suggesting age-related loss of extra splenic sites involved in the maintenance of this cell population (Wasserstrom et al., 2008). Additionally, in children younger than 2 years, $IgM^{+}IgD^{+}CD27^{+}$ B cells can still be found in small numbers in the peripheral blood despite the absence of a distinct anatomical MZ structure (Zandvoort et al., 2001, Weller et al., 2003).

These data suggest that the marginal zone of the spleen, and possibly other lymphoid organs, are the primary sites where IgM memory B cells are maintained.

I.5.4 IgM memory B cell development:

In mice, the unmutated MZB cells develop from a common transitional precursor subject to the cell fate pathway NOTCH2 (Saito et al., 2003). A nonproliferative subset of $CD21^{hi}CD23^{+}$ transitional B cells, dubbed marginal zone precursors (MZP), has been identified as an intermediate subset in the development of MZ B cells (Srivastava et al., 2005, Pillai and Cariappa, 2009). The generation of human $IgM^{+}IgD^{+}CD27^{+}$ B cells was also shown to be NOTCH2- dependent; using an in vivo model of lymphocyte development (Scheeren et al., 2008). Other factors related to cell migration, BCR transduction, BAFF and NFκB pathways are also involved in IgM memory B cell

development (Pillai and Cariappa, 2009). The strength of BCR signalling of developing B cells may constitute a positive selection process in the spleen, where weaker self-antigen affinity favours the development of B cells into MZB cells (Wen et al., 2005).

Following fate determination, these cells undergo a number of SMH rounds and acquire memory cell properties (Weller et al., 2004, Klein et al., 1997). In view of the complete absence of AID in human splenic MZ, an absolute prerequisite for SHM, Willenbrok et al concluded that MZB cells cannot originate in the splenic marginal zone (Willenbrock et al., 2005). Likewise, Shi et al demonstrated the absence of mRNA AID in this subset in the PB (Shi et al., 2003). However, there is some evidence that early post-rearrangement Ig diversification of this subset might take place in humans through induction of SHM during foetal life (Scheeren et al., 2008). Indeed, upon stimulation with CPG, cord blood derived antigen-inexperienced transitional B cells mature in vitro to IgM memory B cells. The maturation is associated with the up-regulation of AID and Blimp1 and their differentiation into plasma cells with IgM specificity against pneumococcal polysaccharides (Capolunghi et al., 2008). To this end, it remains unclear how and where these cells undergo SHM (Weller et al., 2005, Scheeren et al., 2008) and whether this process is part of the early development of this subset or whether it could occur later in the spleen or somewhere else.

I.5.5 IgM memory B cell ontogeny:

The ontogeny of IgM memory B cells is not fully resolved; whether this population derives from precursor transitional B cells or whether they arise during GC B cell

differentiation remains uncertain (Weller et al., 2005, Moens et al., 2008, Tangye and Good, 2007, Kurosaki et al., 2010).

It has been demonstrated that IgM^+IgD^- B cells i.e. IgM only $CD27^+$ memory B cells, undergo SHM before isotype switching and hence, may represent TD CG exit (Tangye and Good, 2007). In addition, IgM^+CD27^+ memory B cells have mutated Bcl-6 gene, a genetic footprint of GC experience, demonstrating that at least some of the IgM^+CD27^+ B cells derive from a GC reaction during a T-cell-dependent response (Seifert and Kuppers, 2009). Therefore, early exit from a GC reaction after SHM and before isotype switching has been proposed as an explanation for the source and mutated state of IgM memory B cells (Klein et al., 1997, Dunn-Walters et al., 1995). Proponents of this theory consider IgM memory B cells to be a direct result of maturation of naive B cells during GC differentiation.

However, IgM only $CD27^+$ memory B cells were recently shown to have a proliferation history and SHM state equal to that of a GC population. IgM memory B cells, on the other hand, have a lower proliferation history and SHM, indicating that while IgM only B cells may be a result of primary GC reaction, IgM memory B cells seem to have a different origin (Berkowska et al., 2011). Furthermore, the identification of functional unmutated IgG memory B cells from Bcl6 deficient mice (Toyama et al., 2002) and in experimental inducible co-stimulator (ICOS) blockade models (Inamine et al., 2005) further strengthens the idea that memory B cells can develop independent of the GC pathway (Kurosaki et al., 2010).

Others suggest that MZB cells, and therefore IgM memory B cells are pre-diversified with a wide range of affinities independent of T cell help, Ag experience and GC reactions (Weller et al., 2005). Weller et al proposed a second T cell independent (TI) pathway in which mutations may occur as part of an intrinsic developmental pathway that is known to occur in sheep (Lucas, 2004). A second round of SHM that takes place a few days following TI challenge may represent selection and activation of intra-clonal cell populations with higher affinity (Weller et al., 2004). In humans almost all MZB (Weller et al., 2005) and IgM memory B cells (Klein et al., 1997) are mutated, albeit to a lesser extent than that of switched memory B cells.

I.5.6 IgM memory B cell function:

Generally, B cell responses are classified as T-dependent (TD) where antigens are processed and peptides presented on MHC class II molecules for recognition by helper T cells and T independent. T-independent (TI) antigens are divided into type I: mitogenic stimuli such as LPS, CpG, or poly-IC that elicit polyclonal B cell activation via Toll-like receptors and type II: polysaccharides (PS) that engage the B cell receptor and thus induce antigen-specific B cell responses (Obukhanych and Nussenzweig, 2006). PS-specific antibodies can mediate opsono-phagocytosis with or without complement mediated lysis, hence facilitating bacterial clearance (Vos et al., 2000).

IgM memory B cells play a major role in protection against bacterial infections, especially Streptococcus pneumoniae in a TI manner (Kruetzmann et al., 2003). In view of their close anatomical proximity to sinuses, IgM memory B cells are the first subset to see the blood-borne bacterial polysaccharides through their interaction with the marginal zone

macrophages and the more recently described B cell-helper neutrophils (Kruetzmann et al., 2003, Guinamard et al., 2000, Koppel et al., 2005, Puga et al., 2012). The response is fast (Lopes-Carvalho and Kearney, 2004); IgM memory cells generate IgM producing plasmablasts within 3 days of a primary response to particulate bacterial antigens (Martin et al., 2001). Indeed, hyposplenic individuals mount poor responses to polysaccharide vaccines (Molrine et al., 1999). Shi et al have shown that IgM memory B cells acquire a plasma cell phenotype i.e. $CD38^{hi}$ $CD20^{-}$ at a higher rate with BCR and CD40 engagement (Shi et al., 2003). The affinity of produced antibodies are enhanced with affinity-increasing replacement (R) mutation in the antigen-binding CDRs (Shi et al., 2003, Klein et al., 1998). Tsuiji et al showed that VH3 antibodies were over represented in IgM memory B cells that bind to Staphylococcus protein A (Tsuiji et al., 2006).

The fast response and recognition of bacterial DNA and LPS through TLR in the absence of cytokines represents the first-line defence from birth with an array of natural antibodies (Shi et al., 2005, Capolunghi et al., 2008). Although this process is T cell independent, IgM memory B cells are better suited to help shape and bring about the adaptive T cell dependent immune response. First, this subset facilitates antigen deposition onto follicular dendritic cells promoting GC formation (Ferguson et al., 2004, Pillai and Cariappa, 2009). Indeed, Guo et al have shown that IgM knockout (IgM-/-) mice have poor GC formation (Guo et al., 2008). Second, it appears that IgM memory B cells are more efficient than follicular B cells in stimulating CD4 T cells, which in turn facilitates the GC reaction (Attanavanich and Kearney, 2004). Third, there is some evidence that IgM memory B cells respond to TD antigen producing IgG as well as

forming GCs (Song and Cerny, 2003). In humanised mouse models, IgM memory B cells produce mainly IgM isotype in response to both capsular polysaccharides and proteins and, IgG, the product of isotype switching, to the higher immunogenicity serotype (Moens et al., 2008). Hendricks et al have found a small number of class-switched MZB cells in the rat spleen, speculating that these cells may provide a rapid source of IgG against a blood-borne pathogen (Hendricks et al., 2011).

IgM memory B cells in essence bridge and co-ordinate the response between innate and adaptive memory. Due to their pre-diversified nature, they react equally to a new antigen as a naïve B cell sharing the same affinity to polysaccharide antigens (Obukhanych and Nussenzweig, 2006). The authors also demonstrated that hapten-protein specific (HS) B cells disappear quickly from the marginal zone after a secondary encounter where specific isotype switched memory B cells exist. Moreover, they also showed that IgG levels regulate the IgM response from HS specific cells (Obukhanych and Nussenzweig, 2006).

MZB cells and hence IgM memory B cells may play a role in the production of natural IgM which has protective and regulatory functions (Ehrenstein and Notley, 2010). It is believed that by virtue of their high avidity and poly-reactivity, natural IgM B cells promote self-tolerance by mopping up excess self-antigen or masking their immunogenic epitopes (Ehrenstein and Notley, 2010).

The central location of the splenic marginal zone allows B cells to clear circulating self-antigens released by apoptotic cells. In fact, it has been shown that the MZ is enriched for self-reactive B cells (Li et al., 2002). Apoptotic cells induce MZB cells to produce IL10,

ameliorating inflammatory arthritis (Gray et al., 2007). Whether this reaction is a result of pooled polyclonal innate-like MZB with weak BCR signalling or Ag specific MZB with stronger BCR signalling is not fully understood. There is some evidence to suggest that it may be Ag specific (Chen et al., 2009). Breaking the tolerance in pathological states such as in SLE leads to clonal expansion of anti-DNA specific MZB cells with dislocation to intra-follicular spaces (Zhou et al., 2011). It is reasonable to propose that auto-reactive MZ or IgM B cells regulate the immune response by mopping up circulating self-antigens, thereby preventing their abundance, and dampening the immune response by secreting the immunomodulatory cytokine IL10. Finally, cytokine production by B cells is important in B cell homeostasis and has been implicated in the pathogenesis of multiple diseases (Wojciechowski et al., 2009).

I.6 CD21 ⁻B cells:

I.6.1 CD21 (CR2) molecule:

Human complement receptor (CR) CD21 (CR2) is a membrane glycoprotein expressed on B cells, follicular dendritic cells and in developing thymic T cells (Fischer et al., 1999). So far, four ligands for CD21 have been identified: complement C3 fragments, gp350 EBV protein, immunoregulatory protein CD23, IFNα and more recently DNA and chromatin in the absence of C3 ligation (Asokan et al., 2006, Asokan et al., 2013). CD21 is an essential molecule for B cell activation and development. CD21 forms a complex with CD19 and CD81 (the B cell coreceptor complex) and stabilises the B cell receptor (BCR), significantly lowering the threshold for B cell activation (Carroll, 1998, Barrington et al., 2005, Cherukuri et al., 2001). The CD19/CD21 complex also enhances responses to T cell dependent immune reactions (Cherukuri et al., 2001, Carroll, 1998), as well as T cell independent responses via binding C3d coated bacterial polysaccharides to IgM memory cells (Zandvoort et al., 2001, Fearon and Carroll, 2000). Consequently, germinal centre (GC) formation is dependent on CD21 expression, on both B cells and follicular dendritic cells, and on the achievement of sufficient B cell activation (Fischer et al., 1998, Carroll, 1999). In addition to its importance in the regulation of mature B cell activation, CD21 has a critical role in immune regulation and self-tolerance induction through the regulation of programmed death of anergic cells with low antigenic affinities and clearing of soluble self-antigens and circulating fragment of DNAs (Barrington et al., 2005, Asokan et al., 2013, Fearon and Carroll, 2000).

I.6.2 CD21 expression by peripheral blood B cells:

In healthy individuals, almost all B lymphocytes in the peripheral blood express CD21 at variable levels with up to 4% of peripheral B cells being $CD21^{-/lo}$ (Moir et al., 2008). IgM memory B cells have the highest expression level of this marker (Zandvoort et al., 2001). CD21 negative subsets include i) pro and pre B cells, although these cells do not normally circulate in the PB (Schwab and Illges, 2001a) ii) early transitional B cells, known to be enriched for auto-reactive clones, also do not express CD21 and only gain CD21 expression once they mature beyond the elimination checkpoints (Suryani et al., 2010) iii) activated memory B cells and plasmablasts (Weiss et al., 2009, Moir et al., 2008, Jego et al., 1999) and iv) apoptotic B cells may also have lower or no expression of CD21 (Segundo et al., 1999), probably consequent on stimulation-induced proteolytic shedding of the molecule (Sengstake et al., 2006, Masilamani et al., 2003). The different subsets of $CD21^-$ B cells can be distinguished based on their surface phenotype. $CD21^-$ transitional B cells are $CD24^{hi}$, $CD38^{hi}$, $CD27^-$, $CD10^+$, IgM^{hi}, IgD^+ and $CD20^+$. Both activated memory cells and plasmablasts are $CD27^+$, $CD10^-$ and double negative for IgD and IgM. While activated memory B cells are $CD24^{hi}$, $CD38^{-/lo}$ and $CD20^+$, the short lived plasmablasts are $CD38^{hi}$ and CD24 and CD20 negative.

I.6.3 Loss of CD21 expression:

Not only has CD21 been shown to be important for normal B-cell functions such as activation of naïve B cells, GC survival, and persistence of B-cell memory, but CD21 is also known to be involved in the negative selection of self-reactive B cells. Downregulation of CD21 expression by B cells is likely to result in an increased BCR

signalling threshold and compromise the ability of $CD21^{-/lo}$ B cells to be activated. In addition, CD21 is able to break anergy in B cells, further suggesting that its down-regulation contributes to an anergic stage. In autoimmunity, the lower CD21 expression raises the activation threshold of B cells and silences self-recognising clones, thereby inducing B cell anergy (Isnardi et al., 2010) and potentially offsetting the impact of CD19 over-expression by autoreactive B cells (Tedder et al., 2005). CD21 downregulation also facilitates B cell apoptosis via the Fas-pathway (Barrington et al., 2005, Charles, 2011). For instance in SLE, up-regulation of CD95 expression correlates with CD95-mediated apoptosis of $CD21^{lo}$ B cells (Warnatz et al., 2002). In addition to the direct effect of lower CD21 expression on B cell activation through CD19 pathway, upregulation of a number of inhibitory receptors that diminish B cell receptor responses have been observed in $CD21^{-}$ B cell populations. Therefore, downregulation of CD21 may be a direct or indirect sign of B cell exhaustion, which in turn further reinforces tolerance in such conditions. Similar findings are also observed in chronic infections such as HIV, HCV and malaria where tolerance may actually be counter-productive (Visentini et al., 2012, Charles et al., 2008, Moir et al., 2008, Weiss et al., 2009). However in some cases, lower CD21 expression may prevent B cell infection by EBV, as infection of B cells is initiated by the interaction of the major EBV glycoprotein gp350 with CD21 on the B cell surface (Shannon-Lowe and Rowe, 2011).

While the developmental lack of CD21 expression by early pro and pre B cells is regulated by DNA methylation, the acquired loss of its expression is not (Schwab and Illges, 2001a, Schwab and Illges, 2001b). B cell stimulation reduces both CD21 mRNA

expression and serine kinase induced production of CD21 proteins (Illges et al., 2000, Shubinsky et al., 1997). A distinct gene profile was found in clonally activated $CD21^{lo}$ B cells including upregulation of IFN-induced, B cell anergy and pro-apoptotic genes (Charles, 2011).

This process is associated in most cases with the expression of an array of inhibitory receptors. Transcriptional profiling of clonal B cells in patients with HCV-associated mixed cryoglobulinaemia (MC) revealed transcriptional features of anergy and apoptosis with abundant transcripts of Stra13, a powerful negative regulator of B-cell proliferation and homeostasis (Visentini et al., 2012).

Loss of CD21 expression has been described both in congenital and acquired conditions. A recent report described the case of a patient with congenital CD21 deficiency who presented with recurrent infections and hypogammaglobulinaemia, resultant from compound heterozygous deletion in the CD21 gene (Thiel et al., 2012). $CD21^-$ B cells are also increased in a number of autoimmune and chronic infection conditions e.g. rheumatoid arthritis (Isnardi et al., 2010), SLE (Wehr et al., 2004), HCV infection (Terrier et al., 2011Charles, 2011), chronic HIV viraemia (Moir et al., 2008), chronic malarial infestation(Weiss et al., 2009), CVID (Rakhmanov et al., 2009), and cGVHD (Greinix et al., 2008).

I.6.4 Low CD21 expression and B cell exhaustion:

A number of investigators have described expansion of $CD21^-$ B cell populations in viral infections and CVID as associated with autoimmune and lymphoproliferative complications (Charles et al., 2008, Terrier et al., 2011, Warnatz et al., 2002, Rakhmanov

et al., 2009). Similar increases in $CD21^{lo}$ B cells were also observed in autoimmune diseases such as RA and SLE (Reparon-Schuijt et al., 2001, Wehr et al., 2004, Isnardi et al., 2010). Interestingly, sCD21 was also significantly decreased in sera of SLE patients. Therefore, it is unlikely that increased shedding of CD21 accounts for the low cell surface expression of CD21 in these patients (Wehr et al., 2004). More recently, Moir et al described a population of $CD21^-$ B cells in HIV positive patients. The subset was designated tissue-like memory B cell in view of their phenotypic resemblance to tonsillar tissue memory B cells (Ehrhardt et al., 2005). Tissue-like memory B cells expressed significantly higher levels of inhibitory receptors such as CD22, CD85J and FCRL4 and a profile of trafficking receptors consistent with migration to sites of chronic inflammation. Their homing receptor profile including CXCR3, CD11c, CCR7, and CD62L is reminiscent of that of virus-specific $CD8^+$ exhausted T in chronic LCMV and HIV infections (Moir et al., 2008). A similar population was also found to be significantly expanded in Plasmodium falciparum exposed and chronically infected Malian adults and children (Weiss et al., 2009).

Despite differences in $CD21^-$ B cells with regards to their mutational status (clonal versus poly clonal) and allocation to different B cell subsets (naïve versus memory), these cells share important features. These populations all display low proliferation and reduced Ca^{2+} response, a hallmark of B cell exhaustion. Although CD21 downregulation may help reduce the deleterious effects of autoreactive antibodies, it also favours the persistence of pathogens by limiting pathogen-specific humoral responses. This may indicate that downregulation of CD21 expression and the associated changes in B cell phenotype is

the result of chronic B cell activation and exhaustion. It is not clear if exhaustion is limited to the Ag specific clone or whether it represents a global B cell phenomenon (Moir et al., 2008, Charles et al., 2008). This is important as exhaustion might potentially be reversible by using a combination of stimuli such as the addition of IL2 and IL10 (Visentini et al., 2012, Moir et al., 2008). Furthermore, by using siRNA to downregulate inhibitory receptor genes such as FCRL4, Kardava and colleagues have demonstrated reversibility of B cell exhaustion in HIV (Kardava et al., 2011). Targeting the exhaustion may thereby improve B cell immune responses against viruses; however, it remains unclear if reversal of B cell exhaustion in autoimmune disorders might aggravate tissue damage.

I.7 B cell and Graft versus host disease:

I.7.1 Stem cell transplantation (SCT):

Stem cell transplantation (SCT) involves the adoptive transfer of stem cells from a donor to replace the host haematopoietic system. The main consideration for SCT is matching for the major histocompatibility antigens known as human leukocyte antigens (HLA). Other important factors in donor selection include, sex, age, ABO blood group and CMV serostatus (Tay et al., 2012). A number of other factors are being recognised to play a role in the outcome of allogeneic stem cell transplantation such as cytokine gene polymorphisms, KIR status and minor histocompatibility antigen mismatches (Gratwohl, 2007, Dickinson and Charron, 2005).

Successful SCT requires the engraftment and homing of stem cells and education of the emerging donor immune system. The process is complicated by the fact that some competent mature donor lymphocytes are infused into the recipient with the graft, despite efforts to deplete them (Daniele et al., 2012). In the adult population, the age related involution of the thymus means that T cell education and tolerisation towards self are compromised (Lynch et al., 2009). Exposure to the hostile transplantation environment created by the conditioning protocol, release of inflammatory cytokines and breakdown in the innate physical barriers affect the maturation and recovery of the immune system (Novitzky and Davison, 2001). These factors contribute to the pathogenesis of graft versus host disease (GVHD).

I.7.2 GVHD- clinical manifestations and classification:

Graft versus host disease remains a major obstacle to the success of allogeneic SCT. The challenge of GVHD stems from difficulties in controlling the full blown disease and the limited understanding of its pathogenesis to allow development of targeted therapies.

GVHD is a clinical syndrome that follows allogeneic SCT; nonetheless, it has rarely been reported in some autologous SCT settings (Zenz et al., 2006). Another similar fatal entity is encountered when competent lymphocytes are transfused during blood product transfusion into immune-compromised HLA haplotype sharing recipients (Sage et al., 2005).

Historically, GVHD is divided based on its time of onset post-transplant into acute (aGVHD), in the first 100 days following SCT, and chronic (cGVHD) beyond this period (Shulman et al., 1980, Martin et al., 1990). The introduction of reduced intensity conditioning (RIC) and donor lymphocyte infusion (DLI) with the delayed emergence of aGVHD and overlap syndromes have changed this concept (Hymes et al., 2012).

In an attempt to standardise the clinical and pathological criteria and to account for the emerging late onset aGVHD and the overlap syndrome, the National Institute of Health published a new consensus criteria for the diagnosis of GVHD **table (I-1)** (Filipovich et al., 2005).

Table I-1: NIH consensus criteria for GVHD diagnosis

Category	Time of symptoms after SCT or DLI (days)	Presence of aGVHD Features	Presence of cGVHD features
Acute GVHD			
Classic aGVHD	<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

Numerous risk factors have been identified for the development of acute and chronic GVHD such as HLA disparity, female to male donation, older age of donor and recipient, and peripheral blood mobilised grafts, TBI, CD3 content of the graft and CMV serostatus (Flowers et al., 2011, Atkinson et al., 1990, Hahn et al., 2008).

Chronic graft versus host disease is a wasting syndrome, which involves specific targeted organs and shares considerable resemblance with autoimmune diseases. It affects 30 to 65% of patients following allo-SCT with 5 year mortality of 30 to 50% (Blazar et al., 2012b). The main target organs for cGVHD include the skin and its appendages, the GI tract, genitalia, mouth, eyes and liver. It is usually associated with cytopenias, autoimmune haemolysis and thrombocytopaenia. Bone marrow failure and red cell aplasia may also ensue in the course of the disease. In the acute disease, the skin, gut and the liver are mainly involved (Filipovich et al., 2005, Ferrara et al., 2009).

Prophylaxis and treatment of GVHD is aimed at addressing the breakdown in self-tolerance and is based on suppressing the immune system. Therefore, the treatment thus far has included corticosteroids, calcineurin inhibitors (e.g. cyclosporin (CsA) (Vogelsang, 2001) and tacrolimus , mTOR inhibitors (rapamycin) (Abouelnasr et al., 2013), mycophenolate mofetil, and monoclonal antibodies against CD25 and TNFα (Rodriguez et al., 2007). Recent experimental treatments for GVHD include tyrosine kinase inhibitors (Olivieri et al., 2011), anti-CD20 (Kim et al., 2010), bortezomib (Koreth et al., 2012), and extracorporeal photopheresis (ECP) (Kuzmina et al., 2009). A number of novel pathway targeting agents and cellular therapies such as mesenchymal stem cells (MSC) are under investigation (Blazar et al., 2012b). However, none of these current therapies are approved by the FDA. More prospective, well designed studies are required to investigate the role of novel therapies in the prevention and treatment of GVHD (Inamoto and Flowers, 2011).

I.7.3 Pathophysiology of GVHD:

Billingham defined the triad of factors required for the development of GVHD. These include i) an immunocompetent graft with viable and functional immune cells. ii) immunological disparity between recipient and donor and, iii) the recipient is immunocompromised and therefore cannot reject the donor cells (Billingham, 1966). The pathophysiology of acute graft versus host disease is thought to happen in a three-step process: i) the conditioning induces tissue damage and the resulting cytokine storm leads to priming of host antigen presenting cells (APC); ii) activation of allo-reactive donor T cells and the subsequent clonal expansion and secretion of IL2 and IFNγ; iii)

inflammation induced by LPS-activated neutrophils and mononuclear phagocytes, with the subsequent recruitment of effector cells such as cytotoxic T cells including Th17 and NK cells. The process culminates in tissue damage of target organs (Ferrara and Reddy, 2006).

While acute GVHD is mediated by the inflammatory environment of the early post-transplant period, little inflammation is involved in triggering of chronic GVHD (Schroeder and DiPersio, 2011). The hallmark of cGVHD is the accumulation of Th-2 polarised $CD4^+$ T cells in the presence of impaired negative selection in the damaged thymus. Th-2 type cytokines lead to the production of pro-fibrotic cytokines such as IL2, IL10 and TGFβ and platelets derived growth factor (PDGF), that promote tissue fibroblast proliferation (Blazar et al., 2012b). More recent studies have underlined the importance of regulatory dysfunction, especially $FoxP3^+$ regulatory T cells, in the development of cGVHD (Rezvani et al., 2006, Zorn et al., 2005). Increasing evidence supports a role for B cell dysregulation in the development of cGVHD (Shimabukuro-Vornhagen et al., 2009). Several autoantibodies have been reported in association with cGVHD and in some cases, have been implicated in the pathogenesis of the disease (Kapur et al., 2008, Svegliati et al., 2007). A potential role for a IL10-producing human regulatory B cell was recently described in some autoimmune diseases (Blair et al., 2010, Iwata et al., 2010). Further insight into the role of B cells, especially the regulatory subsets, is imperative to improve our understanding of cGVHD and to help with the development of novel therapies.

I.7.4 B cell in cGVHD

I.7.4.1 B cell reconstitution following SCT

B cell reconstitution following allogeneic SCT recapitulates B cell ontogeny (Storek et al., 1993). Similar to recovery following B cell depletion, transitional B cells are the first subset to appear in the peripheral blood after SCT. They progressively diminish, while giving rise to naïve B cells (Marie-Cardine et al., 2008), (Anolik et al., 2007). The early period following allogeneic SCT is characterised by supranormal B cell numbers, likely driven by high homeostatic levels of B cell activating factor (BAFF) (Storek et al., 1993, Sarantopoulos et al., 2009). The majority of circulating B cells is antigen-naïve, and the memory compartment remains significantly lower than normal for the first year after SCT (Avanzini et al., 2005, Bemark et al., 2012, Cuss et al., 2006, Storek et al., 1997). Generally, memory B cell recovery following allogeneic SCT and regaining the immunoglobulin repertoire may need many years to reach normalcy (Storek et al., 1997, Avanzini et al., 2005, Velardi et al., 1988). The pre immune repertoire of B cells post SCT resembles that of healthy individuals (Glas et al., 1997). However, there is some evidence that B cells from SCT patients accumulate less antigen-driven T cell-dependent somatic mutations (Glas et al., 2000). Intrinsic B cell deficit or impaired T-B cell cross talk rather than T cell help defect may explain this observation (Glas et al., 2000). Damaged lymph node structure and functional hyposplenism associated with SCT interrupts immune cell interaction (Cuthbert et al., 1995, Sale et al., 1992).

Several variables may influence B cell recovery following stem cell transplantation. Following GCSF mobilized PBSCT, the higher B cell content and the increased activation

state of B cells compared to bone marrow grafts results in faster B cell reconstitution (Abrahamsen et al., 2005, Tayebi et al., 2001). The development of acute and chronic GVHD, the type and duration of immunosuppression as well as the conditioning regimen can affect the kinetics of B cell recovery (D'Costa et al., 2001, Maris et al., 2003).

A crucial aspect of B development is determined by the early checkpoint, at the transitional B cell stage during which time self-reacting B cells are deleted from the peripheral repertoire and self-tolerance is maintained. The survival of auto-reactive B cells is influenced by the strength of receptor cross linking with the Ag (Hartley et al., 1991), the availability of high BAFF levels, and possibly the quality of B cell recovery following transplantation (Lesley et al., 2004). An understanding of normal B cell reconstitution is important to define patterns of B cell reconstitution that may be associated with cGVHD or may be predictive of increased susceptibility to infectious complications (Avanzini et al., 2005).

I.7.4.2 B cells disturbances in human GVHD:

I.7.4.2.1 The role of B cells in GVHD- mouse models:

There is a considerable body of pre-clinical evidence highlighting the role of donor B cells in pathogenesis of chronic GVHD. In an MHC matched murine transplant model, both donor B and $CD4^+$ T cells are required for the development of cGVHD (Zhang et al., 2006, Schultz et al., 1995). The mechanism through which the two immune cells interact and trigger the process is not fully understood.

Recipient and donor APC activate allo-reactive donor $CD4^+$T cells. Activated donor T cells can stimulate donor B cells and facilitate germinal centre formation. Activated B cells expand robustly, undergo SHM, class switch and produce potentially pathogenic allo-antibodies (Srinivasan et al., 2012). In addition, activated B cells express co-stimulatory molecules and in turn can serve as potent APC to allo-reactive donor $CD4^+$ T cells and have the potential to produce cytokines that polarise T cells towards Th2 (Shustov et al., 2000). B cells therefore depend on T cell help to be primed and in turn augment T cell function further. Expanded allo-specific T cells can cause damage thereafter, independent of B cells (Young et al., 2012). B cell activation, however, also occurs independent of T cells in which case B cells themselves can initiate the pathogenic process.

Antigen presentation by recipient APCs is fundamental for the development of cGVHD but is not exclusive to B cells, as depletion of recipient B cells (Matte-Martone et al., 2010) or in fact all APC, does not prevent GVHD (Li et al., 2012a). The currently available

cGVHD mouse models do not mirror human cGVHD, and this deficiency limits the translational value of murine studies of cGVHD (Schroeder and DiPersio, 2011).

I.7.4.2.2 The role of B cells in human aGVHD:

The role of donor B cells in the development of aGVHD is not understood. A recent study from the International Blood and Marrow Transplant Registry (IBMTR) reported that prior treatment with rituximab was associated with less acute GVHD following allogeneic stem cell transplantation (Christopeit et al., 2009), (Ratanatharathorn et al., 2009). These findings are further supported by the recent demonstration that a high concentration of B cells in the graft is associated with more acute GVHD (Iori et al., 2008). Furthermore, Kamble et al reported successful treatment of refractory aGVHD with rituximab in 3 patients (Kamble et al., 2006). On the other hand, post-transplant administration of rituximab failed to have a significant effect on the incidence of acute GVHD in a small cohort of patients with aggressive NHL (Glass, 2008). Moreover, another study reported that higher frequencies of B cell progenitors in the apheresis product are associated with less aGVHD (Michonneau et al., 2009).

I.7.4.2.3 The role of B cells in human cGVHD:

Traditionally, T cells have been the main focus of research in the pathogenesis of GVHD (Inamoto and Flowers, 2011). Recent studies suggest that donor B cells as well as T cells contribute to the immune pathology in cGVHD (Zhang et al., 2006). However, it has been suggested that longer survival of host B cells might be involved in the pathogenesis of cGVHD (Barrett, 2006).

Indeed, at least 35 auto-antibodies have been strongly associated with cGVHD (Kapur et al., 2008). Antibodies to H-Y minor histocompatibility antigens produced in males receiving female grafts correlated with cGVHD development and remission (Miklos et al., 2004, Miklos et al., 2005). The frequent production of auto-antibodies in patients with cGVHD may reflect critical breakdown in peripheral B-cell tolerance after allogeneic HSCT. Whether this phenomenon merely results from B cell recovery in the permissive post-transplant environment or in response to antigenic disparity, ensuing direct tissue damage is not fully known. Direct causal relationship of auto-antibodies in cGVHD has only rarely been demonstrated (Svegliati et al., 2007).

Many factors following SCT may contribute to the survival of autoreactive B cells, such as high levels of BAFF and activated and primed B cells (Granholm and Cavallo, 1992). BAFF is a member of the TNF family, also known as TALL-1, THANK, BlyS and zTNF4, and is a critical B cell survival factor with homeostatic properties. It is secreted by monocytes, dendritic cells and some T cells and is increased following reduction of the B cell count (Saito et al., 2008). It is also increased following B cell depleting therapy (BCDT) e.g. with rituximab (Ansell et al., 2009) and interestingly in the majority of autoimmune conditions (Zhou et al., 2009). It has been shown that under conditions where BAFF levels are elevated, auto-reactive B cells can escape the negative selection process, predisposing to the development of autoimmunity (Lesley et al., 2004). Indeed, Sarantopoulos et al have found that high BAFF to B cell ratio following allogeneic stem cell transplant correlates with the development of chronic GVHD (Sarantopoulos et al., 2009). In addition, B cells derived from cGVHD patients are primed for CpG stimulation and express higher toll-like receptor 9, required for autoantibody production (She et al.,

2007). Other authors have demonstrated that B cells in the cGVHD environment are actually in an activated state (Allen et al., 2012). It has been shown that GCSF mobilised graft may be associated with higher frequencies of activated $CD25^+$ B cells, and a higher risk of cGVHD post-transplant (Tayebi et al., 2001).

The total number of $CD27^+$ B cells was found to be significantly lower in cGVHD patients compared with their no cGVHD counterparts (D'Orsogna et al., 2009, Hilgendorf et al., 2012, Greinix et al., 2008, Corre et al., 2010). Several studies have reported similar observations, but with variable impact on the class-switched and non-switched $CD27^+$ memory B cells. In one study, IgM^+ $CD27^+$ memory B cells were selectively reduced in patients with a history of cGVHD (D'Orsogna et al., 2009). In other studies however, both subpopulations of IgM memory and class-switched memory B cells were numerically and proportionally reduced in cGVHD compared to patients without GVHD (Hilgendorf et al., 2012, Greinix et al., 2008). In contrast, Sarantopoulos et al reported that cGVHD patients maintain their memory B cell compartment in the setting of relative naïve B lymphopenia and high BAFF: naïve B cell ratio (Levine et al., 2012).

Interestingly, abnormal persistence of a high fraction of $CD21^-$ immature/ transitional B cells was observed in patients with cGVHD (Greinix et al., 2008). The $CD21^-$ B cell population correlated with more severe infections post SCT and poorer response to ECP (Kuzmina et al., 2009, Greinix et al., 2008). In addition, the increase in $CD21^-$ B cells correlated with lower serum immunoglobulin levels among cGVHD patients (Kuzmina et al., 2011). The authors concluded that $CD21^-$ transitional B cells are increased and correlate with the severity of cGVHD. However, other groups have reported that

transitional B cells are low in cGVHD patients (Hilgendorf et al., 2012, Storek et al., 1993). Expansion of the $CD21^-$ B cell compartment in the peripheral blood has been reported previously in a number of autoimmune and immune deficiency conditions SLE (Wehr et al., 2004), CVID (Wehr et al., 2008) suggesting that $CD21^-$ B cells may be involved in the pathogenesis of cGVHD as an auto-allo-immune condition.

There is substantial evidence that B cells can both amplify and suppress immune responses by mechanisms that do not involve antibody production. For example, B cells respond to Toll-like receptor (TLR) ligands and present antigens. B cells also organise the structure of lymphoid tissues and regulate lymphangiogenesis. In addition, B cells produce cytokines and can be subdivided into discrete cytokine-producing "regulatory" and "effector" B subsets (Sanz et al., 2007). In the SCT setting, host B cells have been shown to play a protective role in GVHD. After total body irradiation, B cells can secrete IL10, thus inhibiting alloreactive T-cell expansion and subsequent induction of acute GVHD (Rowe et al., 2006). It is therefore possible that B cells with immunoregulatory function may play a role in the pathogenesis of GVHD.

I.7.4.2.4 Chronic GVHD effects on B cells

The relationship between B cells and cGVHD is rather complex. While B cells certainly can play a causative role in cGVHD pathogenesis, cGVHD on the other hand appears to have profound effects on B cell recovery following allogeneic SCT. It has been shown that cGVHD affects B lymphopoiesis and sites of B cell maturation. Chronic GVHD is associated with alloimmune damage to lymph nodes, a major location for antigen encounter and T cell interaction. Altered follicular dendritic reticular cell structure, in

addition to intranodal reversal of CD4/CD8 ratio contribute to the B cell dysfunction observed in cGVHD patients (Sale et al., 1992). Moreover, cGVHD worsens the splenic hypofunction seen in SCT patients further (Cuthbert et al., 1995). There is experimental evidence to support that cGVHD-associated damage to the bone marrow niche impairs B cell lymphopoiesis (Shono et al., 2010). Collectively, the development of cGVHD translates into impaired B cell reconstitution (Storek et al., 1993). In line with this notion, early studies have demonstrated that extensive cGVHD or its treatments are associated with lower bone marrow B cell precursors (Abrahamsen et al., 2005, Storek et al., 2001, Fedoriw et al., 2012). This may explain why cGVHD patients have lower circulating total B cells and immunoglobulin levels compared to patients without cGVHD (Corre et al., 2010, Abrahamsen et al., 2005). A case of almost complete absence of B cell lymphopoiesis attributed to severe chronic GVHD was reported (Takahashi et al., 2002).

I.8 Regulatory B cells

I.8.1 Evidence for regulatory B cells:

Early experimental animal models have identified a potential role of B cells in immune regulation. Transfer of splenocytes lacking B cells in an in vivo mouse model failed to prevent T cell induced delayed-type skin hypersensitivity (Katz et al., 1974, Neta and Salvin, 1974). A series of published reports by Shimamura et al suggested that splenic B cells from mice immunised with sheep erythrocytes suppressed the response of syngeneic recipients to the same antigen via induction of suppressor T cells (Shimamura et al., 1982a, Shimamura et al., 1984, Shimamura et al., 1982b). The later development of B cell deficient mouse models helped to further elucidate the immune-modulatory role of B cells. B cell deficient µMT mice failed to recover from induced experimental autoimmune encephalomyelitis (EAE) (Wolf et al., 1996). Subsequent animal studies demonstrated that B cell regulatory function was mediated through a number of mechanisms including linking antigen presenting cells (APC) to tolerogenic regulatory T cells (D'Orazio and Niederkorn, 1998), regulating the function of pathogenic CD4 T cells(Mizoguchi et al., 2000), inhibiting induced lymphocyte proliferation(Sasaki et al., 1993) or modulating IFN$^{\gamma}$ production by antigen specific T cells (Wijesuriya et al., 1998). However, the mechanism by which B cells or their products contributed to tolerance induction remained unclear. The notion that IL10 production may be essential for regulatory B cell function was demonstrated simultaneously in two different inflammatory models (Mizoguchi et al., 2002, Fillatreau et al., 2002). In the first study, µMT as well as IL10 B cell deficient mice failed to recover from experimental autoimmune

encephalomyelitis (EAE). In the second study, IL10 producing B cells inhibited the progression of spontaneous colitis in a murine model. Similarly, in a collagen induced arthritis mouse model, the adoptive transfer of IL10 producing B cells controlled synovial inflammation in arthritic mice (Mauri et al., 2003). Later work by Yanaba and colleagues indicated that IL10 producing B cells are specialised cells with inducible IL10 production capacity. Measuring 31 cytokines transcript levels, the defined mouse B cell subset uniquely produced IL10 (Yanaba et al., 2009). Subsequently a number of experimental models of chronic inflammatory conditions which helped further studies of different regulatory pathways involved were developed (Mizoguchi and Bhan, 2006).

I.8.2 B cells and immune regulation; the regulatory B cell:

B cells, long known to be mediators of humoral immunity, are increasingly appreciated for other roles including antigen presentation, cytokine secretion and immune response modulation (Allman and Pillai, 2008). The regulatory function of B cells is mediated by a number of mechanisms such as cytokine and Ig production, interactions with other cellular subsets and Ag presentation. Although a number of studies have demonstrated the regulatory function of a bulk of B cells without denoting a specific regulatory subset, it is most likely that one or more B cell subsets possess these regulatory abilities. They are collectively referred to as regulatory B cells. So far, the most extensively studied regulatory B cell population is the IL10 producing B cell.

IL10 producing B cells are directly involved in the suppression of inflammatory immune responses of both the innate and adaptive immune systems (Mizoguchi and Bhan, 2006). IL10 reduces antigen presentation and expression of co-stimulatory molecules, blocks

cytokine production and modifies chemokine receptor expression, culminating in restraint of T cell activation to specific immune responses. Some of the IL10 immunoregulatory function can be attributed to the induction of Tr1 differentiation (Pestka et al., 2004). Although IL10 is produced by a variety of immune cells, IL10-producing B cells appear to play a nonredundant role in immune regulation (Madan et al., 2009). Other cytokines produced by B cells, such as TGF-β, IL12 and IL4, have also been shown to induce immune tolerance (Gonnella et al., 2001, Parekh et al., 2003, Sugimoto et al., 2007, Tian et al., 2001). In addition, regulatory B cells either recruit or act in unison with the wider immune regulatory network in cytokine dependent and independent ways to exert their immunomodulatory effects (Ray et al., 2012). Breg maintain the $FoxP3^+$ regulatory pool, promote the conversion of effector T cells to $FoxP3^-$ $IL10^+$ producing Tr1 cells and recruit $CD4^+$ $CD8\alpha^+$ T cells (Carter et al., 2011, Wei et al., 2005, Ray et al., 2012).

A major role for Breg is the suppression of pathogenic effector immune cells such as Th1 and Th17 (Carter et al., 2012), and modulation of Th2-mediated responses (Fillatreau et al., 2002). This property is mediated via secretion of anti-inflammatory cytokines, in particular IL10, and through direct cell to cell contact. In addition, Breg negatively regulate APC function, thereby breaking the effector inflammatory circuit (Lo-Man, 2011).

Another mechanism by which B cells may regulate immune responses is by inducing apoptosis of pathogenic T cells (Lundy, 2009). A suppressive B cell subset expressing Fas ligand, designated killer B cells, was shown to induce apoptosis of arthritogenic T cells, thereby reducing the inflammation (Lundy, 2009).

B cells may also indirectly contribute to immune regulation through immunoglobulin production, for instance, Ig mediated DC suppression, clearance of pathogenic apoptotic cells and immune complexes and potentially via the production of anti-inflammatory IgG4 (Hussaarts et al., 2011); **figure (I-2).**

I.8.3 Regulatory B cell functions:

Regulatory B cells play a critical role in reducing self-destruction and autoimmunity by controlling autoreactive T cells. It has been observed that IL10 producing B cells control autoreactive CD4 as well as MCMV-specific $CD8^+$ T cells in two different mouse models of adoptive therapy (Knoechel et al., 2005, Madan et al., 2009). Endogenous B cells prevented the differentiation of effector autoreactive T cells that follows the initial expansion of T cells (Knoechel et al., 2005) and played a significant role in recruiting other regulatory cells (Wei et al., 2005). There is evidence that LPS activated B cells with regulatory function suppress APC function, thereby breaking the autoreactive cycle (Tian et al., 2001). Regulatory B cells also help induce or maintain immune tolerance to self-antigens released from apoptotic cells (Gray et al., 2007). Recently, Breg were shown to react to DNA bearing apoptotic cells by IL10 production in a TLR9 dependent way (Miles et al., 2012). Another potential role of Breg is in the regulation of neonatal immune responses, by fostering Th2 biased environment to limit the harmful systemic effects of exacerbated Th1 responses (Sun et al., 2005). In addition to the protective immune suppression of regulatory B cells in variable inflammatory processes, the same property can be exploited by pathogens and tumour cells to escape immune responses (van der Vlugt et al., 2012, Schioppa et al., 2011, Jeong et al., 2012).

Notably, Breg are not detected in the normal state and develop at the sites of inflammation, either in vivo or in vitro, in an Ag specific and non-specific way (Mizoguchi et al., 2002).

I.8.4 Regulatory B cells: polyclonal versus antigen specific

Studies in animal models suggest that regulatory B cells are capable of responding both in an antigen specific as well as in an innate-like polyclonal manner. One could propose that in the antigen specific model (Fillatreau et al., 2002), Breg are recruited to the centre of induced inflammation, in close vicinity to the antigen, and are likely to require T cell help to undergo expansion in the local draining lymph node. This process will require a longer time and the suppressive effect may be limited to the specific antigen only. In the non-specific model, bystander regulatory B cells are induced via their interaction with activated CD154-expressing T cells or pathogen particles such as un-methylated DNA regardless of antigen specificity, and are therefore likely to be more abundant in the periphery to prevent progression of the inflammatory process. In the second scenario, the response is fast and will comply more with the innate immune system. In fact, experimental data support the existence of both proposed mechanisms (Wei et al., 2005, Yanaba et al., 2009).

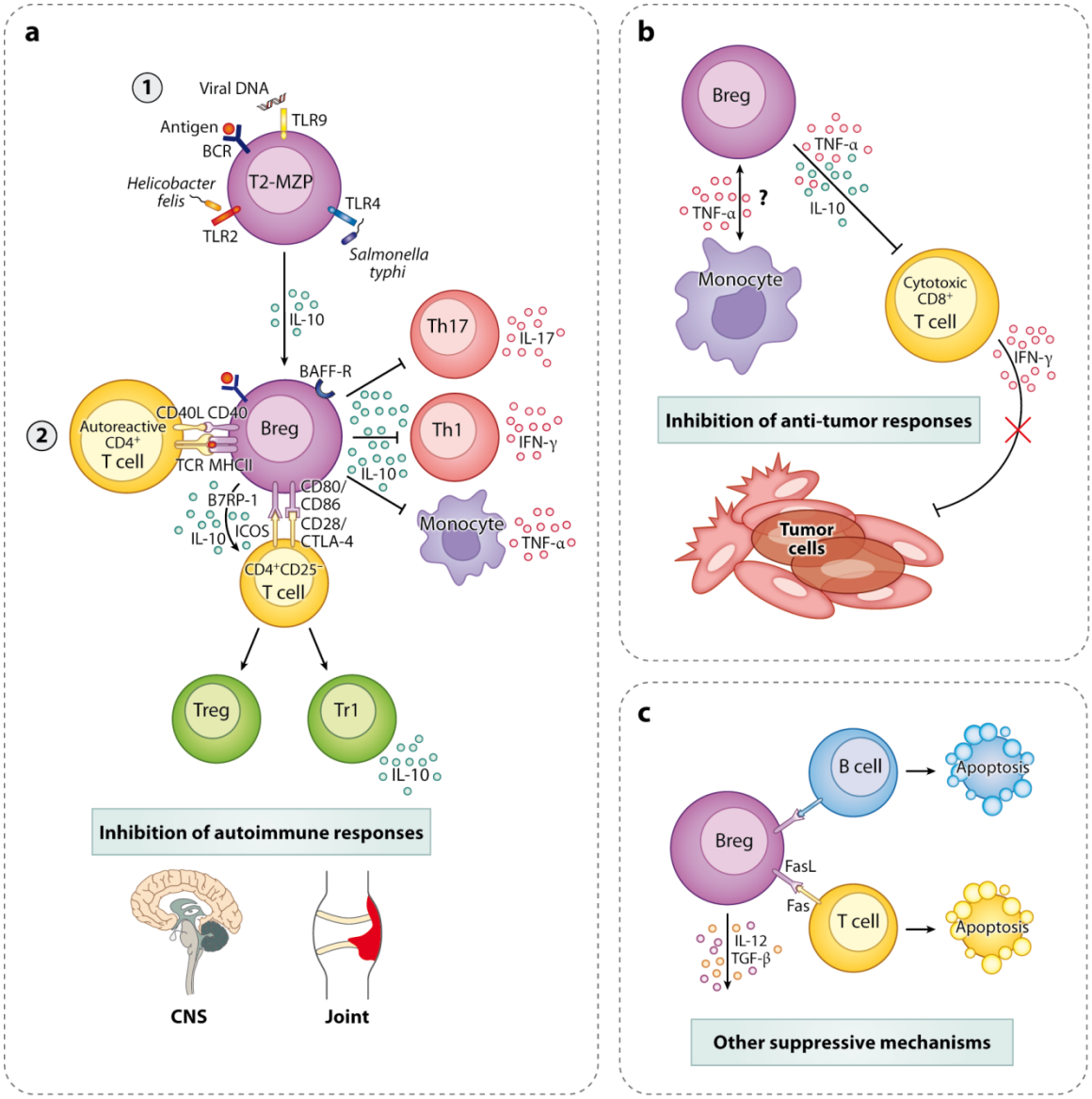


Figure I-2 : Regulatory mechanisms of IL10-producing B cells

A. Different regulatory mechanisms of induced IL10-producing Breg on effector and other regulatory immune cells, **B.** Breg may interfere with tumour clearance through suppressing tumour specific cytotoxic cells and **C.** other potential IL10-independent regulatory mechanisms displayed by B cells. Figure adopted from Mauri et al (Mauri and Bosma, 2012)**.**

I.8.5 Regulatory B cells in mouse models:

Studies in murine models of chronic inflammation including experimental autoimmune encephalomyelitis (EAE), inflammatory bowel disease (IBD) and collagen-induced arthritis (CIA) have elegantly shown that the absence of B cells intensifies the pathological condition, whereas the adoptive transfer of regulatory B cells attenuates the pathology (Yanaba et al., 2009).

I.8.5.1 Induction of regulatory phenotype in B cells:

The activation of regulatory B cells either by challenging with target antigens or using external stimuli is required to induce the regulatory function. A number of studies have shown that engagement of various toll-like receptors (TLR), for instance, TLR2, TLR4 or TLR9 induces IL10 and to a lesser degree IL-6 production by marginal zone B cells, while follicular B cells respond mainly by producing IFNγ and IL6 (Mauri and Bosma, 2012, Barr et al., 2007, Lenert et al., 2005). In addition, multiple studies have identified CD40-CD154 crosslinking as an essential step for Breg activation. Mizoguchi et al, have shown that in a TCRα-/- and αμ-/- mouse model characterised by severe and spontaneous ulcerative colitis mediated by pathogenic $TCR\alpha^{-}$ $TCR\beta^{lo}$ $CD4^{+}$ T cells, adoptive transfer of CD40-/- B cells fails to suppress the pathogenic T cells (Mizoguchi et al., 2000). Although some studies have suggested a role for BCR-mediated pathways, the data remain contradictory (Yanaba et al., 2009, Mauri and Bosma, 2012). Other co-stimulatory molecules such as CD80 and CD86 have also been shown to play a potential role in induction of cellular tolerance in murine models of transplantation. These data suggest

that direct cellular crosstalk may be even more important than cytokine mediated immune suppression (Deng et al., 2007).

I.8.5.2 Regulatory IL10^{+} B cell phenotype(s):

More than one phenotype of B cells have been proposed to define Breg; however, it remains unclear if these subsets of regulatory B cells are distinct or share the same development pathways (Mauri and Blair, 2010). In murine models, IL10 producing B cells were found to be enriched in the spleen and gut-associated lymphoid tissue; with a phenotype similar to MZ B and their precursors T2 MZP B cells i.e. CD19^{+}, CD1d^{hi} (Mizoguchi et al., 2002). Subsequently, a subset of B cells dubbed (B10), expressing CD5 and CD1d^{hi} CD19^{hi} CD21$^{int/hi}$ CD23^{lo}CD24^{hi}CD43$^{+/-}$CD93^{-} was shown to produce IL 10 upon stimulation and reduce inflammation in contact hypersensitivity response and EAE models (Yanaba et al., 2009), (Yanaba et al., 2008, Watanabe et al., 2010, Matsushita et al., 2008). Other groups have defined IL10 producing B cells as marginal zone B (MZB) cells; CD21^{hi}CD23^{-}CD24^{hi}IgM^{hi}IgD^{lo}CD1d^{hi} (Gray et al., 2007), or marginal zone precursors (T2-MZP), which share the same phenotype with the exception of CD23 positivity, reported to be expressed by regulatory B cells in collagen induced arthritis (Mauri et al., 2003, Evans et al., 2007, Blair et al., 2009). In another study Madan et al reported IL10 production in both splenic and lymphoid organ B cells, as well as CD19^{+}CD138^{+} plasma cells (Madan et al., 2009). Similarly, in a recent study implementing IL10 reporter mice, B10 cells, IgM^{hi}CD1d^{hi}CD5^{+}CD19^{hi}CD23^{low}CD38^{hi}B220^{hi}, could differentiate into CD138^{+} B cells, capable of secreting both IgM and IgG (Maseda et al., 2012).

Despite differences observed in the phenotype of IL10 producing B cells, these cells share overlapped expression of some markers such as $CD1d^{hi}$, IgM^{hi}, $CD38^{hi}$ and $CD24^{hi}$. Whether these different Breg are one or more specialised subsets and whether or not they share one origin remains unclear; **figure (I-3).** Breg studies will remain suboptimal until the identification of a specific marker or constellation of markers, or a unique transcriptional factor that can be assigned to Breg, analogous to Foxp3 in Treg.

I.8.5.3 IL10 independent Breg cells:

A distinct subset of suppressive B cells expressing CD5 and Fas ligand, designated killer B cells ($CD5^{+}$, $CD178^{+}$), was shown to induce apoptosis of arthritogenic T cells, thereby reducing inflammation (Lundy, 2009).

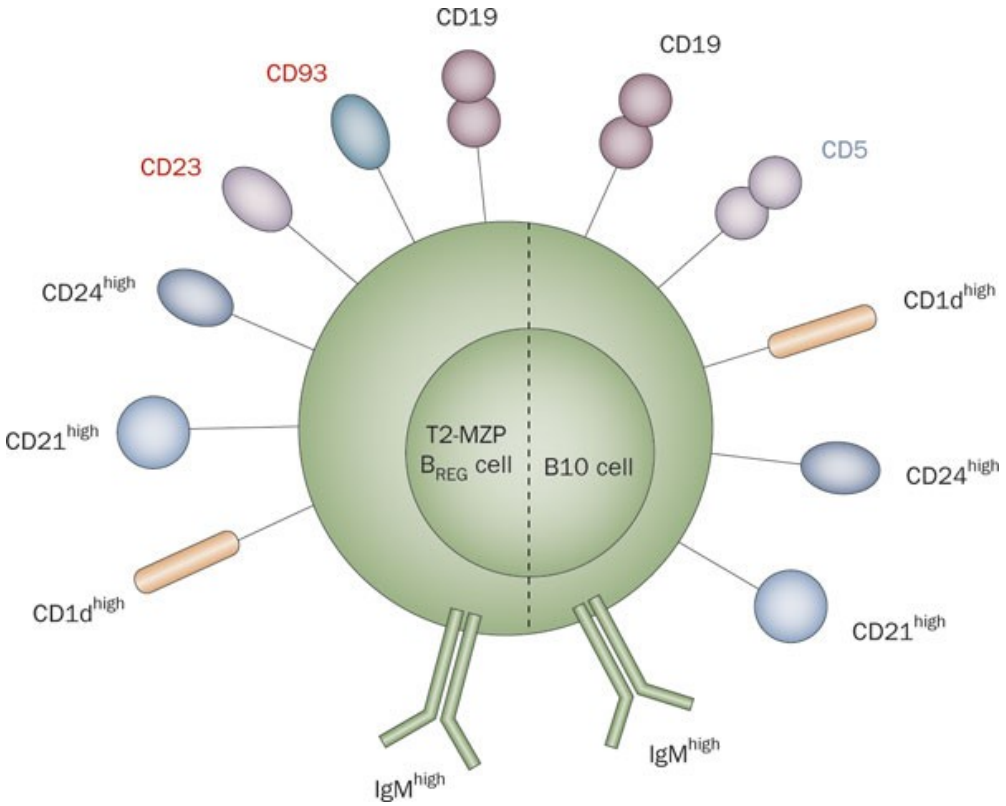


Figure I-3: Phenotypic profiles of IL10-producing Breg in mice

Summarises the phenotypic profile of the two main subsets of IL10 producing Breg in mice; namely, T2-MZP and B10 cells. The colour code depicts shared and unshared surface markers by both cell subsets. Adopted from Mauri et al(Mauri and Blair, 2010a).

I.8.6 Human regulatory B cells:

Similar to murine B cells, human B cells are capable of producing cytokines under different conditions. Human B cells stimulated with CpG and CD40L produce IL10 among other cytokines (Gantner et al., 2003, Wagner et al., 2004).

I.8.6.1 Human IL10^{+} regulatory B cells: subset(s) and phenotype(s)

To date, studies of regulatory B cells in humans have defined these cells as IL10 producing with more than one phenotype.

Evidence for IL10-dependent, B cell mediated immune regulation in humans, was first described by Duddy et al (Duddy et al., 2004). The authors reported that B cells produce distinct profiles of cytokines, depending on the stimulation condition; whereas CD40 ligation alone favoured IL10 production, dual stimulation with CD40L in combination with BCR cross-linking favoured secretion of pro-inflammatory cytokines (Duddy et al., 2004). Subsequently, they showed that IL10 producing B cells are contained within the naive $CD27^-$ B cell population, while memory B cells preferentially produce pro inflammatory cytokines such as IL6, TNFα and lymphotoxin. In another study, naïve B cells (defined as $CD11b^-CD5^-CD27^-$ and IgD^+) from MS patients infected with intestinal helminths were shown to produce more IL10 compared to the subnormal levels of IL10 reported in non-infected MS patients. In vitro, IL10 producing B cells from helminth-infected MS patients were shown to suppress $CD4^+$ T cell proliferation and IFNγ production in response to an MS associated Ag (Correale et al., 2008).

In agreement with this, Blair et al, using sort purified B cell subsets from healthy controls, showed that Breg are enriched within the $CD24^{hi}$ $CD38^{hi}$ transitional (Tr) B cell compartment, and mediate their suppressive function on $CD25^-$ $CD4^+$ effector T cells through the production of IL10 but not TGFβ. Co-culture of transitional B cells with T cells resulted in significant suppression of IFNγ and TNFα production by effector T cells (Blair et al., 2010). Interestingly, Bouaziz et al showed that IL 10 producing human B cells exist across both $CD27^+$ and $CD27^-CD38^{hi}$ transitional B cells(Bouaziz et al., 2010). In their experimental setting, suppression of $CD25^-$ $CD4^+$ effector T cell function was partially IL10 dependent. Similar observations were reported in relapsing remitting MS (RRMS) patients. Whereas in remission, 60% of IL10 producing B cells had a naïve $CD27^-$

phenotype, the majority of Breg during relapsing periods were $CD27^+$ (Knippenberg et al., 2011). Recently, a subset of Breg analogous to mouse B10 was characterised in the peripheral blood of healthy controls by Tedder et al. The group found that IL10 producing regulatory B cells and their progenitors are enriched within $CD24^{hi}$ $CD27^+$ B cells (Iwata et al., 2010). Although no specific inhibitory impact on effector $CD4^+$ T cells was shown, $CD24^{hi}$ $CD27^+$ B cells inhibited proliferation and cytokine production by $CD14^+$ monocytes. The identified B10 subset expressed markers of memory B cells such as CD27 and CD148. Similarly, Miles et al recently reported that in response to DNA bearing apoptotic cells, human IgM^- and IgM^+ $CD27^+$ memory B cells produce significantly more IL10 than $CD27^-$ naïve B cells (Miles et al., 2012).

I.8.6.2 Human regulatory B cells in disease conditions:

Human regulatory B cells have been studied in a number of autoimmune conditions where immune dysregulation is implicated. Mouse models of chronic inflammation have substantiated an indispensable role for B cells in preventing, as well as alleviating, destructive immune responses.

In agreement with the likely involvement of Breg in autoimmune disease, B cells derived from active multiple sclerosis (MS) produced less IL10 compared with normal controls (Duddy et al., 2007, Correale et al., 2008). Interestingly, remission induction using mitoxantrone or following autologous HSCT was associated with restoration of IL10 production by B cells (Duddy et al., 2007). This observation provides a potential role for impaired Breg function in the pathogenesis of MS. Similarly, although Tr B cells, believed to be enriched in Breg, are increased in SLE patients, their IL10 production and

suppressive properties are compromised. Furthermore, Breg from SLE patients are more refractory to CD40 activation (Blair et al., 2010). Idiopathic thrombocytopenic purpura (ITP) is another example of broken self-tolerance with circulating anti-platelets antibodies. In a recent study, Li and colleagues reported reduced IL10 production by B cells, measured by intracellular cytokine staining techniques, in a cohort of ITP patients off treatment. B cells from ITP patients exerted reduced suppressive effect on $CD14^+$ monocytes. Interestingly, $IL10^+$ B cell frequency and their suppressive effect on monocyte function improved in ITP patients who responded to therapy. The authors did not phenotype $IL10^+$ B cells, nor did they sort enriched populations to carry out functional studies. They reported reduced transitional B cell frequencies in ITP patients, and based on previous published studies, they assume that IL0 B cells are enriched within this population (Li et al., 2012b).

In contrast, human B10 cells were found to be increased and exhibited higher IL10 production than healthy controls in 90 patients with a variety of autoimmune disorders including SLE (Iwata et al., 2010). Similarly, higher frequencies of $IL10^+$ B cells were recently reported in patients with untreated active sarcoidosis compared to patients with inactive disease and healthy controls. This was associated with increased BAFF level, hypergammaglobulinaemia and contracted memory and expanded transitional compartments. The authors proposed that these cells act as a "rheostat" to control inflammation (Saussine et al., 2012). In a recent study, spondylarthritis patients were shown to have increased CD5 expressing B cells, mainly naïve and IgM^+ memory B cells, compared with RA patients and HC. Upon in vitro stimulation with anti BCR but not CpG, these cells produced IL10 (Cantaert et al., 2012). The findings of the latter two studies

suggest that the increase in B10 cells may be a compensatory response to restrain the autoimmune process.

Another disease where regulatory B cells might be involved is helminthic infections. There is some evidence that induced Breg might be exploited by some pathogens to evade or dampen the effective immune responses. B cells from MS patients infected with intestinal helminths produced more IL10 than both non-infected MS patients and healthy controls (Correale et al., 2008). In addition, Schistosoma haematobium-infected children had increased PB IL10 producing $CD1d^{hi}$ B cell number compared with uninfected healthy controls (van der Vlugt et al., 2012). Another example comes from the recently published work by Das et al showing that patients with chronic hepatitis B virus infection have higher IL 10 levels as well as increased frequencies of $IL10^{+}$ B cells. IL10 producing B cells were found to be enriched within the $CD19^{+}CD24^{hi}CD38^{hi}$ immature population and suppressed HBV-specific $CD8^{+}$ T cell responses in an IL10–dependent manner (Das et al., 2012). Although increased IL10 availability may protect from extensive liver damage, it will inadvertently suppress viral specific CD8 T cell responses.

Overall, there is ample evidence for the existence of human Breg and their derangement in autoimmunity, although these cells are not yet fully characterised. Whereas impaired Breg function may fail to dampen a self-targeted immune insult, resulting in excessive uncontrolled immune responses, an increase in induced Breg might in turn be exploited by some pathogens to evade effective immune responses. In analogy one may argue that cancer cells may use similar strategies to evade immune surveillance. Furthermore, little data exist on the potential role of B cells in GVHD as an auto-allo-immune condition.

Hypothesis and Aims

The limited understanding of immune mechanisms that underlie the development of graft-versus-host disease (GVHD) hinders our ability to develop effective targeted therapies that could improve the survival of patients undergoing allogeneic haematopoietic stem cell transplantation (HSCT). There is increasing evidence that donor and/or recipient B cells are deranged in GVHD; whether this is a cause or effect is not yet known.

I hypothesise that B cells play a crucial role in induction of chronic GVHD and that the development of chronic GVHD is associated with an abnormal pattern of B cell recovery. I further propose that an imbalance in effector: regulatory B cell ratio during immune reconstitution in the recipient, determines the occurrence of GVHD and autoimmune phenomena frequently seen following SCT.

The specific aims of this thesis are:

1. To design a comprehensive phenotypic and functional B cell panel to study human B cell subsets
2. To evaluate the pattern of B cell recovery in patients with cGVHD by comparing them with SCT patients with no cGVHD and healthy controls.
3. To characterise human IL10 producing B cells in healthy controls and assess their regulatory properties
4. To examine $IL10^{+}$ B cells in patients with cGVHD compared to patients without cGVHD.

Chapter II. Material and methods

II.1 Patients:

Informed consent for the use of patient material was obtained from all patients at Imperial NHS Trust, Hammersmith Hospital. The study was approved by both the National Research Ethics Committee (NREC) and local R&D reviewboard. Peripheral blood mononuclear cells (PBMC) were collected from patients undergoing allo-HSCT prospectively before and at regular time points following allo-HSCT: days 30, 60, 90, 180 days post-transplant.

Informed consent for the use of archived patient material was obtained from all patients at initial presentation to Imperial NHS Trust, Hammersmith Hospital.

II.2 Sample processing:

II.2.1 Isolation of peripheral blood mononuclear cells:

A maximum of 20-30ml of whole blood was collected from patients and healthy controls. PBMC were isolated using density gradient separation technique (Lymphoprep). Briefly, 15 ml of EDTA anticoagulated whole blood was diluted 1:1 with RPMI 1640 media (GIBCO / Invitrogen) in a 50ml falcon tube and 10ml of Lymphoprep (Axis Shield, UK) was gently layered under the diluted blood. After centrifugation at 1800 rpm for 30 min, the interface layer between plasma and Lymphoprep, which contains mononuclear cells, was collected and re-suspended in fresh RPMI 1640. Cells were washed twice with RPMI 1640 media and centrifuged at 1200 rpm for 10 min. PBMC were re-suspended in 10% foetal calf serum (FCS)/ RPMI, cells were counted and cell viability was assessed using Trypan Blue (TB).

II.2.2 Plasma collection:

One heparinised blood sample (7ml) per patient was obtained at each collection time-point. The sample was centrifuged at 2500 rpm for 30 minutes. The clear supernatant was gently aspirated to avoid contamination with blood components. Multiple plasma aliquots per patient were then frozen at -20° C for future batch ELISA assays.

II.2.3 Cell freezing:

PBMC were suspended in freezing media (RPMI-CM with 20% foetal calf serum (FCS) and 20% Dimethyl Sulphoxide (DMSO) (Sigma-Aldrich, UK) on ice and aliquoted into cryovials and stored in -80° C (max 1 week) before transfer to liquid nitrogen.

II.2.4 Cell thawing:

Cryovials of PBMC were transferred from liquid nitrogen on dry ice. Each vial was thawed in a water bath at 37 ºC. Once thawed PBMC were transferred immediately to a 15 ml falcon tube containing 10 ml thawing media (RPMI 20% FCS containing 50,000 unit DNase). PBMC were centrifuged at 1200 rpm for 10 min, pelleted and re-suspended in 1 ml of media for cell count and viability assessment.

II.3 Cell lines- L cells and control cells:

Frozen CD154 (CD40L) expressing transfected fibroblasts (L cells) with their corresponding CD154 negative control (cntl) cells were kindly donated by Dr. Simon Wagner. To ascertain that CD40L is indeed expressed on CD40L transfected L cells, CD154 expression was assessed by Fluorescence-activated cell sorting (FACS) analysis

after staining with CD154-PE mAb, **figure (II-1)**. Cell aliquots were kept in the -80° C freezer and thawed as required following the protocol outlined in II-2.4.

L cells were grown in complete RPMI 1640 (Sigma-Aldrich), supplemented with 12.5 µg/ml Gentamicin, 5 µM HEPES both from (GIBCO/ Invitrogen, Paisley, UK), and 10% FCS. Once cells were confluent in the vented T25 culture flasks, the culture media was removed and the adherent L cells were incubated with 1 to 2 ml of 0.05% Trypsin-EDTA (GIBCO/ Invitrogen Cat. 25300-054) at 37°C for 3 min. L cells were then detached by gentle tapping of the flask and washed by centrifugation. Cells were then frozen as outlined in section II-2.3 and stored at – 80 ° C before transfer to liquid nitrogen. After trypsinisation, the viability of L cells was checked and CD154 was confirmed to exclude any impact of trypsin on CD154 expression (Stelzer et al., 1997). Periodic checks of the CD154 expression were performed to assure that the level of expression was not reduced by frequent passaging. In addition, contamination of L-cells by mycoplasma was intermittently checked for using specific DNA probes and PCR .

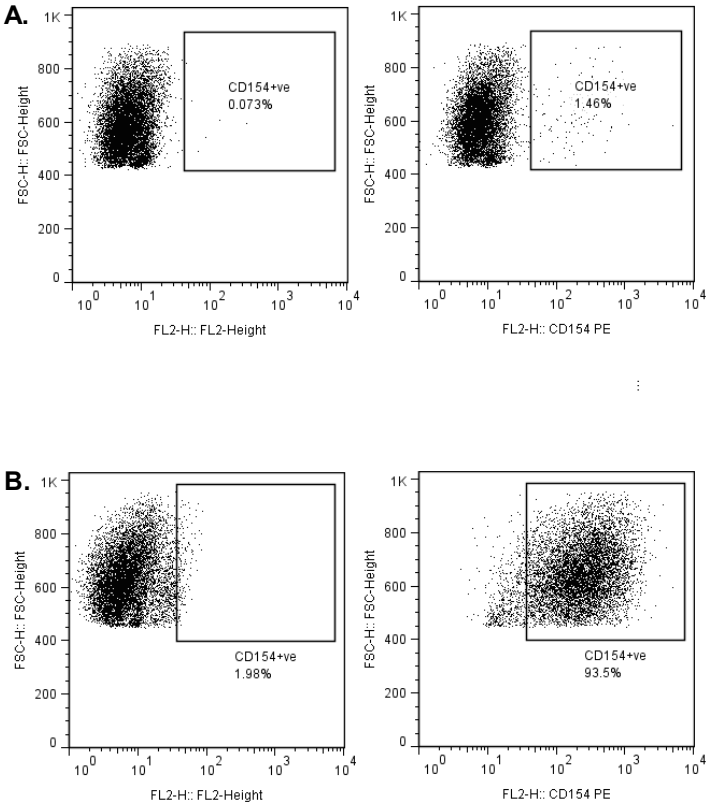


Figure II-1: CD154 expressing L cells and their controls

A. Staining of CD154- cntl cells and **B.** CD154+ L cells with the isotype control (left) and CD154 PE (right).

II.4 B cell culture and activation protocol:

II.4.1 ELISA experiments:

The experiments were performed using $CD19^+$ B cells selected from fresh or frozen PBMC. $CD19^+$ selected total B cells were plated at a concentration of $2x10^5$ cells per well in 200 µl of 10% FCS in complete RPMI 1640 (Sigma-Aldrich), supplemented with 12.5 µg/ml Gentamicin, 5 µM HEPES both from (GIBCO/ Invitrogen, Paisley, UK), in U-bottom 96-well plates (BD Falcon™, Oxford, UK). B cells were stimulated for 72 hours with CpG OND200- 5G (InvivoGen, USA) at a dose of 0.1, 0.2 or 0.8 µg/ml in a humidified incubator at 37 °C with 5% CO2. L cells were irradiated (60 Gy), plated and rested for a minimum of 1 hour and added to $CD19^+$ selected B cells at a ratio of one L cell to 10 B cells (1:10), (from here on referred to as BL condition). In other experiments, $CD19^+$ selected B cells were first incubated with F $(ab')_2$ Goat anti Human IgG+ IgM (H+L) (Jackson ImmunoResearch Laboratories, USA) for one hour to engage the B cell receptor (BCR) followed by L cells (1 L cell: 10 B cell) for 48 to 72h, (from here on referred to as BXL condition). At 72 hours of culture, the plates were centrifuged at 1200 rpm for 5 minutes. A hundred and fifty µl of the supernatant was collected from replicate wells into eppendorf tubes and frozen at - 20°C for batch ELISA analysis.

II.4.2 IL10 quantitative ELISA:

IL10 was assayed using OptEIA ELISA kits (BD Pharningen™, San Diego, CA), following the manufacturer's protocol. Briefly, after washing and blocking of the plate with 50 µl ELISA diluent, 100 µl of sample per well were incubated for 2 h at room temperature. Following five washings, the samples were incubated with 100 µl of the appropriate

detection antibodies for one hour at room temperature. Finally, after seven washings, colour was developed using 100 µl provided (TMB) tetramethylbenzidine reagent per well and incubated for 30 minutes at room temperature in the dark. Fifty microlitres of the stop solution was added and the absorbance was read at 450 nm within 30 minutes of stopping of the reaction. The sensitivities and linear ranges were obtained using the provided standard cytokine and test sample dilutions were determined accordingly, **figure (II-2)**.

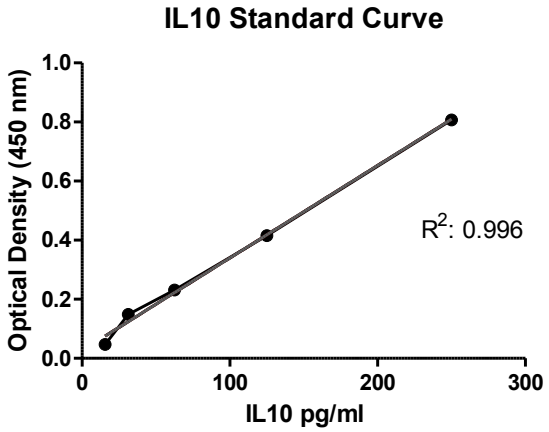


Figure II-2: Example of standard curves for IL 10 detection (n = 6).

II.5 Cell selection

II.5.1 B cell selection:

B cells were selected from fresh and frozen PBMC using human B cell isolation kit II (Miltenyi Biotec Ltd), an indirect magnetic labelling system for the isolation of untouched B cells, following the manufacturer's protocol. Briefly, PBMC were washed using MACS Buffer (autoMACS™ rinsing solution and MACS BSA both from Miltenyi

Biotec Ltd). A total of 10^7 cells were then re-suspended in 40 µl of MACS buffer. Subsequently, 10 µl of Biotin-antibody cocktail against CD2, CD14, CD16, CD36, CD43, and CD235a (Glycophorin A) was added per 10^7 of total cells and the mixture incubated in the refrigerator for 10 minutes. Thirty µl of MACS buffer and 20 µl of anti-Biotin micro-beads were then added to the tube and incubated in the refrigerator for another 15 minutes. A final wash using the buffer was undertaken and the pellet resuspended in 500 µl of MACS buffer. The suspension was applied to primed MS MACS columns (Miltenyi Biotec Ltd) and an additional 3 volumes of the buffer were used to wash the columns. The pass through which contained the purified unlabelled B cells was then centrifuged at 1200 rpm for 10 minutes. The pellet was re-suspended in RPMI/FCS 10% to achieve the required cell concentration. B cell purity was confirmed by staining the cells with anti-CD19 PE (BD Pharmingen™ Cat# 555413) and was consistently between 88.5%-99%, **figure (II-3)**

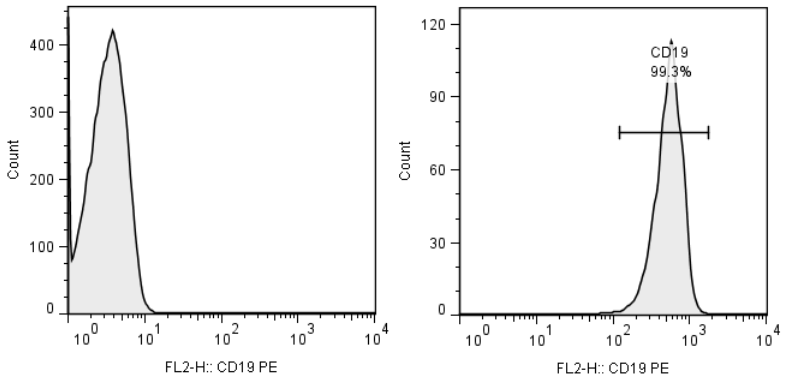


Figure II-3: Purity checks of B cell selection

Purity of selected B cells from a healthy donor stained with CD19 PE and analysed by FACSCalibur.

II.5.2 T cell selection:

II.5.2.1 Total $CD4^+$ T cell selection:

$CD4^+$ T cells were selected from fresh PBMC using human CD4 T cell isolation kit II (Miltenyi Biotec Ltd), an indirect magnetic labelling system for the isolation of untouched $CD4^{+ve}$ T cells, following the manufacturer's protocol.

Briefly, PBMC were washed using MACS Buffer (autoMACS™ rinsing solution and MACS BSA both from Miltenyi Biotec Ltd). A total of 10^7 cells were then re-suspended in 40 µl of MACS buffer. Subsequently, 10 µl of Biotin-antibody cocktail against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ, and CD235a (glycophorin A) was added per 10^7 of total cells and the mixture incubated in the refrigerator for 10 minutes. Thirty µl of MACS buffer and 20 µl of anti-Biotin micro-beads were then added to the tube and incubated in the refrigerator for another 15 minutes. A final wash using the buffer was undertaken and the pellet resuspended in 500 µl of MACS buffer. The suspension was applied to primed MS MACS columns (Miltenyi Biotec Ltd) and an additional 3 volumes of the buffer were used to wash the columns. The pass through which contained the purified unlabelled T cells was then centrifuged at 1200 rpm for 10 minutes. The pellet was re-suspended in RPMI/FCS 10% to achieve the required cell concentration. The purity of the $CD4^+$ T cell purity was confirmed by staining the cells with anti-CD4 PerCP (BD Cat# 555413) and was consistently between 90%-99%; **figure (II-4).**

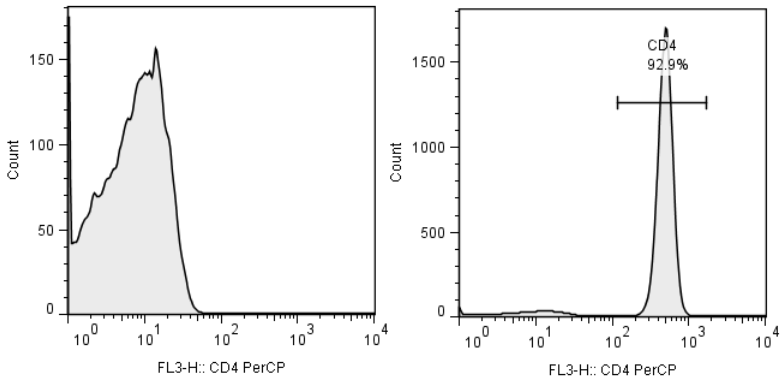


Figure II-4: Purity checks of T cell selection

Purity of selected $CD4^+$ T cells from a healthy donor was determined by staining with CD4 PerCP and analysed by FACSCalibur.

II.5.2.2 $CD4^+CD127^{lo}CD25^-$ and Treg magnetic selection "Treg depletion":

$CD4^+CD127^{lo}CD25^-$ cells were selected from fresh PBMC using $CD4^+$ $CD25^+$ $CD127^{dim/-}$ regulatory T cell isolation kit II human (Miltenyi Biotec Ltd), an indirect two-step magnetic labelling system for the isolation of T^{reg} cells, following the manufacturer's protocol. Briefly, PBMC were washed using MACS Buffer (autoMACS™ rinsing solution and MACS BSA both from Miltenyi Biotec Ltd). A total of 10^7 cells were re-suspended in 40 µl of MACS buffer. Subsequently, 10 µl of Biotin-antibody cocktail were added per 10^7 cells to magnetically remove non $CD4^+$ and $CD127^{hi}$ and the mixture incubated at $+4^oC$ for 10 minutes. Thirty µl of MACS buffer and 20 µl of anti-Biotin micro-beads were then added to the tube and at $+4^oC$ for 15 minutes.

A subsequent wash using the buffer was undertaken and the pellet suspended in 500 µl of MACS buffer. The final suspension was applied to LD MACS columns (Miltenyi Biotec

Ltd) and 3 volumes of the buffer used to wash the columns. The flow-through which is enriched for $CD4^+$ cells was then centrifuged at 1200 rpm for 10 minutes. In step two, the pellet was re-suspended in 90 µl of MACS buffer followed by 20 µl of CD25 MicroBeads. The mixture was incubated for 15 min on ice and finally washed and re-suspended in 500 µl of MACS. This cell suspension was passed through an LS MACS magnetic column. The pass through contains untouched $CD4^+$ cells depleted of Treg ($CD4^+CD127^{lo}CD25^{hi}$) and the column contains labelled T^{reg} that were flushed out by firmly pushing the plunger into the column. The cells are then checked for viability by staining in Trypan blue, counted and seeded in plates as required.

The purity of both subsets was confirmed by staining the cells with anti-CD25, anti-CD4 and anti-CD127 and was found to be consistently between 80-90%; **figure (II-5).**

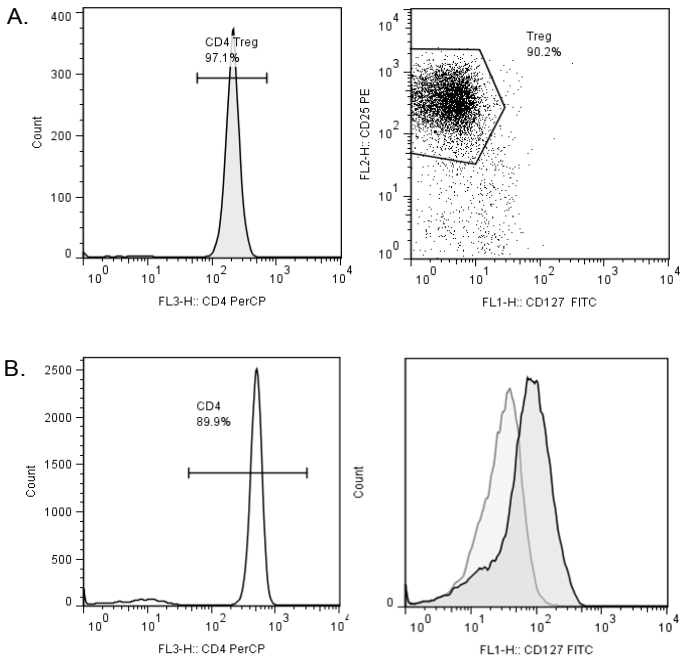


Figure II-5: Purity check of Treg selection
A. Purity of selected Treg cells from a healthy donor was determined by staining with CD127 FITC, CD25 PE and CD4 PerCP. **B.** Phenotype of the flow through (depleted of Treg) shows CD4 purity of 90% and lower CD127 expression ($CD127^{lo}CD4^{+}$) (**grey**) compared to total $CD4^{+}$ T cells (**black**)

II.6 Cell sorting:

II.6.1 Sample preparation and sorting:

B cells subsets:

Cell sorting was performed using BD FACS Aria IIu at the FACS core facility, Respiratory Sciences, St Mary's hospital.

Briefly, freshly prepared PBMC were stained with 2 µL of FITC anti-CD27 DakoCytomation (# F7178), 10 µL of PC7 anti-CD19 BC/Immunotech (# 3628) and 2 µL

of PerCP cy5.5 anti-IgM (# 561285) for 30 min. Positive controls for each individual parameter were included in addition to the negative unstained control. One microlitre of Propidium Iodide (PI) was added to samples prior to sorting to assess their viability. All samples were filtered prior to sorting to prevent aggregates clogging the 70 µm nozzle. A concentration of 10^7 cells/ml of sorting medium was determined to achieve a sorting efficiency of > 80%. In view of the rarity of some target populations and to reduce sorting time and improve cell viability, enrichment for $CD19^+$ cells using a B cell Miltenyi isolation kit **(section II-5.1)** was undertaken in some experiments.

The gating strategy for B cell subset sorting is presented in **figure (II-6)**. Four $CD19^+$ B cell subsets, namely IgM memory ($CD27^+$ $IgM^{+/hi}$), transitional B cells ($CD27^-$ $IgM^{+/hi}$), switched memory ($CD27^+$ IgM^-) and naive B cells ($CD27^-$ IgM^+) were sorted in purity sorter mode; **figure (II-7)**. Purity checks were undertaken at the end of each sort to ensure high purity population; typically 99%.

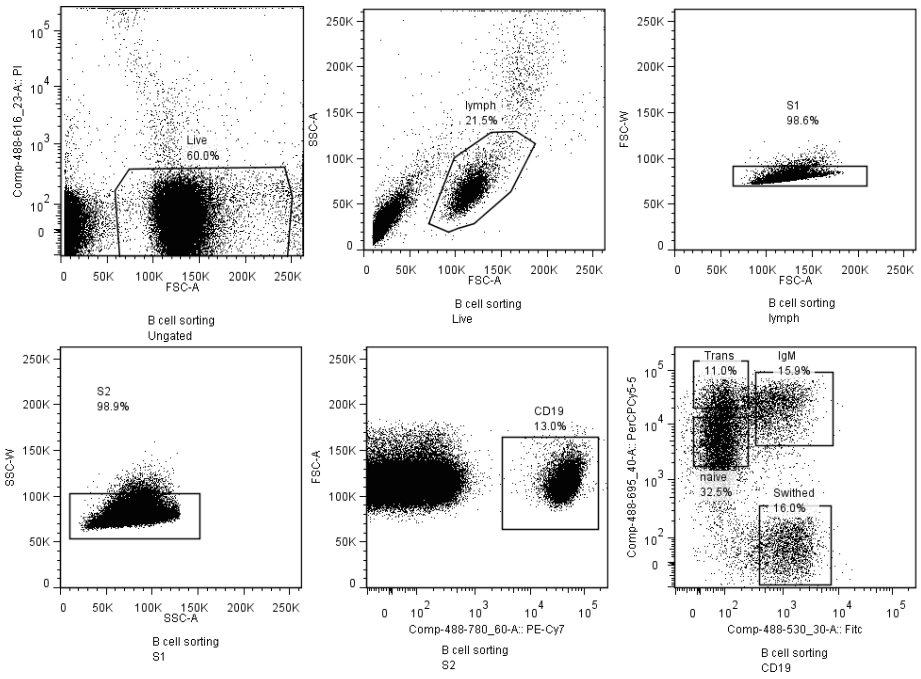


Figure II-6: Gating strategies of B cell sorting

Multicolour flow cytometric gating strategy for B cell subset sorting on BD FACS ARIA IIu. Following a lymphocyte gate, cell doublet and dead cell discrimination, live cells (PI^-) are further gated for $CD19^+$ B cells. $CD19^+$ B cells are then sorted based on CD27 and IgM expression into 4 subsets, namely IgM memory, transitional, naïve and switched memory B cells.

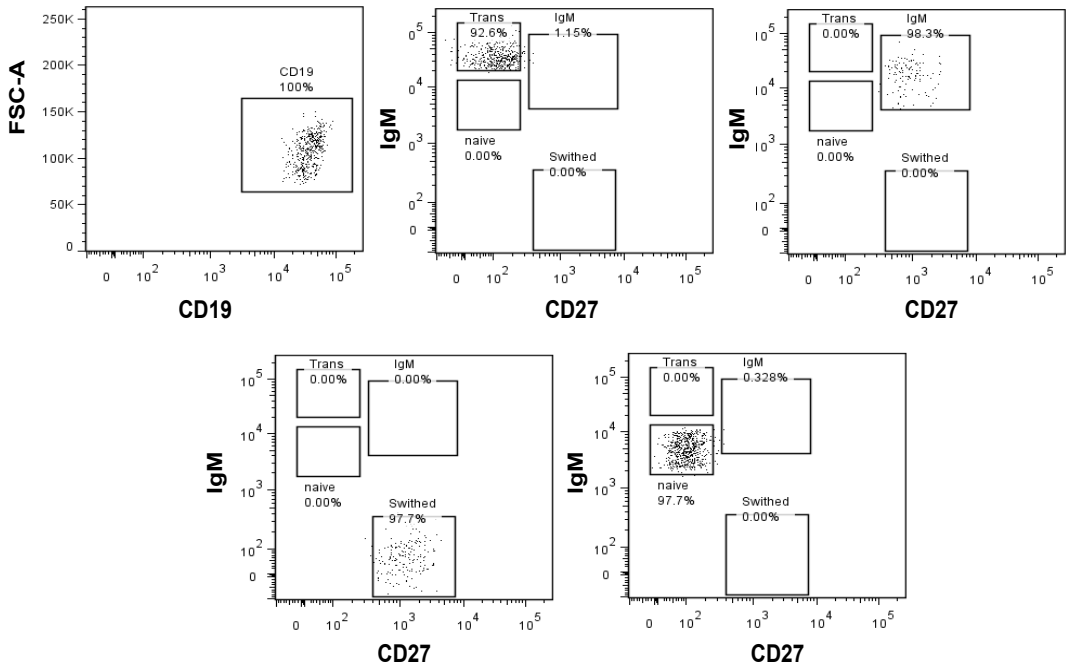


Figure II-7: Purity checks of B cell sorting

FACS plots demonstrating the high purity of sorted B cell subsets are shown within the $CD19^+$ gate

The sorted population were collected in 12 X 75 mm sterile polypropylene tubes containing MACS buffer solution and transferred to culture media (10% FSC in RPMI-1640 complete media) to minimise apoptosis.

II.7 Proliferation Assay:

Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a cell permeable division tracking dye that is retained within cells and covalently couples to intracellular molecules. The dye allows the monitoring of lymphocyte proliferation, both in vitro and in vivo, in view of the progressive halving of CFSE fluorescence within daughter cells following each cell division (Hawkins et al., 2007).

Freshly isolated PBMC were re-suspended in PBS at cell concentrations of 2.5 to 5 × 10^6/ml. For each independent experiment, CFSE (65-0850; eBioscience, USA) was diluted from a stock solution (500µg/ml; stored at -20^{o}C in DMSO) to reach final concentration of 2 µM. This was achieved by adding 2 µL of aliquoted CFSE per each ml of prepared sample and mixing well; in experiments where the sample volume was greater than 5 ml, cells were vortexed following the addition of CFSE to achieve uniform cell labelling. After incubation for 10 minutes at room temperature in the dark, 2 to 3 volumes of neat FCS were added to the tube for 5 minutes to stop the labelling process. Labelled cells were seeded into a flat-bottom 96-well tissue culture plates at a concentration of 2x10^5 per well in 10% FCS/RPMI and incubated for 96h at 37ºC with 5% CO2.

FACS analysis was undertaken after 96 hours on the FACSCalibur to assess cell proliferation. CFSE labelled unstimulated cells, incubated for the length of the experiment, served as negative control, and stimulated cells as positive controls. The proliferation of the test lymphocytes was interpreted relative to the negative and

positive controls. The percentage of dividing cells, proliferation index and rounds of divisions were calculated using FlowJo's, Treestar flow cytometry analysis software, proliferation platform; **figure (II-8)**.

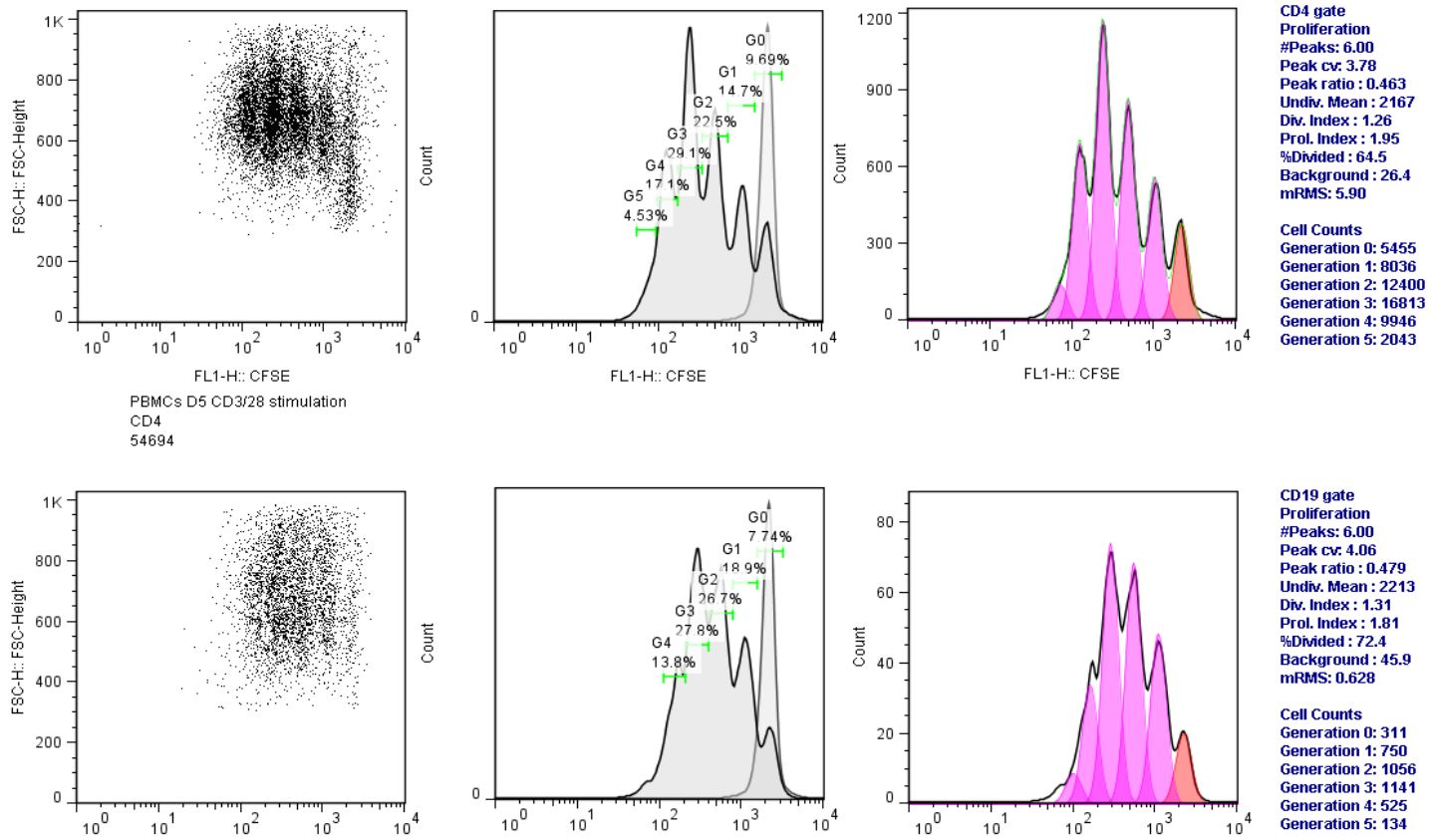


Figure II-8 : CD4 and CD19 proliferation assay

Proliferation of gated $CD4^+$ (top) and $CD19^+$ cells (bottom) following stimulation of PBMC with anti-CD3 and anti-CD28 beads. Dot plots and histograms show the serial dilution of CFSE as with each cell division, the CFSE is divided between the two daughter cells. FlowJo is used to generate data related to the proliferation index.

II.8 Phenotypic and functional characterisation of B cells using flow cytometry:

II.8.1 Intracellular cytokine assay for detection of IL10$^+$ B cells and phenotyping:

The experiments were performed using either CD19$^+$ selected B cells derived from fresh or frozen PBMC or total PBMC. To determine the kinetics of IL10 production using intracellular cytokine assay, CD19$^+$ selected B cells or total PBMC were stimulated with irradiated L cells at a ratio of 1:10 for 6, 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 to eight hours of the incubation. The aim was to determine the incubation time points at which maximal IL10 production could be achieved without major activation induced alterations of B cell phenotype.

To investigate whether T cells are capable of inducing B cells to produce IL10, PBMC were stimulated with CD3/CD28 Dynabeads® Human T –activator (Invitrogen, Oslo, Norway) for 72h in a humidified incubator at 37 °C with 5% CO2. At the end of the culture duration, T cell activation defined as upregulation of CD154 was examined by flow cytometry with anti CD154 PE and CD4 PerCP. Using intracellular cytokine assay, IL10 production was assessed on gated CD19$^+$ B cells.

II.8.2 Phenotypic analysis of IL10 producing B cells:

For $IL10^{+}$ B cell characterisation PBMC were stimulated with irradiated L cells for 12 to 15 hours. PMA (50 ng/ml), Ionomycin (250 ng/ml) (Sigma Aldrich) and Brefeldin A (5 µg/ml) (Golgi-plug Sigma Aldrich) were added for the last 6-8h of the culture. Cells were harvested, washed in staining buffer (1x PBS, 2% heat-inactivated FCS, 0.1% Sodium Azide) and incubated for 20 min at room temperature with a cocktail of CD19-PE, CD3 PerCP, (BD Biosciences), CD22 FITC, CD24 FITC, CD5 FITC, BAFFR FITC, CD1d PE, CD27 PE, IgD PE, CD40 PE and IgM PerCP Cy5.5, all from BD Pharmingen™, CD21 FITC and FCRL4 PE (BioLegend) and CD38 PE Cy7 (eBioscience). Cells were then washed with staining buffer and fixed/permeabilised for 30-45 min at 4°C with intracellular staining kit (eBioscience). At this stage, 20 µl of FcR Blocking Reagent, Miltenyi Biotec (120-000-442), was added. Without washing, cells were incubated for 30 min at 4°C with the following antibodies: 10 µl of FITC conjugated anti-TNFα, 10 µl of FITC conjugated IFN-γ or 5 µl of FITC conjugated IL-2 and 0.5 µl of either APC conjugated IL10 or IgG2aK isotype antibodies. All incubations were performed in Perm-wash solution. Cells were washed twice with perm-wash and re-suspended in staining buffer at approximately 0.5-1 $x10^5$ cells per tube prior to acquisition using BD FACSCalibur (Becton Dickinson). Data analysis was performed using FlowJo® software; **figure (II-9)**. The volume of IL10 monoclonal antibody was determined in dose titration experiments. The volume which resulted in maximal mean fluorescence intensity (MFI) and maximal percentage of positively stained cells with minimum background (negative control) was selected.

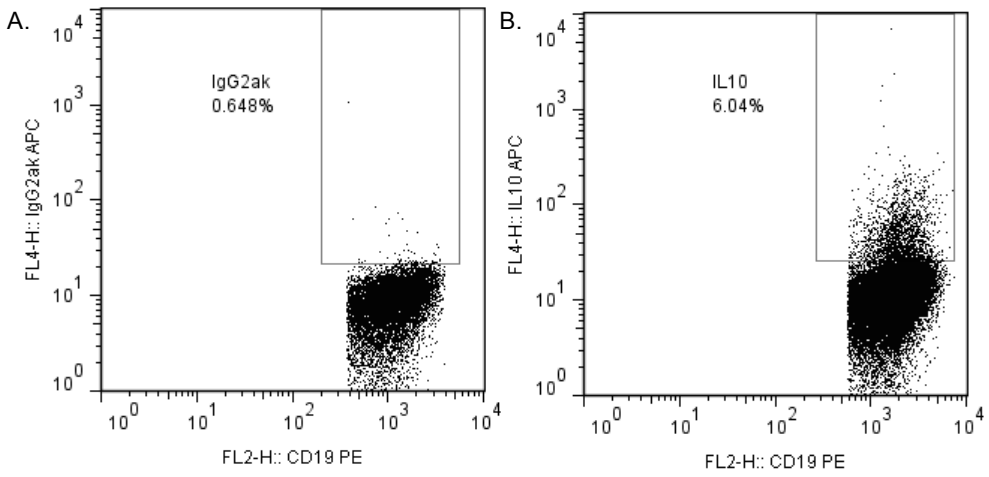


Figure II-9: Intracellular staining of IL10

A representative FACS plot illustrating intracellular IL10 production when PBMC from a healthy control were stimulated with L cells from a healthy donor for 48 h. A. IgG2aκ isotype control B. IL10

II.8.3 Phenotypic characterisation of B cells:

B cell Phenotyping using an extended panel of surface antibodies:

The relatively unique multidimensional nature of flow cytometry allows for particular cell populations to be identified through the use of specific staining and various gating strategies. The selection of fluorochromes was optimised in light of the commercially available options and the instrument configuration; lasers and detectors and the intended cell markers.

A panel of 9 antibodies (as defined in Table II-1) was used to determine the following B-cell subsets:

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

II.8.3.1 Antibody titration:

Each individual antibody was titrated to determine the concentration at which high resolution of the desired populations was achieved with minimal loss of population positivity. The optimum titre or dilution is the concentration which gives the best staining with minimum background / nonspecific binding (Hulspas et al., 2009).

Briefly, serial dilutions of each antibody were made in 3 fold increments starting from 3 times the recommended volume, if the concentration was unknown. Whenever the concentration of the tested Ab was provided by the manufacturer, the titration started from three times the recommended quantity and down to the desired final dilution. Samples and experimental conditions, including staining methods and cell numbers,

were identical to that of the intended experiment. Unstained controls were used to set up the flow cytometer and define the negative populations. Histograms were generated for each antibody dilution and gates were drawn based on the unstained control and the clarity of the positive peaks. However, when the positive population was small and/or the separation from the negative events was not clear enough to ascertain the gates, bivariate plotting against forward or side scatter was used. The gates were manually adjusted at higher concentrations to make for the right-shift of the background accompanying the high concentrations especially when there was clear delineation of the different populations.

Using FlowJo, the MFI and percentages of positive and negative populations as well as the standard deviation of the negative population were determined.

The stain index (S.I) takes into account the width of the background and the distance from the positive population (Hulspas et al., 2009) and is defined as MFI of the positive population – MFI of the negative population (background)/2x standard deviation of the background (Hulspas et al., 2009) **figure (II-10).**

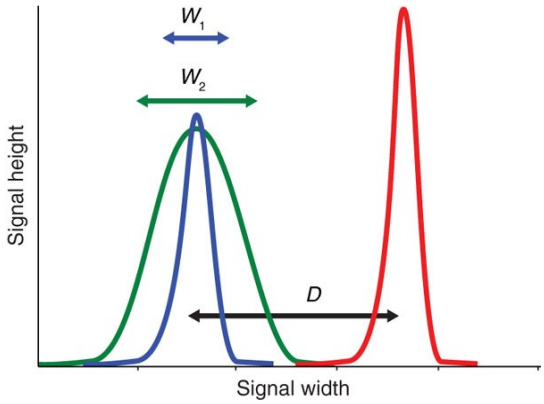


Figure II-10 : stain index

Stain index: The effective brightness of an antibody depends on the distance (D) between the positive (red) and the negative (blue, green) populations and the width of the negative population (W); adopted from reference.(Maecker, 2009)

The calculated stain index and median fluorescence intensity acquired from single stained samples of the studied antibodies' serial dilutions were then plotted against the antibody concentrations; **figure (II-11).** The concentration with the highest stain index, an MFI close to the saturation segment on the MFI curve and the smallest drop in the percentage of the positive portion was chosen for each individual antibody.

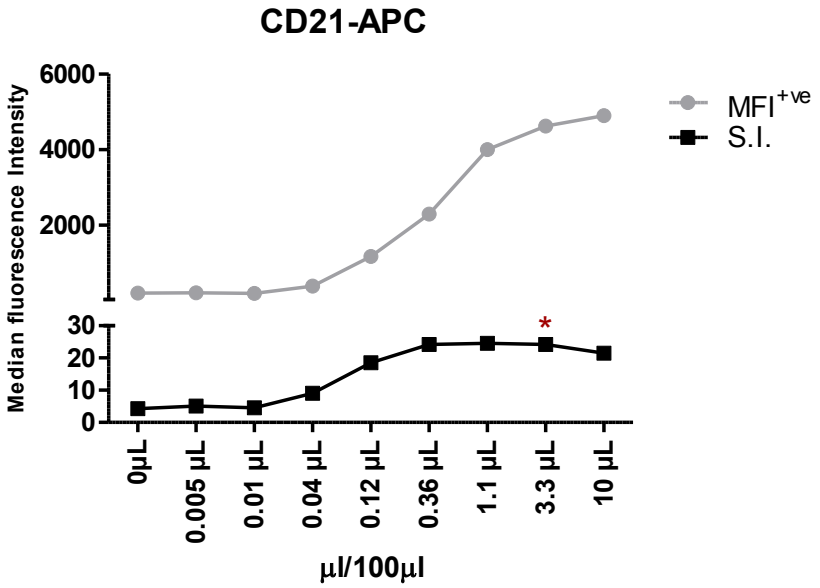


Figure II-11: Antibody titration: CD21 APC

An example of antibody titration: CD21 APC. MFI of the positive population (MFI^{+}) and S.I. are plotted against increasing CD21 APC volumes. The asterisk indicates that 1 to 3 µl/100µl of the antibody is the optimum concentration as it represents a high S.I. and MFI before the saturation plateau.

II.8.3.2 Cell staining and FACS acquisition:

Briefly, approximately 1×10^6 of fresh or thawed PBMC were stained with 1µl of the viability marker invitrogen™ LIVE/DEAD Fixable Aqua Dead cell stain Kit cat# L34957 (L_D) for thirty minutes in the dark at room temperature. Cells were then washed and incubated with the cocktail of mAb **(table II-1)** at the titrated concentrations in x1 PBS in the dark for a further 30 min at room temperature. Subsequently, stained cells were washed once more before acquisition on a three-laser BD™ LSRII (BD Biosciences).

Instrument setup was standardised to reduce experiment-to-experiment variation. Cytometer Settings & Tracking (CST) beads (Becton Dickinson) were run daily, by the core facility laboratory, St Mary's Hospital, at the pre-optimised detector PMT voltages.

Colour compensation was accomplished by using a mixture of negative and positive antibody-capture beads (BD^{TM} Comp Beads) singly stained with the panel antibodies. Before sample acquisition, PMT values were adjusted using unstained cells to exclude auto-fluorescence and to control the intensity of the background; normally by restraining the negative population below log 10^2. The single-stained beads were then run and checked individually to ensure that each stain was the brightest in its own channel before acquisition of 5000 events per tube.

The compensation matrix of acquired control tubes was computed using FACSDiva (automated and acquisition defined) and exported with FACS files or using FlowJo compensation editor at later analysis. Manual compensation was rarely used and only on occasions when optimal adjustment of the spectral overlap between the different fluorochromes did not allow compensation by the software.

Data analysis was performed using FlowJo (v.7.6.5) software (Tree Star) on live cell gate as shown in **figure (II-12).**

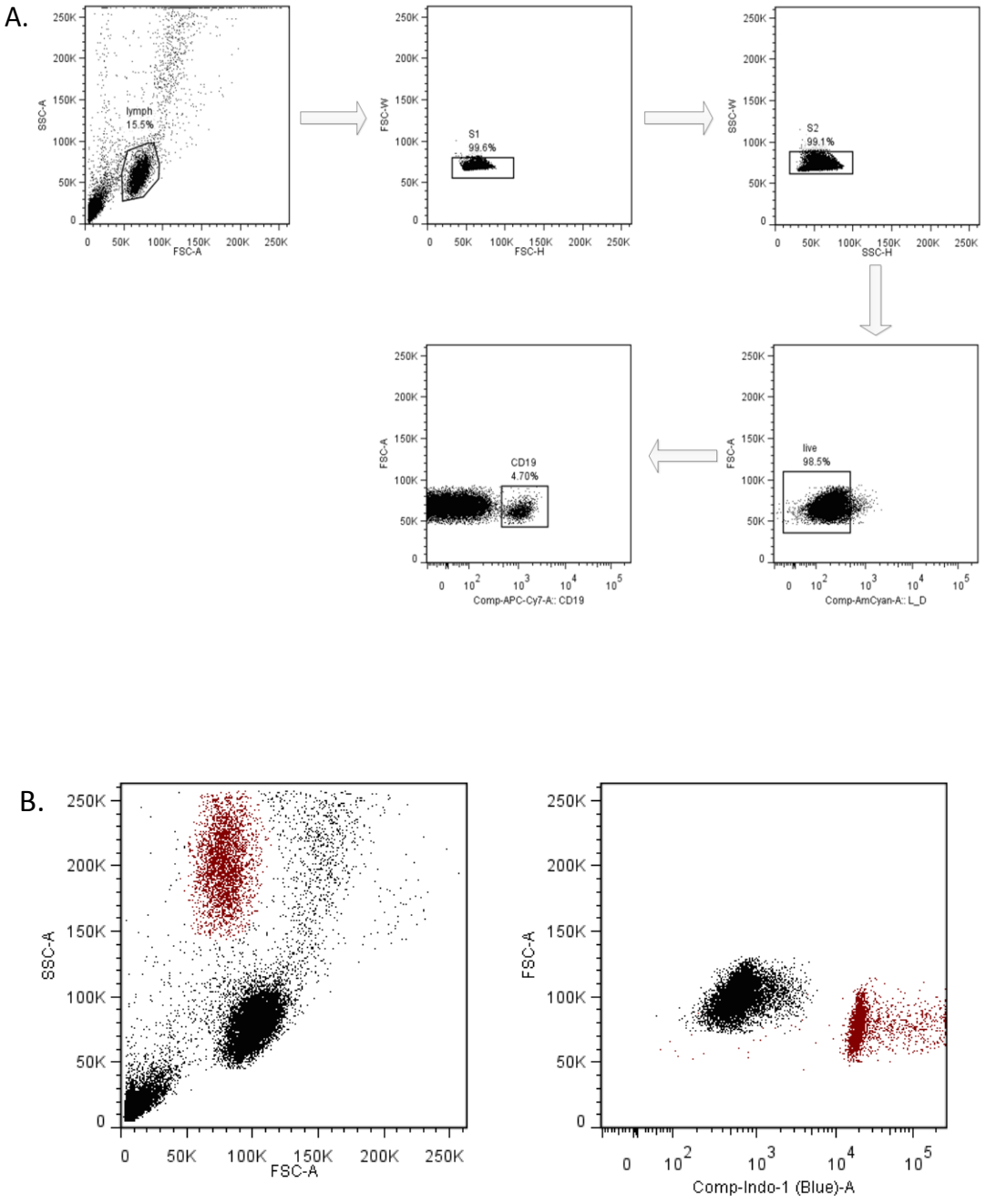


Figure II-12 : Gating strategies of B cell surface phenotyping

A. The gating strategy is presented here: Following a lymphocyte gate, cell doublet and dead cell discrimination, live cells (L_D^-) are further gated for $CD19^+$ B cells. **B.** Dot plots of live/dead positive (L/D) control. PBMC are exposed to high temperature in boiling water bath (positive control). The right dot plot illustrates the dead population in red, that uptakes the dye with the corresponding location on the forward and side scatter dot plot on the left.

Table II-1: antibodies used for B cell characterization:

Antibody	Volume µl	Manufacturer	Description	Clone
CD19 APC H7	5	BD Pharmingen™	Pan B cell marker (BCR activation)	SJ25C1
CD21 APC	3	eBioscience	complement component receptor 2	HB5
CD24 FITC	5	BD Pharmingen™	B cell proliferation and maturation	HB5
CD27 PE	5	BD Pharmingen™	TNF-receptor superfamily	M-T271
IgD v450	5	BD Horizon™	Surface antibody	IA6-2
IgM PerCP cy5.5	3	BD Pharmingen™	Surface antibody	G20-127
CD38 PE Cy7	3	eBioscience	Ectoenzyme (calcium signalling)	HIT2
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.9 In vitro suppression Assay:

II.9.1 Proliferation Suppression:

To assess the suppressive properties of sorted B cell subsets on the proliferation of CD4+ve T cells, B cells were co-cultured with CFSE stained CD4 T cells in vitro.

Total $CD4^+$ or $CD127^{-/dim}$ $CD4^+$ T cells were magnetically selected as described in **section II.5.2.2**, and labelled with CFSE (section II.8). Labelled cells were seeded in 96-well flat-bottom tissue culture plates and FACS sorted B cell subsets including IgM memory, switched memory and naïve were added to separate wells at a B cell-to-T cell ratio of 1:1 in a total volume of 200 µL of 10% FCS in supplemented RPMI medium; maximum cell count in each well was $2x10^5$ per well. T cells were activated by adding Dynabeads® human T- activator CD3/CD28 (InvitroGen, Oslo, Norway) at a concentration 2µl/$8x10^5$ cells following the manufacturer's protocol. CFSE stained T cells cultured with no stimulation (negative control) and CFSE stained T cells cultured with CD3/CD28 coated beads (positive proliferation control), were included in each experiment for reference. Furthermore, selected Treg were co-cultured with tested autologous CD4 T cells as quality control of suppression, as shown **table (II-2).** The plate was then incubated at 37ºC in the presence of 5% CO2 for 96 hours, at which point the cells were harvested from the wells and transferred into FACS tubes. Following one wash, cells were stained with APC anti-CD4 and PE anti-CD19 (both from BD Biosciences) for 20 min and acquired on BD FACSCalibur.

Table II-2: experimental conditions for CFSE suppression assay

Condition	$CD4^+$ / $CD4^+$ $CD25^-$	CFSE	CD3/CD28	Subset
Negative Control	+	+	-	-
Positive proliferation control	+	+	+	-
Positive quality control	+	+	+	Treg
Condition 1	+	+	+	IgM memory
Condition 2	+	+	+	Switched memory
Condition 3	+	+	+	Naive

II.9.2 Suppression of cytokine production:

To assess the suppressive effects of sorted B cell subsets on cytokine production by $CD4^+$ T cells, magnetically selected total $CD4^+$ or $CD127^{-/dim}$ $CD4^+$ T cells (**II.5.2**) were seeded into a 96-well flat-bottom tissue culture plate, to which the FACS sorted B cell subsets, namely IgM memory, switched memory and naïve B cells were added at a B cell-to-effector T cell ratio of 1:1 as described in **II.9.1**. Negative and positive controls were included with each experiment for reference as shown in **table (II-3).** The plate was then incubated at 37ºC in the presence of 5% CO2 for 48 hours. Brefeldin-A (BFA) (Sigma 5mg/ml DMSO cat# B-7651) was added for the last 5 hours of the culture at a final concentration of 10µg/ml. At the end of the culture period, the cells were harvested from the wells and transferred into FACS tubes. Following one wash, cells were stained in a 2-step process; the first step involved surface staining with anti-CD19 PE, CD4 PerCP

(both from BD Biosciences) for 20 min. Cells were fixed and permeabilised using the eBioscience FoxP3 Staining Buffer Set (cat.72-5776) and eBioscience Permeabilisation Buffer (1x) (cat. 00-8333) as per manufacturer's protocol. For the second step, intracellular antibodies IFNγ FITC and TNFα APC were added for 25 minutes. After the last wash, samples were acquired on BD FACSCalibur and analysed using FlowJo.

Table II-3: Experimental conditions for cytokine production suppression assay

Condition	$CD4^+$ / $CD4^+$ $CD25^-$	CD3/CD28	Subset
Negative control	+	-	-
Positive proliferation control	+	+	-
Positive quality control	+	+	Treg
Condition 1	+	+	IgM memory
Condition 2	+	+	Switched memory
Condition 3	+	+	Naive

II.9.3 IL10 blocking:

To assess whether suppression could be mediated through IL10 from B cells, IL10 receptors were blocked using Purified NA/LE rat anti-human CDw210 (BD Pharmingen™ cat# 556011). The antibody was added to the wells at a concentration of 2 µg /ml on day 0 of the culture. Production of IFNγ and TNFα by stimulated CD4 T cells was assessed in the presence or absence of IL10 blocking antibody as described in **section II.9.2**.

Chapter III. B cell subset and characterization of CD21^{-} B cells in healthy controls

III.1 Introduction

B cells are divided into a number of subsets ranging from immature to mature terminally differentiated cells. Immature B cells with non-self-specificity survive the scrutiny of immune surveillance and differentiate into more mature stages in the periphery. Knowledge of B cell subsets and their phenotypic characteristics is essential for studying B cell involvement in both health and disease. A number of B cell classifying schemes have been used to identify B cells subsets; Bm1-Bm5, CD27/IgD and CD24/CD38, **figure (III-1 A to D)**. Correlation between different classifications has been addressed by Sanz et al (Sanz et al., 2008).

Bm1-Bm5 classifies B cells in the human tonsil based on relative expression of IgD and CD38 (Bohnhorst et al., 2001). Five categories are separated: virgin naïve cells (Bm1: IgD^{+}CD38^{-}); activated naïve cells (Bm2: IgD^{+}CD38^{+}); pre-GC cells (Bm2`: IgD^{+}CD38^{++}); GC cells (Bm3-centroblasts and Bm4-centrocytes, both are IgD^{-}CD38^{++}); and memory cells (Bm5: IgD^{-}CD38$^{+/-}$) which are further divided into early Bm5 (CD38^{+}) and late Bm5 (CD38^{-}). All memory B cells (BM5) are IgD^{-} which makes this scheme inherently unable to recognise IgM^{+}CD27^{+} memory B cells. Moreover, IgM memory B cells which are IgD^{+} will fall into the Bm1 or Bm2 region. Bm1-Bm5 can be therefore applied to identify PB B cells with the exception of GC B cells which do not normally circulate.

The second classification is based on the expression of CD27 and IgD (Maurer et al., 1992). This strategy implements CD27 as a universal marker of human memory B cells to distinguish between $CD27^+$ memory and $CD27^-$ antigen-naïve B cells. In the peripheral blood, naïve as well as transitional B cells are $CD27^-IgD^+$, therefore, this classification does not distinguish naïve and transitional B cells. Furthermore, the $CD27^-$ fraction also includes isotype switched $CD27^-$ IgD^- memory B cells. $CD27^+$ memory cells are subdivided into IgD^+ (usually co-expressed with IgM^+ or alone in a minor fraction of memory cells) and IgD^- isotype switched (predominantly IgG^+ or IgA^+). A major limitation of these two classifications is that they do not identify transitional B cells as an independent category.

Another scheme uses CD24 and CD38 (Carsetti et al., 2004) to classify B cells into four subsets: $CD24^{hi}CD38^{hi}$ transitional B cells, $CD24^+CD38^+$ naïve B cells, $CD24^{hi}$ $CD38^{lo}$ memory B cells and $CD24^-CD38^{hi}$ plasmablasts. Memory B cells are defined in one population regardless of their isotype status. Immature/ transitional B cells can be further characterised based on other markers expression such as IgM, CD10 and CD21 (Suryani et al., 2010, Carsetti et al., 2004, Palanichamy et al., 2009).

The above-mentioned classifications do not include CD21 expression to define B cell subsets despite the fact that $CD21^{-/lo}$ B cells have be shown to be expanded in a number of diseases. In health, there is scanty literature about this cell population. Four B cell groups have been reported to lack CD21 expression; namely, activated memory cells, early transitional B cells, plasmablasts and exhausted B cells (Moir et al., 2008).

In this chapter I aim to validate an extended panel of monoclonal antibodies against a number of B cell antigens, in addition to CD21, CD10, IgM and IgD to define B cells more comprehensively. Titration of the monoclonal antibodies is crucial to optimise the separation of cell populations and to assure reproducibility and comparison of the phenotype plots. I implemented the panel to phenotype B cell subsets in the PB of healthy individuals. I also defined $CD21^-$ B cells more comprehensively.

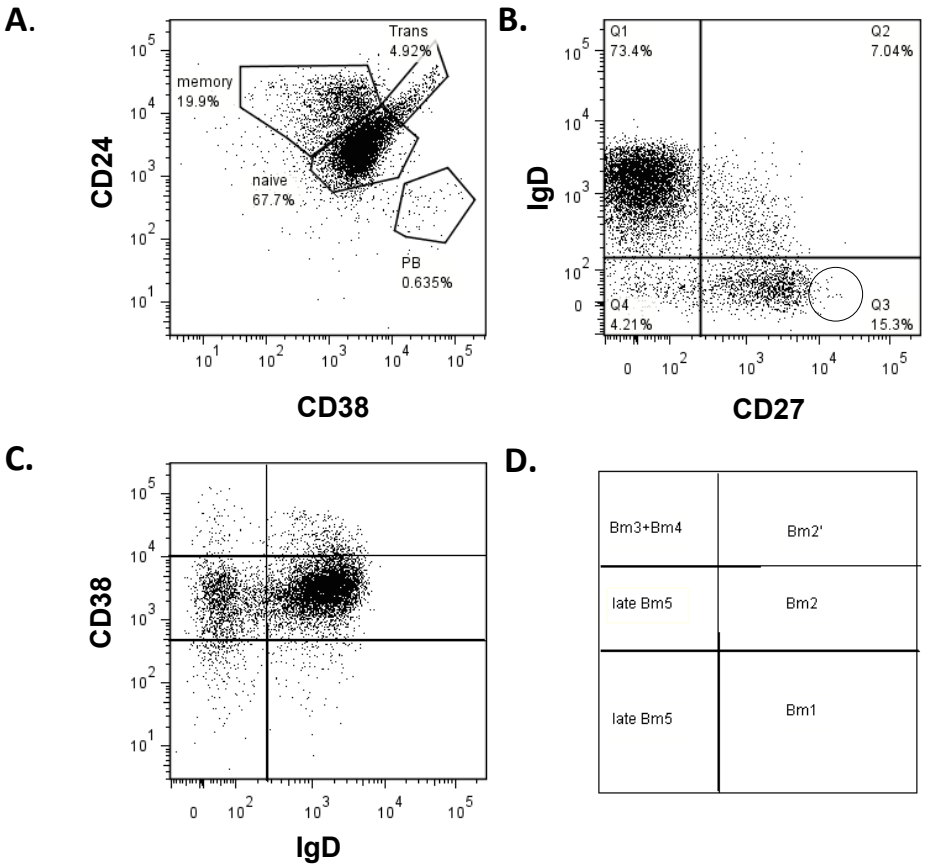


Figure III-1: $CD19^+$ B cell classifications

Gated $CD19^+$ B cells are plotted according to the three classifications **A**. CD24/CD38: Transitional (Trans), memory, naïve and plasmablasts (PB). **B.** IgD/CD27: naïve (Q1), IgM memory B cells (Q2) and switched memory B cells (Q3) with IgD^-CD27^{hi} PB encircled. **C.** Bm1-Bm5 classification with **D.** schematic representation and subsets.

III.2 Results

III.2.1 Titration of monoclonal antibodies and flow cytometry settings

Each antibody was titrated as outlined in **section II.8.3.2** The same number of PBMC was stained with serial dilutions of anti-CD21 APC. Standard lymphocyte gating and subsequent gating of $CD21^+$ and $CD21^-$ populations was performed. Gates were adjusted according to positive and negative populations. Standard deviation of the negative population and the Median Fluorescence Intensity (MFI) of both negative and positive populations were calculated using the FlowJo software. The stain index was calculated as $[(MFI^{pos} - MFI^{neg})/ 2xSD^{neg}]$ and a scatter plot of Antibody Concentration versus Stain Index was generated. The concentration of mAb that yielded the maximum Stain Index with the best separation of negative and positive populations, while achieving a high positive frequency, was considered the optimal volume to be used in subsequent experiments. As shown in **figure (III-2)**, 1 µl of anti-CD21 APC, associated with the highest stain index (SI=24.5), was considered the optimal volume to use per 100 µl of sample.

Similar analyses were performed for each of the remaining antibodies and optimal concentrations were determined. Finally, the chosen mAbs concentrations based on single staining titrations were combined in one panel, **table (III-1).**

Table III-1: titrated antibodies' volumes in the final master mix

Reagent (µL)	CompBeads	Master mix
CD24 FITC	3	1
CD27 PE	3	1
CD19 APCH7	1	.5
CD38 PC7	1	1
CD21 APC	1	1
CD10 QDOT	.4	1
IgM PerCP-Cy5.5	1	1
IgD v450	1	1
Total antibodies volume (µL)	11.4	7.5
Sample volume (µL)	0	92.5
Final volume (µL)	50	100

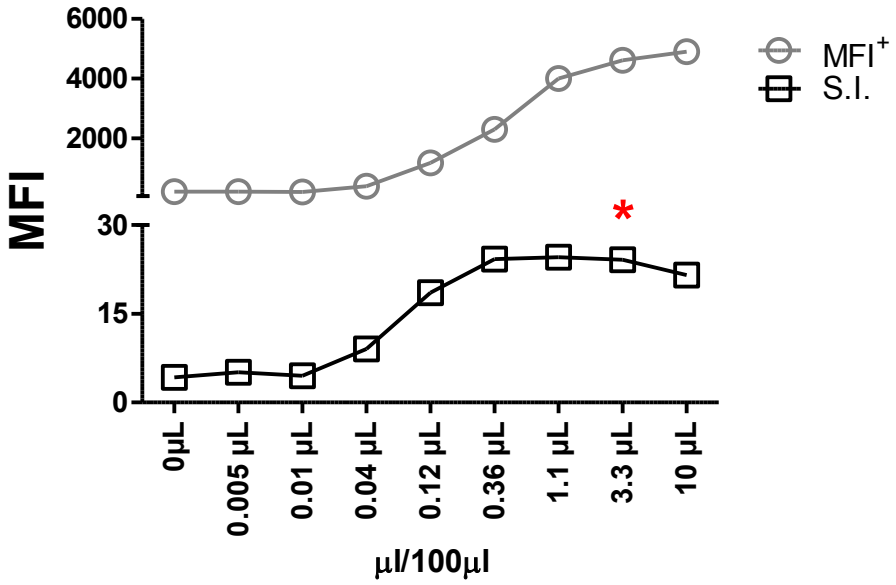


Figure III-2: Stain index and MFI: CD21 APC

Stain index and median fluorescence intensity (MFI) titration curves for anti CD21-APC: 5×10^5 cells were stained with serial amounts of anti CD21-APC. Unstained cells were used as negative control. Calculated stain index and MFI of the positive population were plotted against the corresponding antibody volume. Three microlitres was determined to be the optimal volume as it corresponds to the highest S.I. and MFI.

III.2.2 Characterisation of human B cell subsets and panel validation

III.2.2.1 Transitional B cells

Using the titrated panel of mAbs, and in keeping with published data, $CD24^{hi}CD38^{hi}$ transitional B cells were mostly $CD27^{-}$ (~ 97%), IgD^{+} and IgM^{hi}. Around 32% (21.5%-42.6%) of gated $CD24^{hi}CD38^{hi}$ B cells expressed CD10, indicating that these cells are the most immature subset in the PB, **figure (III-3).** In studies of B cell repopulation, the most immature transitional cells were shown to be $CD10^{+}$ with the highest expression of CD24 and CD38. In keeping with these data, $CD10^{+}$ transitional B cells in my samples also expressed high levels of CD24 and CD38. Dividing transitional B cells phenotypically into T1 and T2 revealed that T1 were more immature, expressing CD10 and completely lacking CD21; **figure (III-4).**

Around 20% (11.5%- 23.1%) of $CD24^{hi}CD38^{hi}$ transitional B cells did not express CD21. Interestingly, the majority of $CD10^{+}$ transitional B cells were negative for CD21. Gaining CD21 expression marks the transition to more mature transitional and naïve B cells and follows the known T1 to T2 pattern.

I also correlated the phenotype of $CD24^{hi}CD38^{hi}$ transitional B cells with the other two classification systems; **figure (III-5 A).** $CD24^{hi}CD38^{hi}$ B cells overlaid the Bm2` pre-GC region defined by IgD and CD38 of Bm1-Bm5 classification. With respect to the IgD/CD27 scheme, $CD24^{hi}CD38^{hi}$ transitional B cells were mainly contained within the $CD27^{-}IgD^{+}$ naïve B cells; although they did not fall within a distinctive population, as transitional B cells express lower IgD than naïve cells. In contrast, transitional B cells fell within the $CD27^{-}IgM^{hi}$ B cells when analysed in relation to IgM and CD27. This distinct location

makes it possible to sort an enriched transitional population using IgM and CD27, **figure (III-7).**

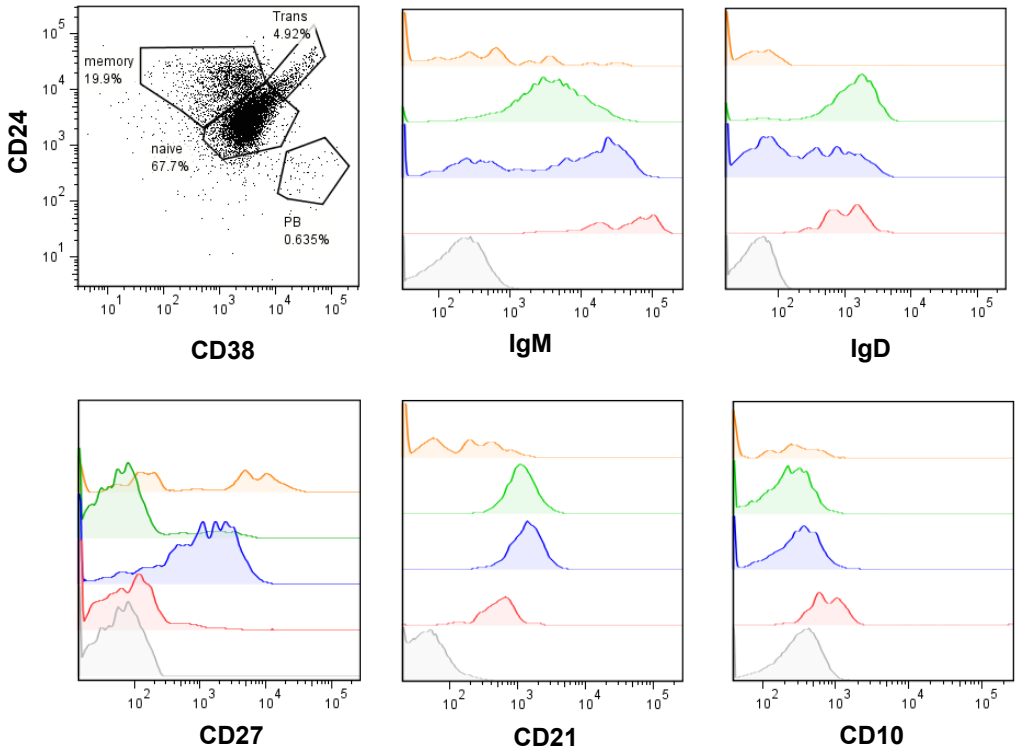


Figure III-3: Surface expression of B cell markers

Surface expression of CD10, CD21, CD27, IgM and IgD of $CD24^{-}CD38^{hi}$ plasmablasts (yellow), $CD24^{+}CD38^{+}$ naïve B cells (green), $CD24^{hi}CD38^{lo}$ memory B cells (blue) and$CD24^{hi}CD38^{hi}$ transitional B cells (red). Fluorescence minus one controls were used to determine positive populations (grey).

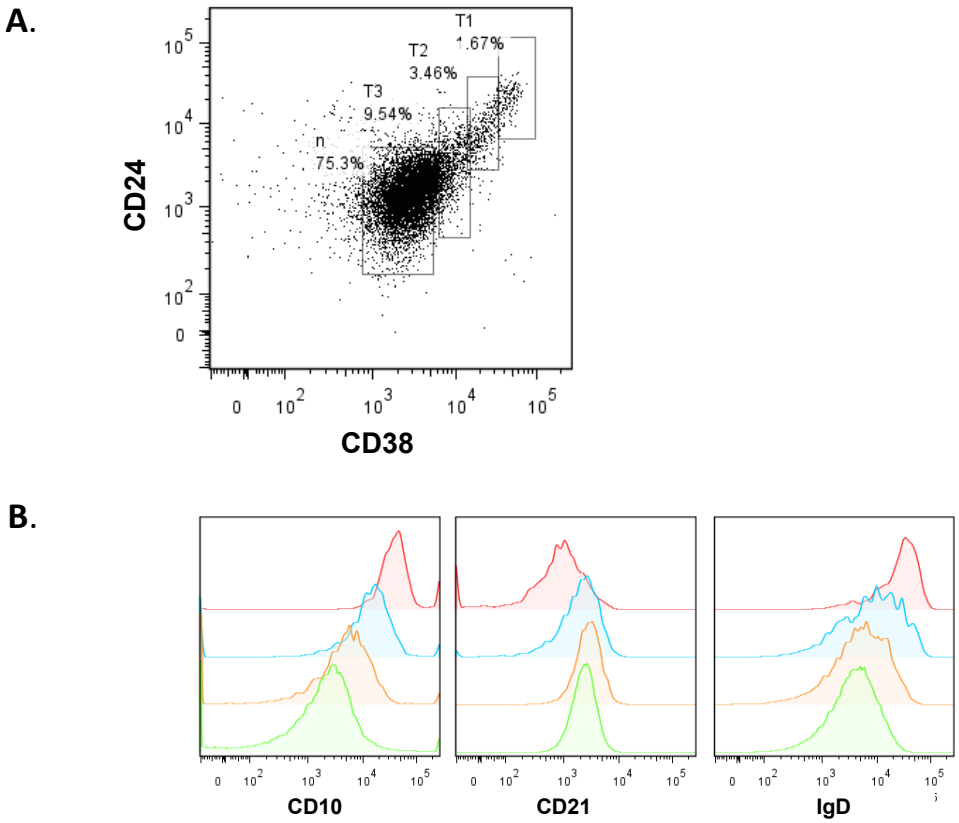


Figure III-4: Transitional B cell subsets: T1, T2 and T3

A. Transitional B cells are gated into T1, T2, T3 based on CD24 and CD38 expression in a reconstituting patient post SCT. T1 B cells, most immature transitional cells, have the highest expression of both CD24 and CD38. **B.** Histograms compare the differential expression of CD10, CD21 and IgD. T1 (red) are mostly $CD10^{+}CD21^{-}$ and express highest level of these markers. T2 (blue) gain gradual expression of CD21 and lose CD10 expression as they mature. T3 (yellow) and naïve (green) share similar expression of these markers

III.2.2.2 Naïve B cells

Using the optimised antibody panel, and in keeping with previously published reports, the majority (~ 95%) of gated $CD24^{+}CD38^{+}$ naive B cells were $CD27^{-}$, IgD^{+}, IgM^{+}, $CD10^{-}$ and $CD21^{+}$, **figure (III-3).**

$CD24^{+}CD38^{+}$ naïve B cells mainly overlaid Bm2 activated naïve B cell and early Bm5 regions in relation to IgD and CD38. Only a minority of the gated $CD24^{+}CD38^{+}$ naïve B cells correlated with Bm1 early naïve B cells. In fact, gated Bm1 ($IgD^{+}CD38^{-}$) B cells were divided between $CD24^{hi}CD38^{hi}CD27^{+}$ memory and $CD24^{+}CD38^{+}CD27^{-}$ naïve B cells, which challenges the labelling of Bm1 as naïve B cells. With regard to the IgD/CD27 classification scheme, $CD24^{+}CD38^{+}$ naïve B cells were expectedly within the $CD27^{-}IgD^{+}$ naïve B cell subset **figure (III-5 B).** In addition, naïve B cells fell within $CD27^{-}IgM^{+}$ B cells when analysed according to IgM and CD27, **figure (III-7).**

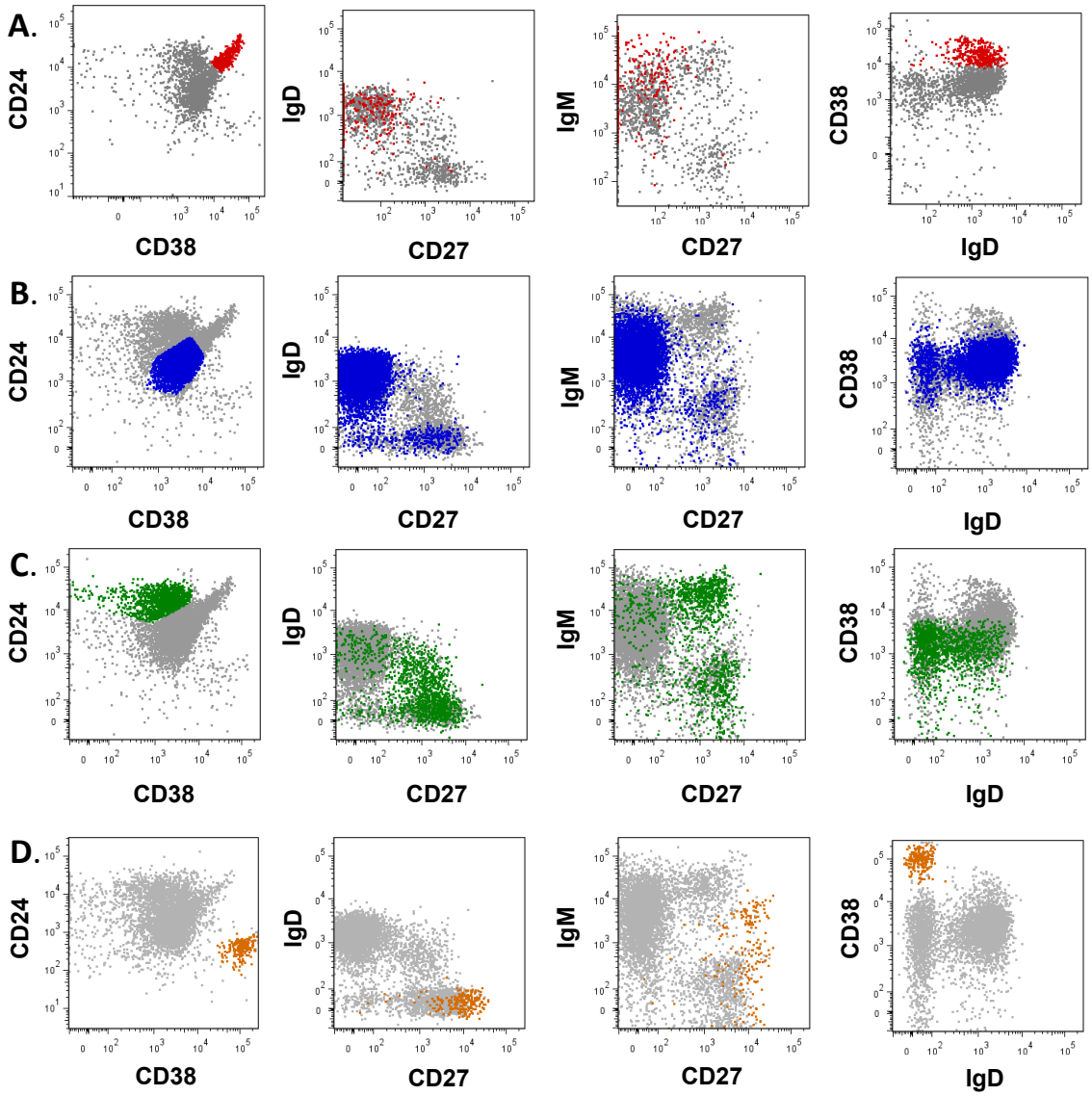


Figure III-5: B cell subsets correlation with different B cell classifications

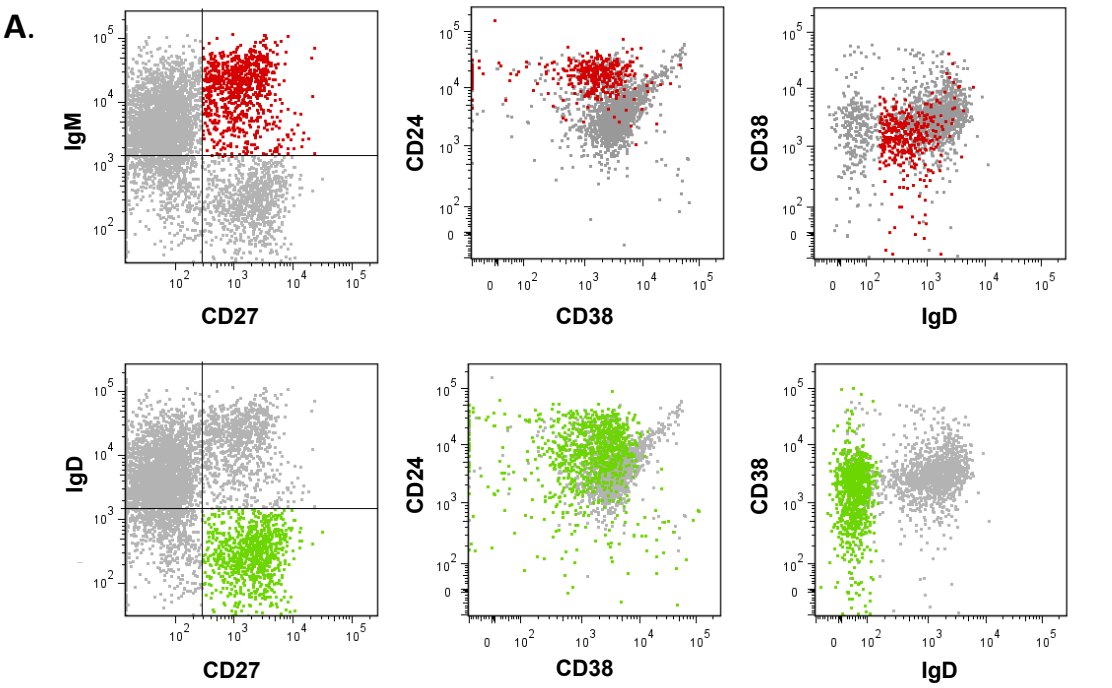
Colour coded gating of B cell subsets according to CD24/CD38 with their phenotypic correlation with IgD/CD27, IgM/CD27 and IgD/CD38 classifications; **A.** transitional (red), **B.** naïve (blue), **C.** memory (green) and **D.** plasmablasts (yellow).

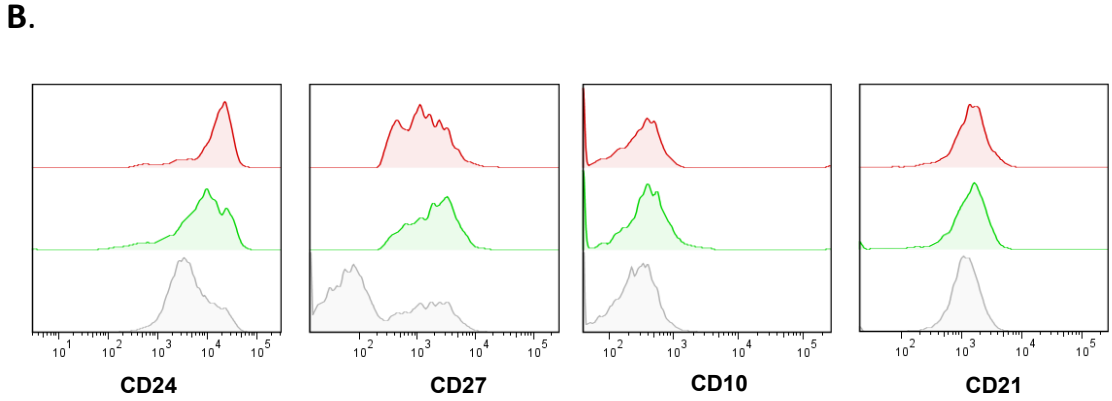
III.2.2.3 Memory B cells

In keeping with published phenotype, ~ 85% of gated $CD24^{hi}CD38^{-}$ memory B cells were $CD27^{+}$ and mostly $CD10^{-}$ and $CD21^{+}$**, figure (III-3).** Around 20% of the $CD27^{-}$ fraction was double negative for IgM and IgD suggesting that they are $CD27^{-}$ memory B cells. Adding the $CD27^{-}$ memory B cells, around 13% of cells of gated $CD24^{hi}CD38^{-}$ B cells were phenotypically naïve B cells. This gating strategy doesn't allow the distinction between switched and non-switched memory B cells. Further analysis revealed that around 40% were switched memory B cells and remaining proportion IgM^{+} or $IgM^{+}IgD^{+}$ cells.

$CD24^{hi}CD38^{-}$ memory B cells overlaid early and late Bm5 memory B cell and some of Bm1 regions. As regard to the IgD/CD27 classification scheme, $CD24^{hi}CD38^{-}$ memory B cells were expectedly within the $CD27^{+}$ quadrants; both switched and non-switched memory B cells, **figure (III-5 C).** Using IgM and CD27, memory B cells overlaid $CD27^{+}IgM^{+/hi}$ non-switched memory B cells and $CD27^{+}IgM^{-}$ switched memory B cells, **figure (III-7).**

$IgM^{+}IgD^{+}$ and $IgM^{-}IgD^{-}$ memory B cells were examined for CD24, CD38 and CD27 expression. Non-switched memory cells expressed higher CD24, measured by MFI, (12200 vs. 7626; p=0.02), lower CD27 (966 vs. 2700; p=0.0006) and similar CD38 (1197 vs. 1143, p= 0.6) respectively, **figure (III-6A).** Both subsets were superimposed on each other in the $CD24^{hi}CD38^{-}$ memory region. When compared with regards to IgD and CD38, Bm1-Bm5 classification, $IgM^{-}IgD^{-}$ memory B cells overlaid early and late Bm5 subset and $IgM^{+}IgD^{+}$ memory B cells fell between Bm1 and Bm5, **figure (III-6B).**


A.
IgM
CD27
CD24
CD38
CD38
IgD
IgD
CD27
CD24
CD38
CD38
IgD


B.
CD24
CD27
CD10
CD21

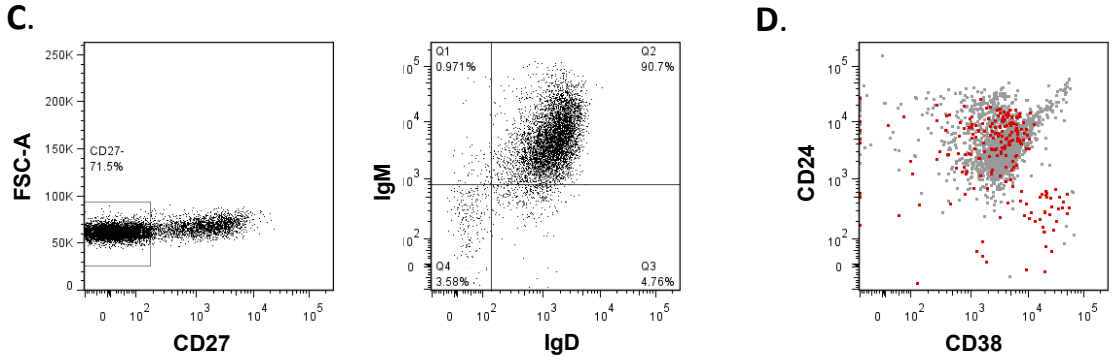

C.
FSC-A
CD27-
71.5%
CD27
IgM
Q1
0.971%
Q2
90.7%
Q4
3.58%
Q3
4.76%
IgD
D.
CD24
CD38

Figure III-6: Memory B cells

A. $IgM^{+}CD27^{+}$non switched (red) and $IgM^{-}CD27^{+}$ switched (green) memory B cells are overlaid on total $CD19^{+}$ B cells (grey) with their corresponding representation in CD24/CD38 and IgD/CD38 schemes. Switched memory B cells fall under early and late Bm5, while non-switched memory B cells are superimposed on Bm1, naïve cells, and partly on Bm5. **B.** Switched memory B cells express higher CD27, lower CD24 and equivalent CD21 and CD10 compared with non-switched memory B cells. **C.** $CD27^{-}$ memory B cells were determined from total $CD19^{+}$ cells and as being double negative for IgD and IgM. **D.** CD27- memory B cells (red) and total CD19 (grey) overlay: $CD27^{-}$ memory B cells are superimposed mainly on memory and plasmablast regions when plotted against CD24 and CD38.

Although CD27 is used as a universal B cell memory marker, around 5% of PB B cells are $CD27^{-}$ B cells(Sanz et al., 2008). My panel allows the study of this subset based on isotype status; IgM and IgD, as well as assessing CD24 and CD38 phenotype. I found that around 3% (1.2%-4.6%) of peripheral blood B cells were $CD27^{-}$ and double negative for IgM and IgD. This population was mainly $CD24^{hi}CD38^{-}$ or $CD24^{-}CD38^{hi}$ which substantiates the memory origin of these cells. Of interest, the presence of $CD27^{-}$ cells within the $CD24^{-}CD38^{hi}$ plasmablast region suggests that these memory cells may also differentiate into effector cells, **figure (III-6C and III-6D).**

III.2.2.4 Plasmablasts

$CD24^{-}CD38^{hi}$ plasmablasts expressed higher levels of CD27 compared with memory B cells. Most of the gated plasmablast population did not express IgM or IgD in keeping with their function. In addition, this population did not express CD10 or CD21; **figure (III-3).**

$CD24^{-}CD38^{hi}$ plasmablasts B cells mainly fell over Bm3+4 in relation to IgD and CD38. With regard to the IgD/CD27 classification scheme, plasmablasts were observed in

$CD27^{hi}IgD^{-}$ position and $CD27^{hi}IgM^{-}$ B cells when analysed according to IgM and CD27, **figure (III-5D).**

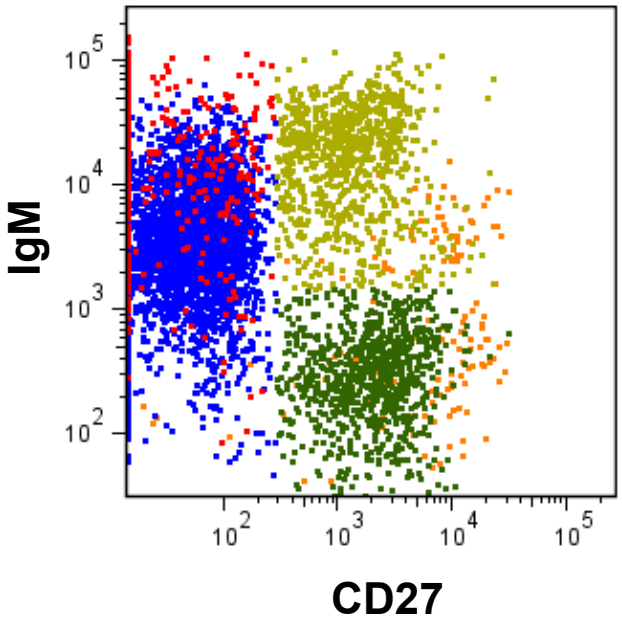


Figure III-7: IgM/CD27 B cell classification scheme

Colour coded B cell subsets plotted according to IgM and CD27 expression. Five distinct populations can be enriched transitional (red), naïve (blue), switched (dark green), non-switched (light green) and plasmablasts (yellow)

III.2.2.5 $CD21^{-}$ B cells

Gated $CD19^{+}CD21^{-}$ cells were first analysed for the expression of CD27. $CD27^{+}$ cells were mainly distributed between $CD24^{hi}CD38^{lo}$ activated memory cells and $CD24^{-}CD38^{hi}$ plasmablasts. $IgM^{+}IgD^{+}$ non-switched and $IgM^{-}IgD^{-}$ switched memory phenotypes were observed within this population. The $CD27^{-}$ fraction overlaid $CD24^{hi}CD38^{hi}$ transitional B cell and extended into $CD24^{+}CD38^{+}$ naïve B cell. A second population of $CD21^{-}CD27^{-}$ cells

was also observed. These cells were $CD24^{-}CD38^{-}$ and mostly negative for CD10, **figure (III-8).** The latter phenotype is reminiscent of exhausted B cells described in chronic HIV viraemia (Moir et al., 2008).

The observed subpopulations discussed above varied between healthy subjects. More detailed analysis was hindered by the low number of events detected in the $CD21^{-}$ B cells subpopulations in view of the low frequencies of total $CD21^{-}$ B cells in PB of healthy individuals.

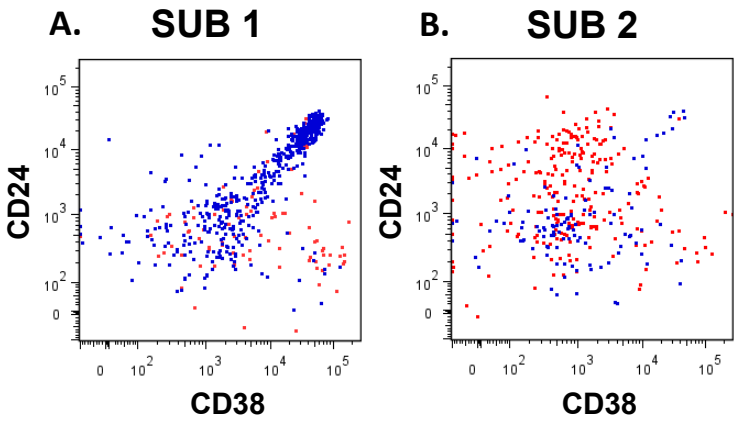


Figure III-8: $CD21^{-}$ $CD19^{+}$ B cells inter-individual variation

$CD21^{-}$ $CD19^{+}$ B cells from 2 healthy representatives were subdivided according to CD27 expression into $CD27^{+}$ (red) and $CD27^{-}$ (blue). **A.** SUB1; The majority of $CD21^{-}CD27^{-}$ B cells fell under $CD24^{hi}CD38^{hi}$ transitional B cells and some under naïve B cells. $CD21^{-}CD27^{+}$ fraction in this case were mainly $CD38^{hi}CD24^{-}$ plasmablasts. **B.** SUB 2; Fewer $CD21^{-}CD27^{-}$ (transitional) and more in $CD24^{-}CD38^{-}$ (exhausted cells). $CD21^{-}CD27^{+}$ fraction were mainly $CD24^{hi}CD38^{lo}$ activated memory cells and $CD38^{hi}CD24^{-}$ plasmablasts in addition to a proportion in the $CD38^{-}CD24^{-}$ quadrants.

III.2.3 Peripheral blood B cell subset frequencies

17 healthy volunteers (10 males and 7 females) were phenotyped using the extended panel. The median age of the volunteer cohort was 33.5 y (21 to 47).

III.2.3.1 B cell subsets

On average $CD19^{+}$ B cells comprised 5.6% (3.34%-11.4%) of peripheral blood lymphocytes. Antigen naïve; transitional ($CD24^{hi}Cd38^{hi}$) and $CD24^{+}CD38^{+}$ naïve B cells constituted 4.5% (2.7%-7.6%) and 60% (42.2%-74.6%) of the total $CD19^{+}$ B cells respectively.

Antigen experienced B cells; memory B cells ($CD24^{hi}CD38^{lo}$) and $CD24^{-}CD38^{hi}$ circulating plasmablasts comprised 26.8% (17.6%-47.1%) and 0.9% (0.3%-4.43%) of total $CD19^{+}$ B cells, respectively. Likewise, $CD27^{+}$ memory B cells constituted 29.3% (12.7%-43.8%) of PB $CD19^{+}$ cells. This population was further divided according to IgM and IgD expression into four subsets: double negative isotype switched (median 45.7%, range 19.7%-63.6%), double positive IgM memory B cells (median 45.29%, range 29.2%-75.7%), IgM only expressing (median 6.15%, range 1.65%-11.7%) and IgD only expressing (median 3.02%, range 1.45%-5.5%) of total $CD27^{+}$ memory B cells, **figure (III-9).**

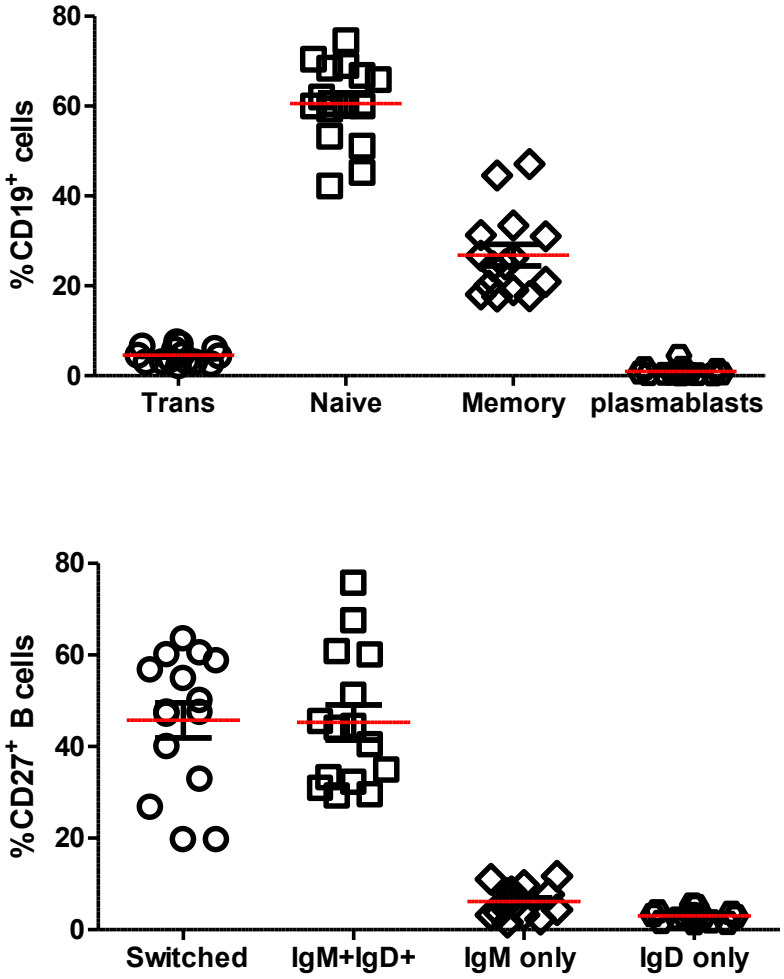


Figure III-9: B cell subsets in healthy controls

FACS analysis of B cell subsets in healthy controls (n=16). A. B cell subset frequencies out of total $CD19^+$ B cells defined as per CD24/CD38 scheme. B. $CD27^+$ memory B cells subdivision according to the expression of IgM and IgD. Percentages of the four subpopulations out of $CD27^+$ memory B cells, IgD only, IgM only, double positive and double negative are shown.

III.2.3.2 CD21$^-$ B cells

The majority of circulating B cells expresses CD21; an essential molecule for B cell activation and function. Expanded populations of CD21$^-$ B cells have been observed in chronic inflammatory conditions. Therefore, we studied the phenotype of CD21$^-$B cells and their representation in the peripheral blood of healthy volunteers.

Approximately, 3.5% (1.02%-7.54%) of circulating CD19$^+$ B cells lacked CD21 expression; CD21$^-$CD19$^+$. CD21$^-$ B cells were gated on B cells that lacked CD21 expression using an FMO control for CD21. Further analysis of this population revealed that 45.3% (18.8%-71.10%) were CD27$^+$, suggesting that a proportion of this population have undergone somatic hypermutation. The majority [65.04% (34.2-84.6%)] of CD21$^-$CD27$^+$ B cells carried switched isotype i.e. negative for both IgM and IgD. This phenotype most likely represents the activated memory B cells. In addition, it includes the CD27^{hi}IgM$^-$IgD$^-$ plasmablasts that naturally lose CD21 expression.

The remainder of CD21$^-$ B cells [54% (28.9-81.2%)] did not express CD27 i.e. these cells are Ag naïve B cells. Almost half expressed IgM, IgD or both which makes this population likely to be transitional B cells. The other half [46.69% (15.10-72.50%)] did not express IgM or IgD. This phenotype did not fall within any of the 'conventional' B cell subsets; however, it shared similarity with exhausted B cells described in chronic HIV patients.

III.2.3.3 Gender has no effect of B cell subsets

Next, I compared the frequency of B cell subsets based on gender (10 males and 7 females), median age 33.5 (21-45) vs. 34 (24-55); p=0.58. I found no significant difference in the percentage of memory B cells defined as both $CD24^{hi}CD38^{lo}$ and $CD27^{+}$ (35% vs. 43.8%; p=0.59) and naïve B cells defined as $CD24^{+}CD38^{+}$ and $CD27^{-}$ B cells (62% vs. 45.2%; p=0.8) in females compared to males, respectively. In addition, the frequency of total $CD19^{+}$ B cells, $CD21^{-}$ B cells, transitional B cells and plasmablasts were comparable between the two groups.

III.2.3.4 Effect of Cryopreservation of the frequencies of B cell subsets

Aliquots of same healthy control samples were frozen in order to study the effect of cryopreservation on the extended phenotype panel. Samples were frozen in liquid nitrogen for a minimum of 2 months before performing the surface staining.

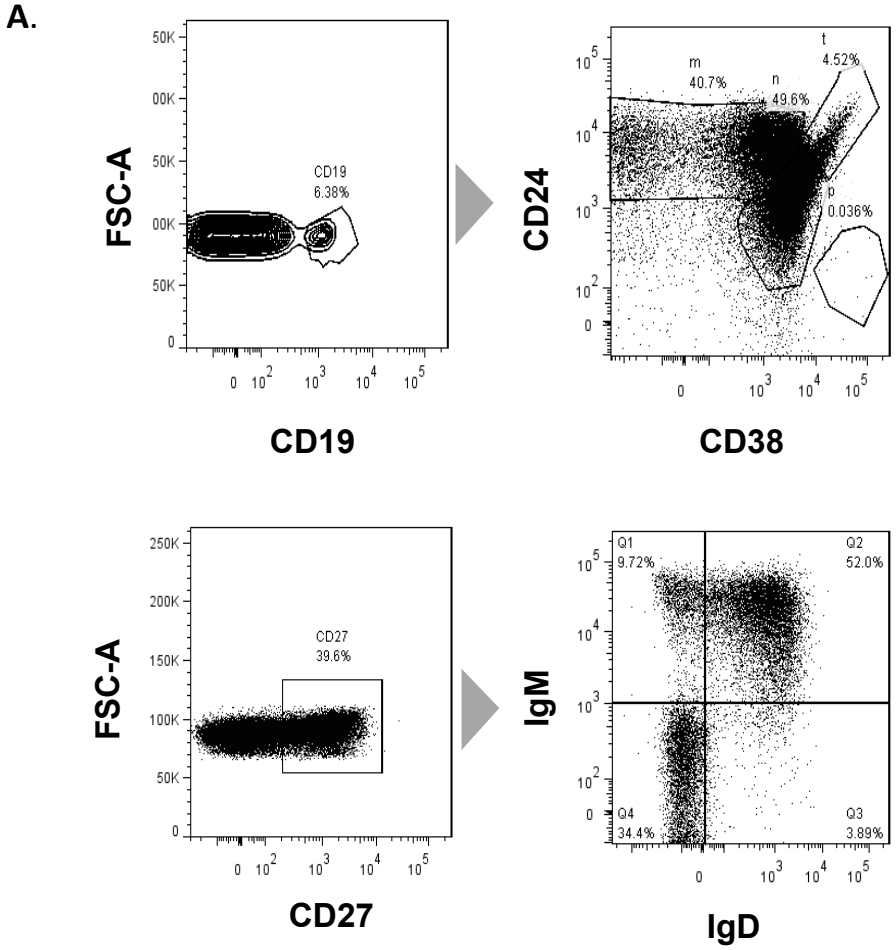
Frozen samples yielded comparable FACS plots with distinctive populations similar to the freshly obtained ones, **figure (III-10A)**. There was significantly more $CD19^{+}$ B cells, out of lymphocyte gate, in the frozen samples compared with their corresponding fresh ones; 8.06% (4.9%-21.05%) vs. 4.6% (3.34%-11.4%) ; p=0.005. Out of the $CD19^{+}$ gate, lower frequency of $CD27^{+}$ events was observed in the frozen than the fresh samples, 23.9% (11.5%- 41.3%) vs. 29.1% (12.7%- 43.8%); p=0.0004. However, the difference was concordant i.e. in one direction; **figure (III-10B)**.

The frequency of B cell subsets were also compared between frozen and fresh samples based on CD24 and CD38 expression. There was no significant difference between the observed frequencies of transitional B cells; p=0.47, naïve B cells; p=0.75 and memory B

cells; p=0.8. The frequency of $CD24^{-}CD38^{hi}$ plasmablasts was significantly lower in the frozen samples compared to their corresponding fresh counterparts; $p<0.05$.

Although $CD27^{+}$ $CD19^{+}$ B cells were lower in frozen samples, the representation of both $IgM^{+}IgD^{+}$ memory B cells and switched memory B cells was comparable. The frequencies of $CD10^{+}CD19^{+}$ and $CD21^{-}CD19^{+}$ B cells were not different between frozen and fresh samples; **figure (III-10).**

Overall, these results validate our panel to study frozen patient samples which are prospectively collected and cryopreserved for batched processing.



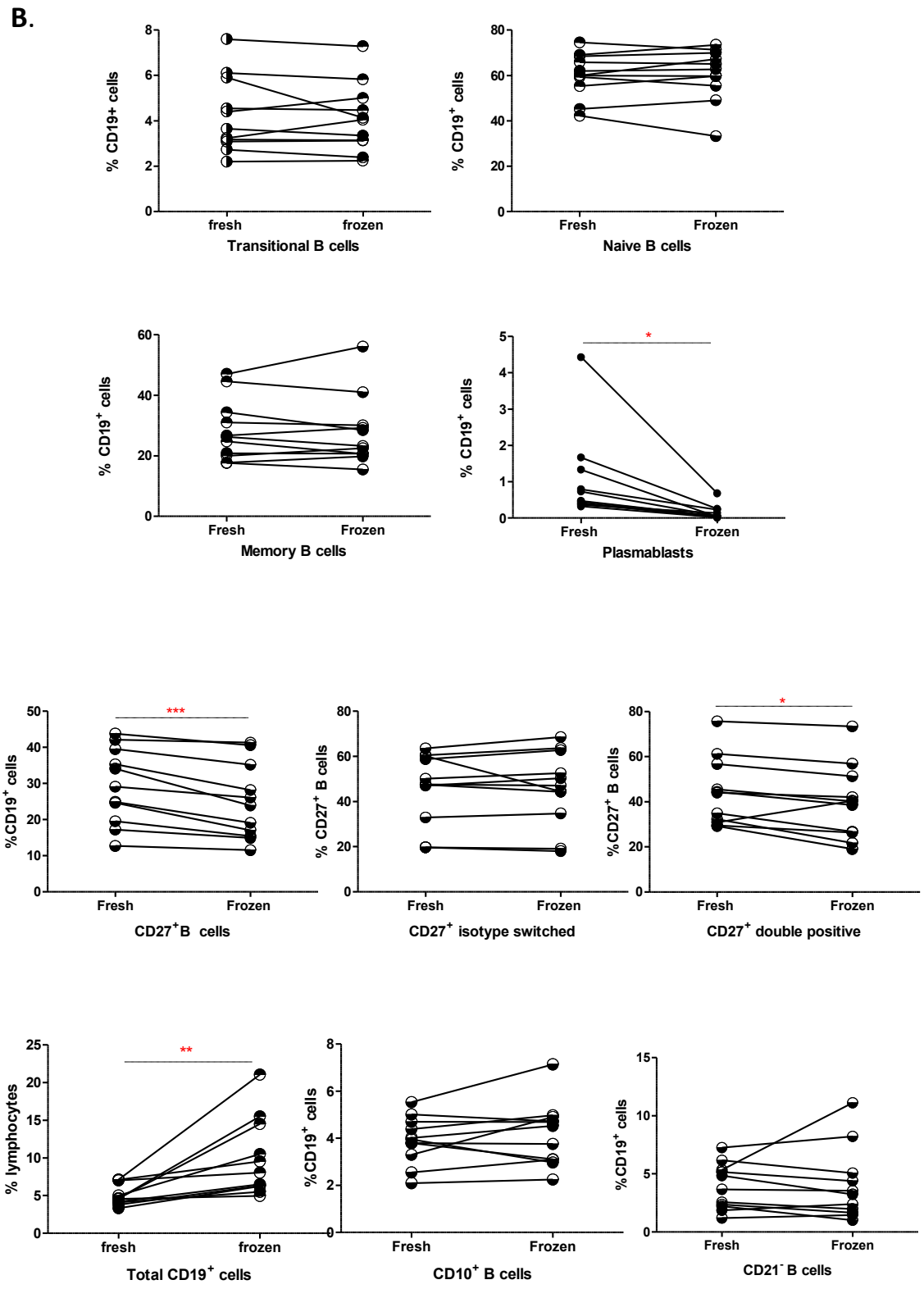


Figure III-10: Cryopreservation effect on B cell subset phenotypes

Cryopreservation effect on B cell subset phenotypes. **A.** Data acquired from a representative frozen sample are depicted in various bivariate plots. Frozen samples produce interpretable plots and distinctive populations similar to fresh samples. **B.** Cumulative data comparing different B cell subsets between paired fresh and frozen samples, n=10. The results were analysed with two-tailed nonparametric paired t test analysis, *P<0.05, **P<0.01.

III.3 Discussion

Different schemes have been used to classify B cells based on different B cell markers. Correlation between different subsets defined by one scheme with the same subset defined by another is not clear. A recent review by Sanz and colleagues has addressed the heterogeneity of memory B cell phenotypes in these classifications (Sanz et al., 2008).

Flow cytometry provides a powerful tool to study cell populations through the use of specific staining and various gating techniques. Titration of antibody is crucial to achieve optimal interpretable plots with the possibility to quantitate cell marker expression. Using the right amounts of the antibodies is particularly important to control background staining and to enhance the separation of negative and positive events (Baumgarth and Roederer, 2000).

I implemented an extended phenotypic panel to correlate defined subsets by one scheme with the other two. The majority (~85%) of $CD24^{hi}CD38^{lo}$ cells defined as memory B cells in the CD24/CD38 classification expressed CD27, a universal B cell memory marker. Moreover, the $CD27^{+}$ fraction of the $CD24^{hi}CD38^{lo}$ population was distributed among IgD^{+} and IgD^{-} as well as IgM^{+} and IgM^{-} B cell subsets when examined according to the IgD/CD27 and IgM/CD27 classifications. A fifth of the $CD27^{-}$ fraction was double negative for IgD and IgM, which make these cells likely to be $CD27^{-}$ memory B cells. The remaining $CD27^{-}$ cells expressed IgM and IgD which classifies them as naïve B cells according to other schemes. This finding validates the CD24/CD38 definition for memory B cells, with the provision that a minority of naïve B cells may fall within this

gate, however this classification fails to distinguish switched and nonswitched memory B cells.

$CD24^{hi}CD38^{lo}$ and $CD27^{+}$ memory B cells overlaid the early and late Bm5 region with a minority of events spilling into the bm2 naïve region. A major limitation of Bm1-Bm5 classification for memory B cells (Bm5 group) is that it does not recognise IgM^{+} memory B cells as this population also expresses IgD. Interestingly, we found that IgM memory B cells had higher CD24 and lower CD27 expression, true to their lower level of somatic hypermutation (Weill et al., 2009). Plasmablasts, a minor circulating population (Piatosa et al., 2010) defined as $CD24^{-}CD38^{hi}$ overlaid $CD27^{hi}$ IgD^{-}, $CD27^{hi}IgM^{-}$ and Bm5. Interestingly, gated $CD24^{-}CD38^{hi}$ included isotype switched $CD27^{-}$ B cells which may indicate that $CD27^{-}$ memory cells differentiate towards plasmablasts.

$CD27^{-}$ memory B cells form about 5% of total B cells (Sanz et al., 2008). Around 3% (1.2%-4.6%) of peripheral blood B cells were $CD27^{-}$ and double negative for IgM and IgD. This population was mainly $CD24^{hi}CD38^{-}$ memory B cells, or $CD24^{-}CD38^{hi}$ plasmablasts, which substantiates their memory origin. Of interest, finding $CD27^{-}$ cells within the $CD24^{-}$ $CD38^{hi}$ plasmablasts region may support that these memory cells also differentiate into effector cells. This analysis is based on the negative expression of IgM and IgD rather than positive expression of IgA or IgG.

$CD24^{hi}CD38^{hi}$ transitional B cells were mostly $CD27^{-}IgM^{hi}$ and IgD^{+} in keeping with published data (Palanichamy et al., 2009). In my study 40% of $CD24^{hi}$ $CD38^{hi}$ transitional B cells were $CD10^{+}$, marking the more immature cells (Carsetti et al., 2004). Interestingly, the majority of $CD10^{+}$ transitional B cells were also $CD21^{-}$, another feature of immature

transitional B cells (Suryani et al., 2010). Of note, I found that $CD24^{hi}$ $CD38^{hi}$ transitional B cells superimposed on Bm2' pre-germinal centre cells of the CD38/IgD classification and on $IgD^{+}CD27^{-}$ naïve B cells, which makes these classifications unsuited to study transitional B cells.

Using the IgM/CD27 marker combination was equivalent to IgD/CD27 in separating the different B cell subsets, with one main difference. Transitional B cells ($CD24^{hi}$ $CD38^{hi}$) were superimposed in the IgM^{hi} region of the $IgM^{+}CD27^{-}$ naïve B cell compartment, which allows this combination to identify four subsets and therefore be used as a sorting panel.

$CD21^{-}$ B cells constituted around 4.5% of peripheral B cells in keeping with published data (Moir et al., 2008). Further analysis of $CD21^{-}CD19^{+}$ B cells revealed that this population could be allocated to 4 subsets. $CD27^{+}$ cells were mainly distributed among $CD24^{hi}CD38^{lo}$ activated memory cells, both isotype switched and nonswitched, and $CD24^{-}CD38^{hi}$ plasmablasts. The $CD27^{-}$ fraction constituted mainly transitional B cells and another population bearing phenotypical resemblance to exhausted B cells described in HIV patients (Moir et al., 2008). More detailed analysis was hindered by the low number of events in each $CD21^{-}$ B cell subpopulations in view of the low frequencies of total $CD21^{-}$ B cells in the PB of healthy individuals.

Finally, subset frequencies obtained by my panel were in keeping with published data (Palanichamy et al., 2009, Sanz et al., 2008). In our cohort, there was no statistical difference observed between age matched genders with regards to each subpopulation. A number of reports did not detect any gender-specific differences in total $CD19^{+}$ B cells

(Jentsch-Ullrich et al., 2005). To the best of my knowledge, no studies compared B cell subsets between genders.

Cryopreservation led to increased percentage of $CD19^+$ events and reduced percentage of $CD27^+$ memory B cells and plasmablasts. However, the difference was concordant and overall the panel produced distinctive populations. It is plausible that plasmablasts, defined as $CD38^{hi}CD24^-$, may not withstand the freezing process relative to naïve B cells. Importantly, I observed no effect of freezing on CD21 expression on B cells. A few studies reported conflicting effect of cryopreservation on total $CD19^+$ B cell frequency. There was no effect on $CD19^+$ percentage in thawed samples, however freezing increased CD19 MFI in one study (Romeu et al., 1992, Rosillo et al., 1995). In another study, cryopreservation was associated with lower $CD19^+$ frequency (Tollerud et al., 1991). Again to the best of my knowledge, no studies compared the effect of cryopreservation on B cell subset frequencies.

Overall, the panel produced FACS plots and cell population frequencies comparable to published data. Furthermore, it provides a powerful tool to study B cell subsets and correlate the different schemes available for B cell classification.

Chapter IV. IL10 producing B cells

IV.1 Introduction

IL10 production as a trademark of regulatory B cells was introduced simultaneously in two different inflammatory models (Mizoguchi et al., 2002, Fillatreau et al., 2002). To date, IL10 producing B cells remains the most studied regulatory B cell population.

In animal models, the regulatory potential of B cells is demonstrated in vivo by the amelioration or recovery of model-associated inflammatory conditions. A number of mouse models have been used for this purpose (Mizoguchi and Bhan, 2006). Early studies showed that lack of B cells, either by transferring inoculum, e.g. splenocytes, lacking B cells (Katz et al., 1974, Neta and Salvin, 1974) or generating B cell deficient knockout (KO) mice (Wolf et al., 1996) led to more severe inflammatory disease.

Interleukin 10 produced by total B cells was shown to be instrumental in immune regulation using IL10 KO mice (Mizoguchi et al., 2002), chimera mice (Fillatreau et al., 2002); where IL10 deficiency is limited to B cells, or by demonstrating the loss of B cell suppressive effect when B cells from IL10 KO mouse were transferred to normal recipient mice (Mauri et al., 2003). These studies investigated B cells as a whole and no phenotype was attached to IL10 producing regulatory B cells.

Insight into the IL10 producing B cell phenotype was possible by using intracellular IL10 staining technique and surface phenotyping or by measuring IL10 in supernatants of purified B cell subsets. Yanaba and colleagues found that IL 10-producing B cells shared features common to marginal zone (MZ), T2-MZ precursors, and $CD1d^{hi}$ $CD5^{+}$ B-1a B cells

(Bouaziz et al., 2008). Others detected higher amount of IL10 in the supernatant of purified MZ B cells in comparison with follicular B cells (Gray et al., 2007). A number of phenotypes have been reported by different groups for $IL10^+$ regulatory B cells (Mauri and Blair, 2010a).

In humans, two main phenotypes for $IL10^+$ regulatory B cells have been reported, namely transitional and $CD27^+$ memory B cells. In the study by Dr. Mauri's group, $IL10^+$ Breg were found to be enriched within the $CD24^{hi}$ $CD38^{hi}$ transitional B cell compartment. In an in-vitro culture system, sorted transitional B cells displayed a suppressive effect on autologous effector CD4 T cells and reduced the production of $IFN\gamma^+$ and $TNF\alpha^+$ by activated $CD4^+$ T cells (Blair et al., 2010). Conversely, Iwata and colleagues reported that $IL10^+$ Breg are enriched within the mature $CD24^{hi}$ $CD27^+$ memory B cell subset. Although sort-purified $CD27^+$ B cells failed to suppress $CD4^+$ T cells directly, they exerted their suppressive effect through inhibiting cytokine production by $CD14^+$ monocytes (Iwata et al., 2011). Other groups reported IL10 production across the transitional and memory compartments (Bouaziz et al., 2010). In these studies, $IL10^+$ B cell frequencies were examined in a number of autoimmune conditions where immune dysregulation is implicated, with variable findings.

Based on current available techniques and in the absence of specific cell markers, it is not possible to accurately define the phenotype of $IL10^+$ B cells. Searching for unique markers that enable purifying regulatory B cells to allow their study is highly warranted.

The aim of my project was to optimise IC staining protocols for $IL10^+$ B cells and to test their functional properties by assessing their suppressive activity in vitro.

IV.2 Results

The purpose of this chapter is firstly to define human IL10 producing B cells in the peripheral blood and secondly to examine their suppressive properties against autologous $CD4^+$ T cells. All data are presented as mean± SE and the unpaired two tailed T test is used unless otherwise specified.

IV.2.1 In vitro production of IL10 by B cell

IV.2.1.1 IL10 production by B cells from fresh PBMC:

Human B cells have been shown to produce distinct patterns of cytokines under conditions designed to mimic bystander B cell activation (CD40 alone), or full dual stimulation (BCR and CD40). BCR cross-linking with T cell help via CD154 (CD40L) leads to pro-inflammatory cytokine production, reflecting B cell reaction to a specific antigen, whereas stimulation via CD40L alone is associated with an anti-inflammatory cytokine profile (Duddy et al., 2004).

Using the method outlined in **section II.4.2**, I found that un-stimulated selected B cells (mean purity 92%± 3.75) from 3 healthy subjects produced either no detectable cytokine or only small amounts of IL10 (mean 25±25 pg/ml, n=3). Of note, irradiated CD154 expressing fibroblasts (L cells) and their control counterpart, $CD154^-$ fibroblasts (cntl cells), produced only negligible amounts of IL 10 (mean 6.5± 6.5 and 21± 12 pg/ml respectively, n=3).

When selected B cells, from 7 individuals, were stimulated with L cells alone at a ratio of 1:10 (BL), high levels of IL10 (mean 4433± 803 pg/ml; n=7) were detected in the culture

supernatant compared with B cells cultured with cntl cells (mean 695± 140 pg/ml; n=3, p= 0.018), (5 independent experiments). Although dual stimulation, consisting of BCR crosslinking and co-culturing with L cells (BXL) induced IL10 production, the levels of cytokine measured in the supernatant were significantly less than that achieved following stimulation with CD40L alone (mean 636±274 pg/ml; n=7, p=0.0008); **figure (IV-1).**

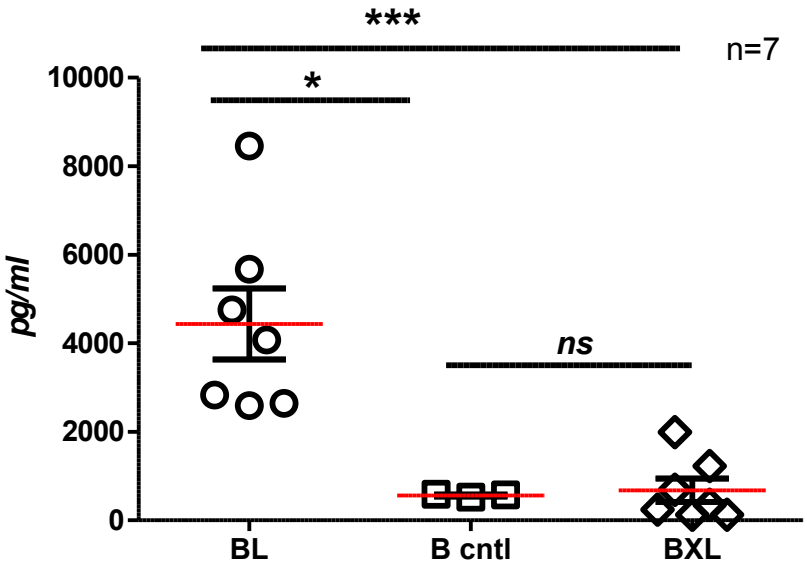


Figure IV-1: IL 10 ELISA

Levels of IL10 measured by ELISA in the supernatant of purified B cells cultured for 72h with L cells, (BL), control cells, (Bcntl) and with L cells and anti-human IgG + IgM (H+L) (BXL). Purified B cells from healthy donors stimulated with L cells alone produced higher amount of IL10 compared with dual stimulation. Data were compared by statistical analysis with the two-tailed nonparametric t test analysis; $*p < 0.05$, $***p < 0.0001$.

I also observed high inter-individual variations in IL10 production. Notably, however, the intra-individual variability was minimal and the levels of IL10 produced following stimulation of B cells from the same individual in repeated experiments was highly reproducible IL 10.

IV.2.1.2 Effect of cryo-preservation on in vitro IL10 detection by ELISA:

I tested the effect of freezing on IL10 production. A proportion of PBMC from three donors were frozen at -80^{o}C for a minimum of four weeks. After thawing, the cells were rested overnight in a humidified incubator and B cells were selected as outlined in **section II.5.1** The IL10 levels measured in the supernatant of selected B cells cultured with CD40 alone and following the dual stimulation protocol were (mean IL10 646± 264 pg/ml and 40± 21.9 pg/ml; n=3) respectively. Although measured levels of IL10 were lower in the frozen runs compared to their freshly prepared counterparts, the IL10 production pattern remained consistent among the three tested subjects. These results suggest that frozen samples can be used for cytokine detection provided that all samples are subjected to the same freezing and thawing conditions.

IV.2.2 Intracellular staining for interleukin 10 in human B cells:

Based on ELISA experiments, I used stimulation with L cells to optimise IL10 intracellular staining of both selected and gated CD19^{+} B cells. Protein transporter inhibitors were added to enhance IL10 signals by blocking extracellular transport of the cytokine. Intracellular cytokine staining for IL10, IFNγ, and IL2 were carried out according to methods described in **II. 8. 1**.

IV.2.2.1 Optimising negative controls for IL10 intracellular staining

A number of known negative controls were explored, namely, manufacturer recommended isotype control, (IgG2aκ), co-culture with $CD154^-$ cntl cells (PBcntl), unstimulated control incubated with BFA alone (unstimulated, BFA), FMO where anti-IL10 antibody was not added, and appropriately stimulated B cells without the addition of BFA (stimulated, no BFA).

All tested negative controls produced comparable plots with clear separation of negative and positive populations; **figure (IV-2)**. For most of the subsequent experiments I used isotype or unstimulated controls incubated with BFA alone.

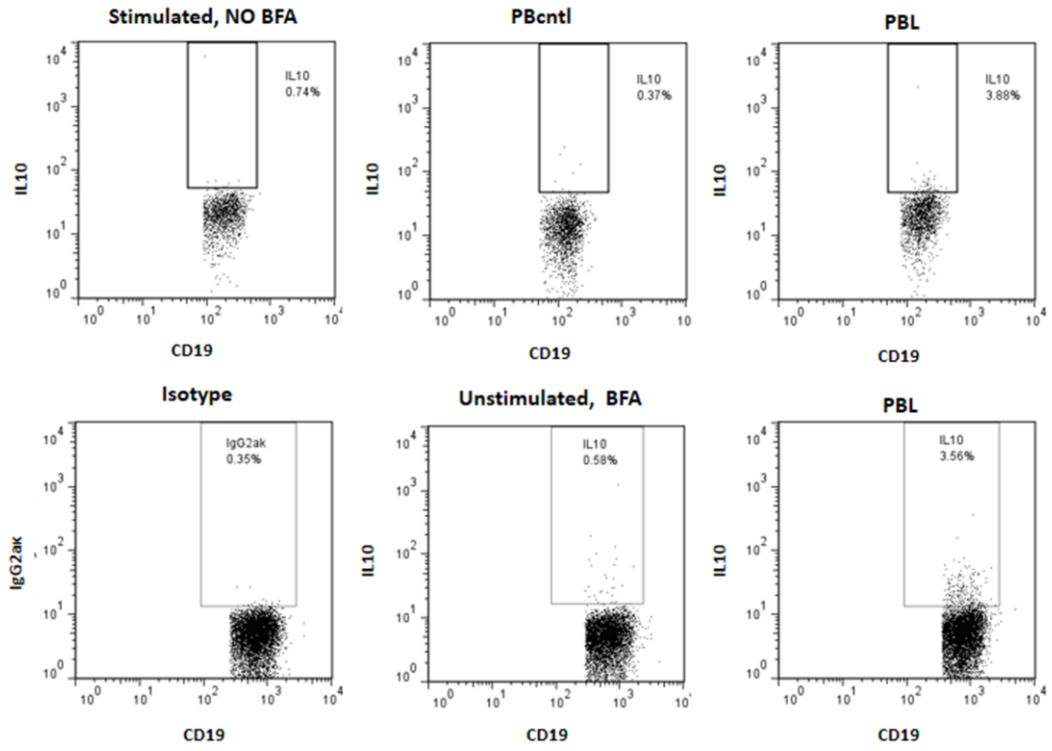


Figure IV-2: Comparison of intracellular staining negative controls

FACS plots compare gating strategies of $IL10^{+}CD19^{+}$ B cells based on available negative controls. Isotype control: PBMC were stimulated for the same duration with L cells and PMA, Ionomycin and BFA were added for the last 6 hours of culture and subsequently stained with isotype control, IgG2aκ. Stimulated, no BFA control; cells were stimulated with L cells, PMA and Ionomycin and were stained with anti-IL10; BFA was not added to this control. Bcntl: PBMC were cultured with $CD154^{-}$ fibroblasts for same duration and stained with anti-IL10 after the addition of PMA, Ionomycin and BFA for the last 6 hours of culture. Unstimulated cells, PBMC cultured without L cells for 48h, were stained with anti-IL10 after the addition of BFA alone for the last six hours of the culture.

IV.2.2.2 Optimisation of IL10 intracellular staining technique

In order to optimise IL10 intracellular staining I tested a number of incubation lengths with L cells. The addition of Brefeldin A, PMA and Ionomycin for the last 6 to 8h of the culture period, and permeabilisation using formaldehyde containing solution, **section II, 8, 1**, for a minimum of two hours prior to adding intracellular antibodies were crucial. At 24 and 48h, an $IL10^{+}$ population from $CD19^{+}$ B cells was reproducibly obtained in comparison with the negative control; **figure (IV-3A)**. Furthermore, IL10 producing B

cells were negative for IL2, IFNγ and mostly for TNFα production, suggesting that IL10 producing B cells represent a distinct cell population, **figure (IV-3B)**.

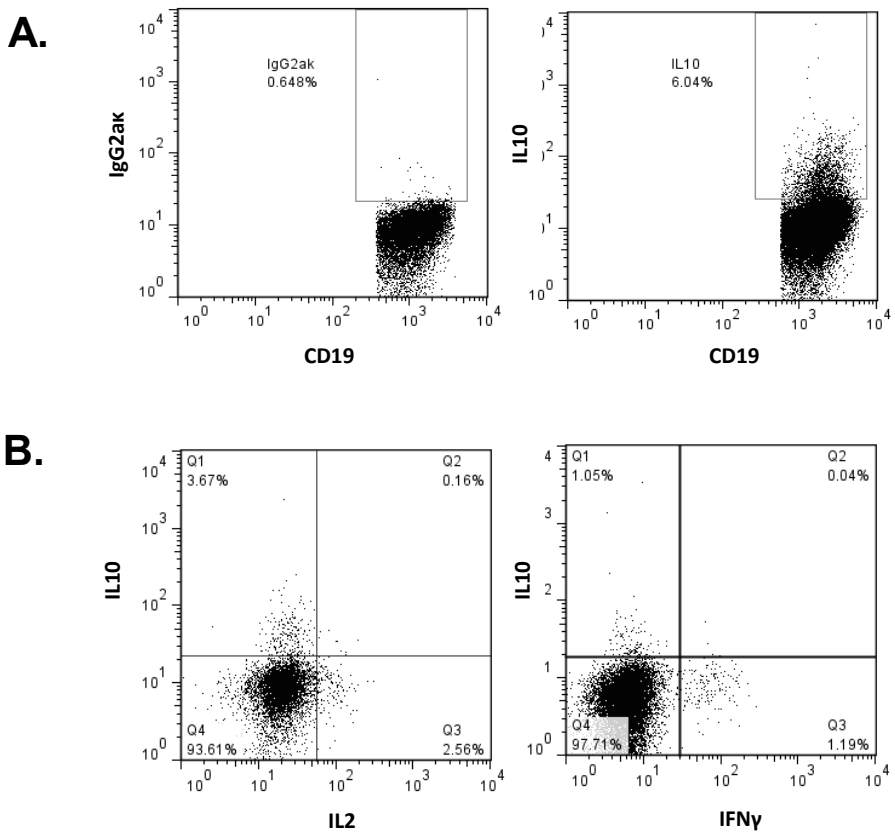


Figure IV-3: IL10 IC staining of CD19$^+$ B cells

A. Representative FACS plot of IL10 IC staining in gated CD19$^+$B cells. PBMC from healthy donors were cultured for 48 hours with L cells. PMA, Ionomycin and BFA were added for the last 6 hours of culture. Cells were then stained with CD19-PE and CD3-PerCP mAb and intracellularly stained with IL10-APC mAb or with an appropriate isotype control and acquired on FACSCalibur cytometer. Isotype control was used to determined IL10$^+$CD19$^+$ gate. **B**. IL10$^+$B cells represent a distinct cell population. Intracellular staining for IL10, IL2 and IFNγ of B cells stimulated with L cells. IL10$^+$ B cells are negative for IL2 and IFNγ.

IV.2.3 Kinetics of inducible IL10 production using intracellular staining

Next, I studied the kinetics of $IL10^+$ B cells following in vitro stimulation with anti-CD3/ anti-CD28 activated $CD4^+$ T cells and L cells.

IV.2.3.1 Anti-CD3/ anti-CD28 stimulated CD4 T cells upregulated surface expression of CD154

First, I confirmed that in vitro stimulation of PBMC with anti-CD3/anti-CD28 beads induced $CD4^+$ T cells to upregulate CD154 expression, **figure (IV-4 A)**, which in turn stimulated B cells to produce IL10. At baseline a negligible fraction of unstimulated $CD4^+$ T cells expressed CD154 (0.2%± 0.02). Induced expression of the molecule was time dependent with the highest level of expression of CD154 achieved at 72h, (58.3%± 4.9). Activation of cultured B cells was confirmed on flow cytometry as increases in forward and side scatter.

IV.2.3.2 Stimulation with activated T cells

PBMC obtained from three healthy controls were stimulated with anti-CD3/anti-CD28 beads and analysed for intracellular IL10 on gated $CD19^+$ B cells at 24, 48, 72, and 96 h.

The frequency of $IL10^+$ $CD19^+$ B cells observed in the negative control (BFA) was almost the same at each time point; on average 0.29%± 0.12. Stimulating B cells with activated T cells induced a time dependent increase in $IL10^+$ $CD19^+$ B cell frequencies (0.66%± 0.14, 2.37%± 0.37, 4.08%± 0.19 and 2.3%± 0.22) at 24, 48, 72 and 96 h respectively. Interestingly, $IL10^+$ B cells percentage correlated with the percentage of $CD154^+CD4^+$ T cells in the culture system; **figure (IV-4 B)**.

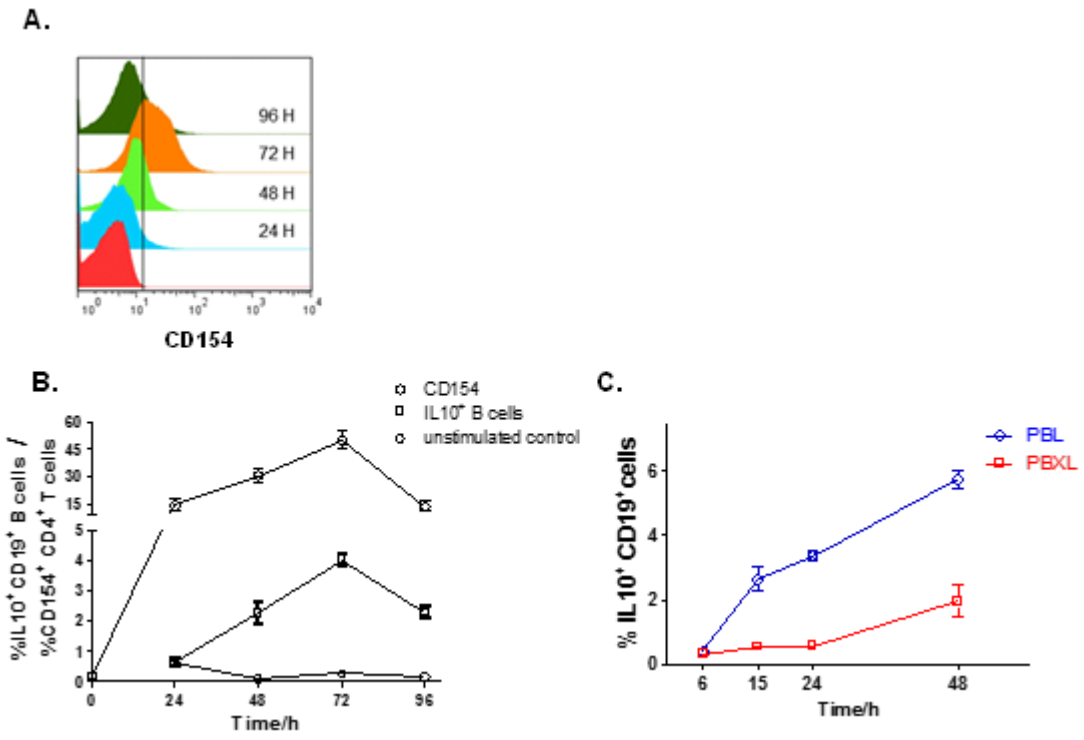


Figure IV-4: Inducible expression of CD154 by activated CD4$^+$T cells

A. Inducible expression of CD154 by activated CD4 T cells. PBMC were incubated with anti-CD3/ anti-CD28 beads and gated CD4+ cells were assessed by surface staining for CD154 expression. The highest level of expression was observed at 72 hours which declined thereafter. **B**. Kinetics of IL10$^+$CD19$^+$ B cells stimulated with activated T cells. The frequency of IL10$^+$ CD19$^+$ B cells correlated with that of CD154 expression and compared with unstimulated PBMC control. **C**. Kinetics of IL10$^+$CD19$^+$ B cells stimulated with L cells alone and L cells with anti-BCR using Intracellular IL10 staining. The highest frequency of IL10$^+$CD19$^+$ B cells was observed at 48 h in a time dependent manner. In comparison, B cells stimulated with dual stimulation PBXL produced less IL10.

IV.2.3.3 Stimulation with L cells with and without anti-BCR

In a minimum of three independent experiments, PBMC obtained from healthy controls were cultured with irradiated L cells (PBL) as well as with L cells and anti-BCR (PBXL) and analysed for intracellular IL10 on gated CD19$^+$ B cells at 6, 12, 24 and 48h.

After six hours of incubation with L cells, BFA, Ionomycin and PMA, only 0.4% ± 0.09 of total PB $CD19^+$ B cells stained positive for IL10. The observed frequency of $IL10^+$ $CD19^+$ B cells was not different from that seen with BFA alone or BXL conditions (0.33%± 0.07 and 0.32%± 0.09) respectively. Stimulating B cells with CD154 alone (PBL) induced a time dependent increase in $IL10^+$ $CD19^+$ B cells (2.1%± 0.3, 3.3%± 0.06 and 5.73± 0.31) at 12-15, 24 and 48h respectively; **figure(IV-4 C)**. In contrast, the PBXL condition led to frequencies of $IL10^+$ $CD19^+$ B cells above baseline only after 48 hours of incubation (1.9%± 0.49). In keeping with ELISA findings, the percentage of $IL10^+$ B cells detected at 48 hours was significantly higher in B cells stimulated with the BL condition when compared with BXL; p= 0.02.

These results indicate that at normal resting state, an inducible population of $IL10^+$ B cells exists in the PB. Moreover, the induction of IL10 production is condition and time dependent.

IV.2.4 Comprehensive phenotyping of human $IL10^+$ B cells

I sought to determine the phenotype of $IL10^+$ B cells using a number of 4-colour surface antibody panels, previously shown to be expressed by murine IL10 producing regulatory B cells. I found that stimulating PBMC with L cells for 15h is optimal; it allowed sufficient numbers of IL10-producing B cells to be detected for rare events analysis by flow cytometry without significant change in overall B cell phenotype. The phenotype of B cells started to change after 24 hours of incubation, characterised by down-regulation of CD24 and up-regulation of CD27 and appearance of a plasmablast population, **figure (5)**.

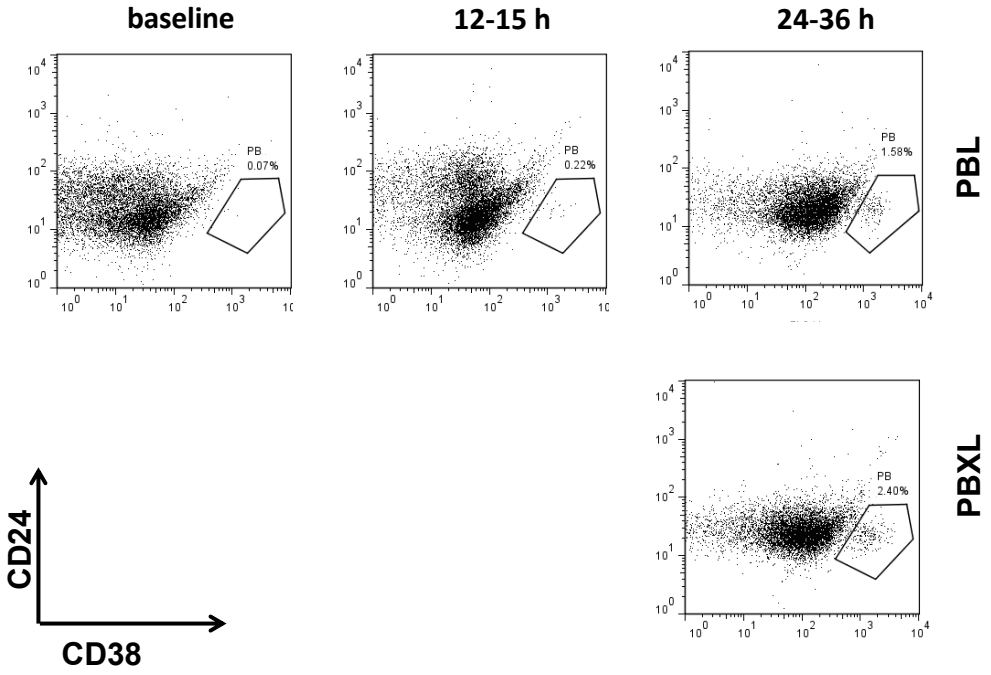


Figure IV-5: Determination of optimal time point for IL10$^+$ B cell study

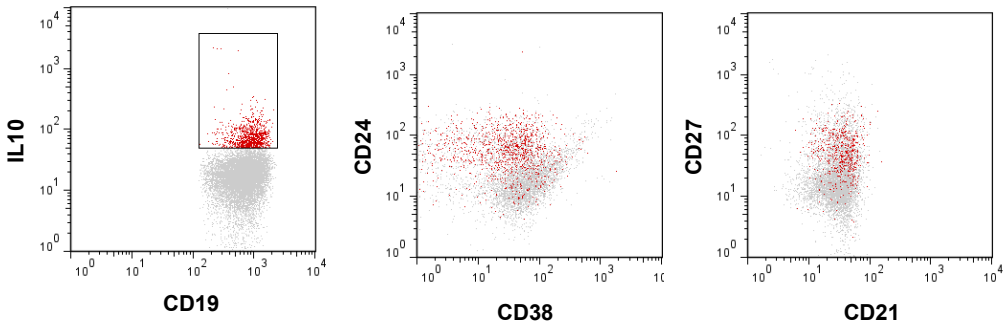
Determination of the optimal time point for studying IL10+ B cells. Gated CD19+ B cells are analysed for CD24 and CD38 expression at baseline, 12-15 hours and 24-36 hours. CD24-CD38hi plasmablasts are gated and compared between the three time points. A distinct population starts to appear at 15-25 hours (~1.5%) when stimulated with L cells alone; top, and (~2.5%) with dual stimulation; bottom, associated with overall reduction of CD24 expression.

Analysis of three independent experiments and six healthy controls showed that IL10$^+$ CD19$^+$ cells (IL10$^+$ cells) were positive for CD1d, CD20, CD21, CD22, CD40, CD268 (BAFFR), and HLA-DR with the majority expressing CD27, CD24, IgD and IgM. Although almost all IL10$^+$ cells expressed high levels of CD25 reminiscent of regulatory T cells, this was associated with CD69 expression suggesting that CD25 upregulation may be activation induced; **figure (IV-6A and 6B)**. Interestingly, the majority of IL10$^+$ cells lacked CD23 expression and had low or no expression of CD38. Compared to IL10$^-$ CD19$^+$ B cells, IL10$^+$

cells had higher expression of CD24, IgM and CD1d. I calculated median fluorescence intensity (MFI) of CD1d for both $IL10^{+}$ and $IL10^{-}$ populations since all B cells expressed CD1d. $IL10^{+}$ B cells expressed higher CD1d compared with the $IL10^{-}$ B cells (CD1d MFI; 62.5 vs. 44.5; p=0.05). Both $IL10^{-}$ and $IL10^{+}$ $CD19^{+}$ B cells expressed similar levels of CD5.

$IL10^{+}$ cells were enriched within the $CD24^{hi}$ $CD38^{-/lo}$ memory cell compartment; [72%; (65.5-81%)]. In our setting, the majority of $IL10^{+}$ B cells were $CD27^{+}$, which further supports their memory phenotype. In addition, the co-expression of IgD and high level of IgM indicates the non- switched nature of these cells. Furthermore, $IL10^{+}$ cells lacked CD23 expression. Collectively, the majority of $IL10^{+}$ B cells were $CD24^{hi}$ $CD38^{-/lo}$ $CD27^{+}$ IgD^{+} IgM^{hi} $CD23^{-}$, a pattern of expression suggestive of IgM memory B cells or marginal zone B cells. I tried to combine these markers in one extended panel to confirm this phenotype; however, attempts to combine five colour flow cytometry with intracellular staining were unsuccessful due to the poor separation of $IL10^{-}$ IL and IL 10^{+} populations; (data not shown).

A.



B.

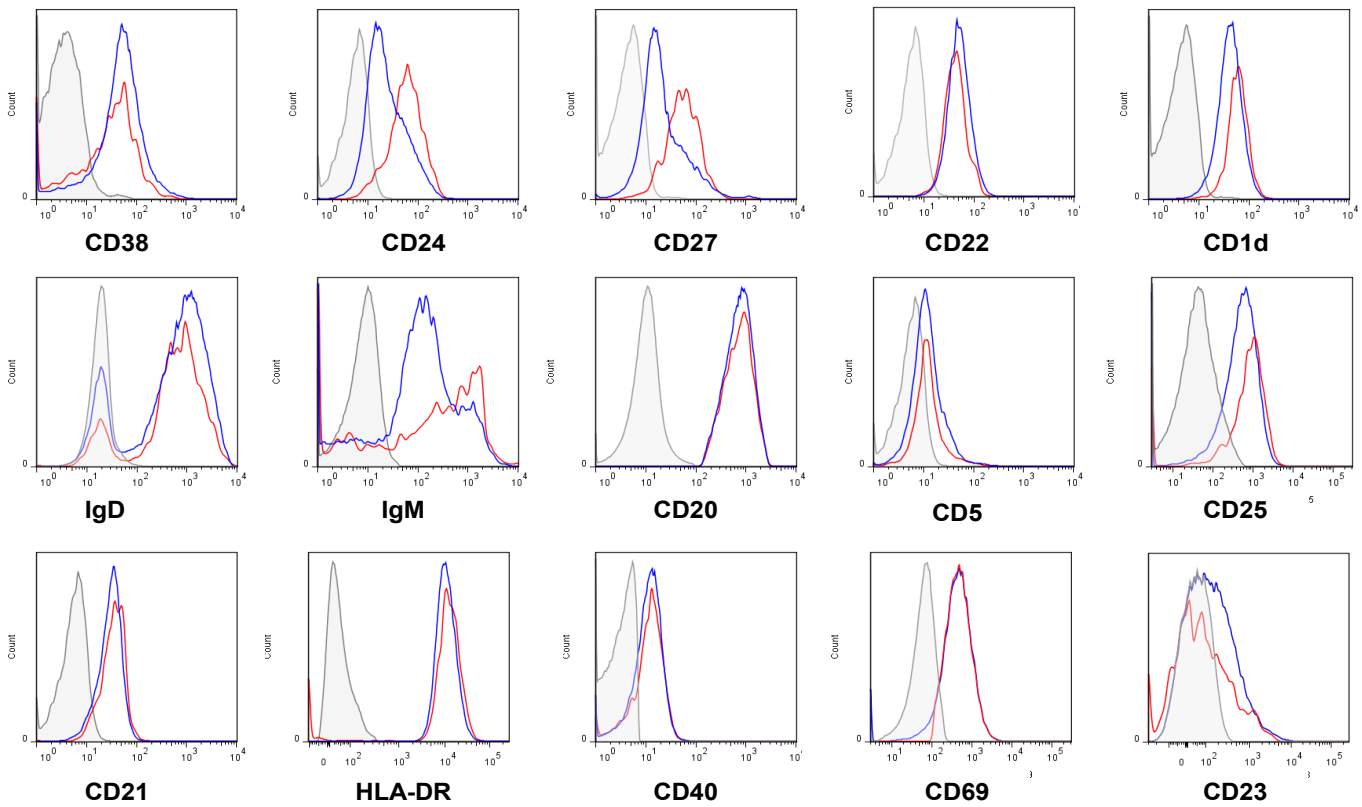


Figure IV-6: Phenotypic characterisation of IL10^{+}CD19^{+} B cells

Phenotypic characterisation of IL10^{+}CD19^{+} B cells. **A**. A representative bi-dimensional FACS plot of IL10^{+} CD19^{+} B cells (red) relative to IL10-CD19^{+} B cells (grey). PBMC from healthy donors were cultured for 15 hours with L cells. PMA, Ionomycin and BFA were added for the last 6 hours of culture. Cells were then stained with CD19, CD24, and CD38 or CD19, CD27 and CD21 mAbs and intracellularly stained with IL10 mAb or with an appropriate isotype control. **B**. Extended phenotyping of IL10^{+} B cells (red) compared with IL10- B cells (blue) and isotype control (grey)

IV.2.5 Effect of cryopreservation on IL10 IC staining

Aliquots of PBMC from 6 healthy controls were frozen for later intracellular staining. After thawing, cells were rested for a minimum of 6 hours and stimulated thereafter with irradiated L cells at a ratio of 1:10 for 24 h. BFA, Ionomycin and PMA were added; **section II, 8, 1**, for the last 6 to 8 h of the incubation. Tested samples were stained with CD19, CD24, CD38 and IL10. Frozen samples yielded FACS plots with distinctive populations comparable to the freshly processed ones. Furthermore, similar to the freshly stained samples, cells stained positive for IL10 were also enriched within $CD24^{hi}$ $CD38^{h}$ **figure (IV-7A)**. Frequencies of $IL10^{+}$ achieved from the frozen samples were not different to their non-frozen counterparts; (2.81% vs. 2.86%; p=0.5) respectively; **figure (IV-7B)**.

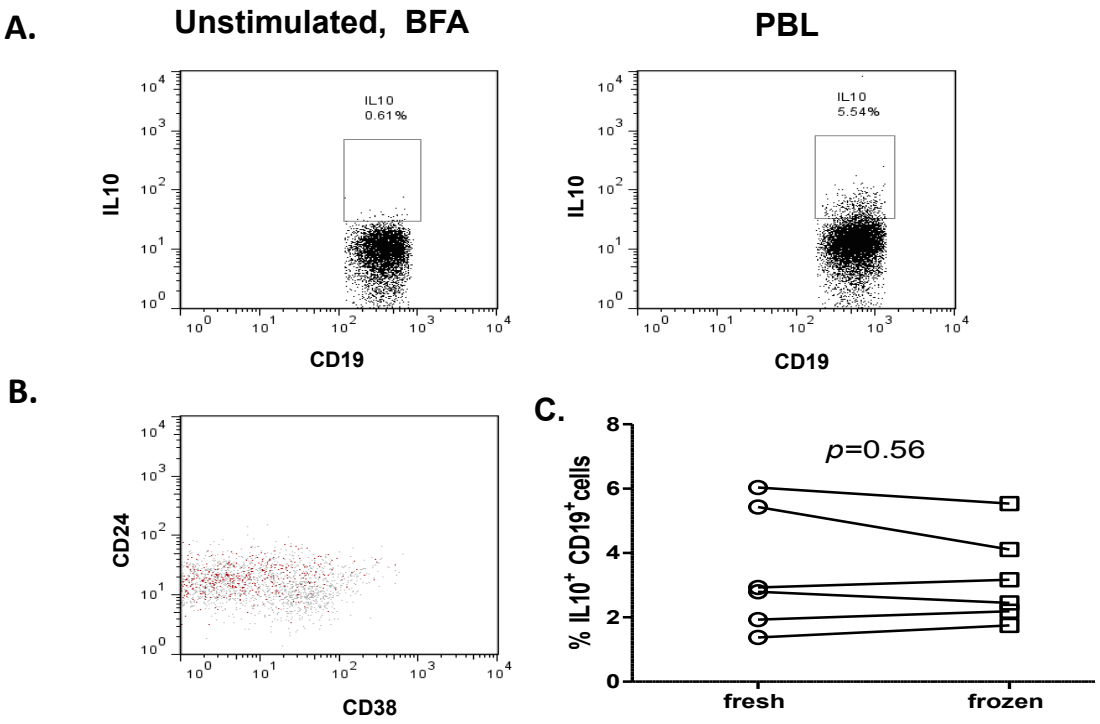


Figure IV-7: IL 10 IC staining in frozen samples

A. FACS plots from a representative frozen sample show $IL10^+$ $CD19^+$ B cells gated based on unstimulated control. PBMC were stimulated for 48 hours with L cells (PBL) and PMA, Ionomycin and BFA were added for the last 6 hours of the culture. **B**. $IL10^+$ B cells are also $CD24^{hi}CD38^-$ (red) compared with total $CD19^+$ B cells (grey) similar to that observed with freshly processed samples. **C.** $IL10^+$ B cell frequencies from paired fresh and frozen healthy control samples (n=6), there was no significant difference in the frequencies of $IL10^+CD19^+$ B cells between fresh and frozen samples. The results were compared with two-tailed nonparametric paired t test analysis.

IV.2.6 Functional assays

Regulatory B cells modulate immune responses through a variety of mechanisms. The regulatory function has been demonstrated in a number of published studies with in vitro suppression assays of T cell cytokine production and/or proliferation. To date, IL10 production by a B cell subset remains by and large the hallmark of regulatory B cells. Therefore, I examined whether or not our identified population, enriched in $IL10^+$ B cells, possessed regulatory properties. I designed an in vitro culture system whereby sorted B cell subsets were incubated with purified autologous $CD4^+$ T cells. The proliferation and pro-inflammatory cytokine production, namely IFNγ and TNFα, of CD4 T cells following in vitro stimulation was assessed by flow cytometry and compared with that of other T cells cultured with different B cell subsets as detailed in **section II, 9, 1 and II, 9, 2.**

IV.2.6.1 Sort purification of B cell subsets

I employed a B cell sorting strategy based on the identified $IL10^+$ B cell phenotype. As demonstrated in **IV.2.4** above, $IL10^+$ cells expressed a phenotype suggestive of IgM memory B cells. $CD27^+$ B cells can be divided according to class switching recombination into switched memory B cells, IgM^- IgD^-, non-switched, IgM^+ IgD^+, and two minor populations IgM only and IgD only memory B cells. The phenotype $CD27^+IgM^+$ defines almost all non-switched memory B cells. Therefore, we used a 4 colour sorting panel including CD19, CD27, IgM and PI. This panel allows the separation of four B cell subsets starting from a viable $CD19^+$ cell gate, namely, $CD27^-$ IgM^{hi} transitional, $CD27^-$ IgM^+ naïve, $CD27^+$ $IgM^{+/hi}$ IgM memory B cells and $CD27^+$ IgM^- switched memory B cells. The population $CD27^-$ IgM^{hi} cells are enriched for transitional B cells, however, may also

include naïve B cells. The set sorting gates for naïve and transitional populations took into account this fact and were well separated into high IgM, for transitional and intermediate IgM, for naïve B cells within the $CD27^-$ B cells. The purity of each subset was excellent: naïve 96.5%; (92%-99.7%), transitional 91%; (82.2%- 93.5%), switched 95.8% (91%- 99.4%) and IgM memory B cells 93.7%; (92%-96.6%), **figure (IV-8).**

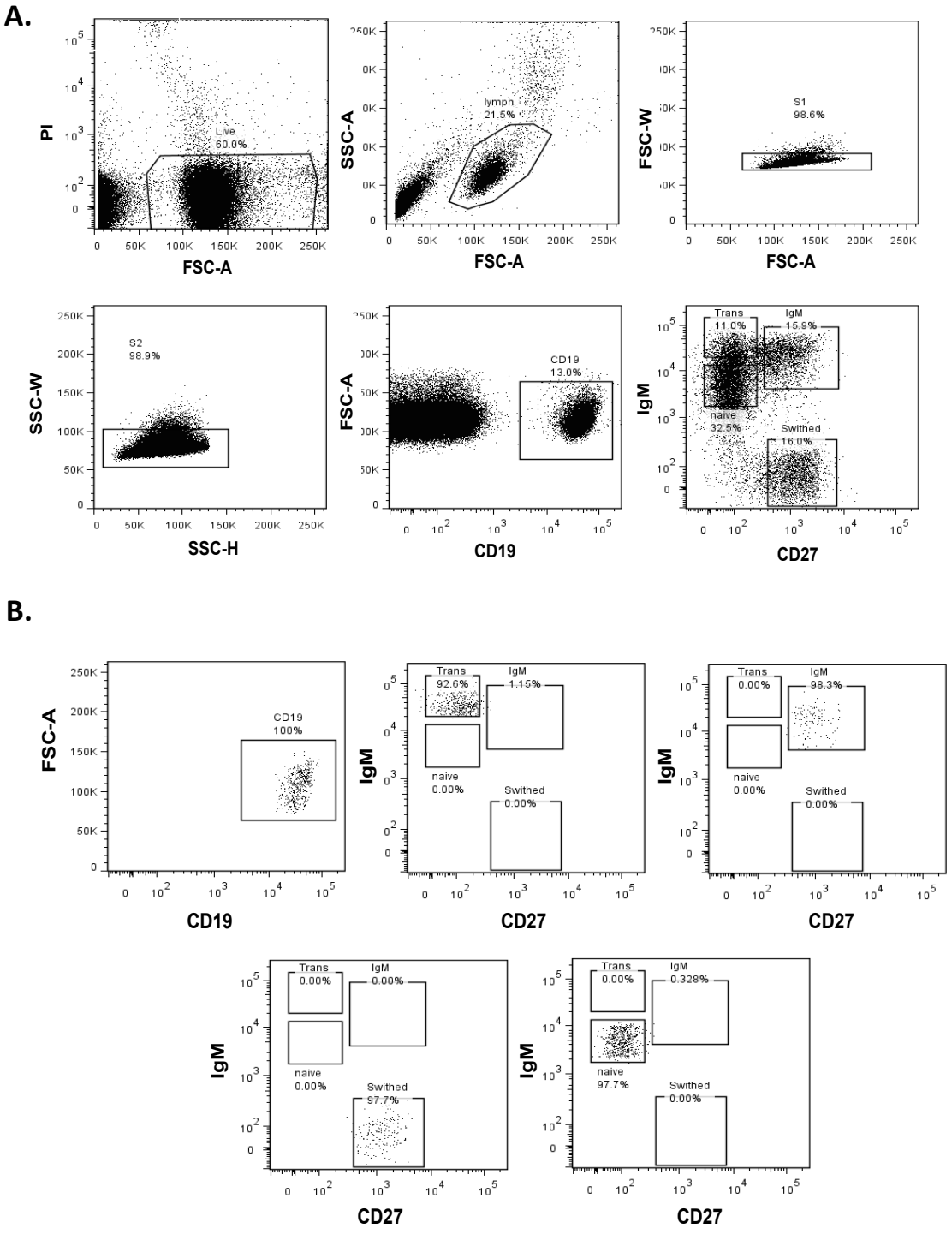


Figure IV-8: Gating strategy of B cell sorting

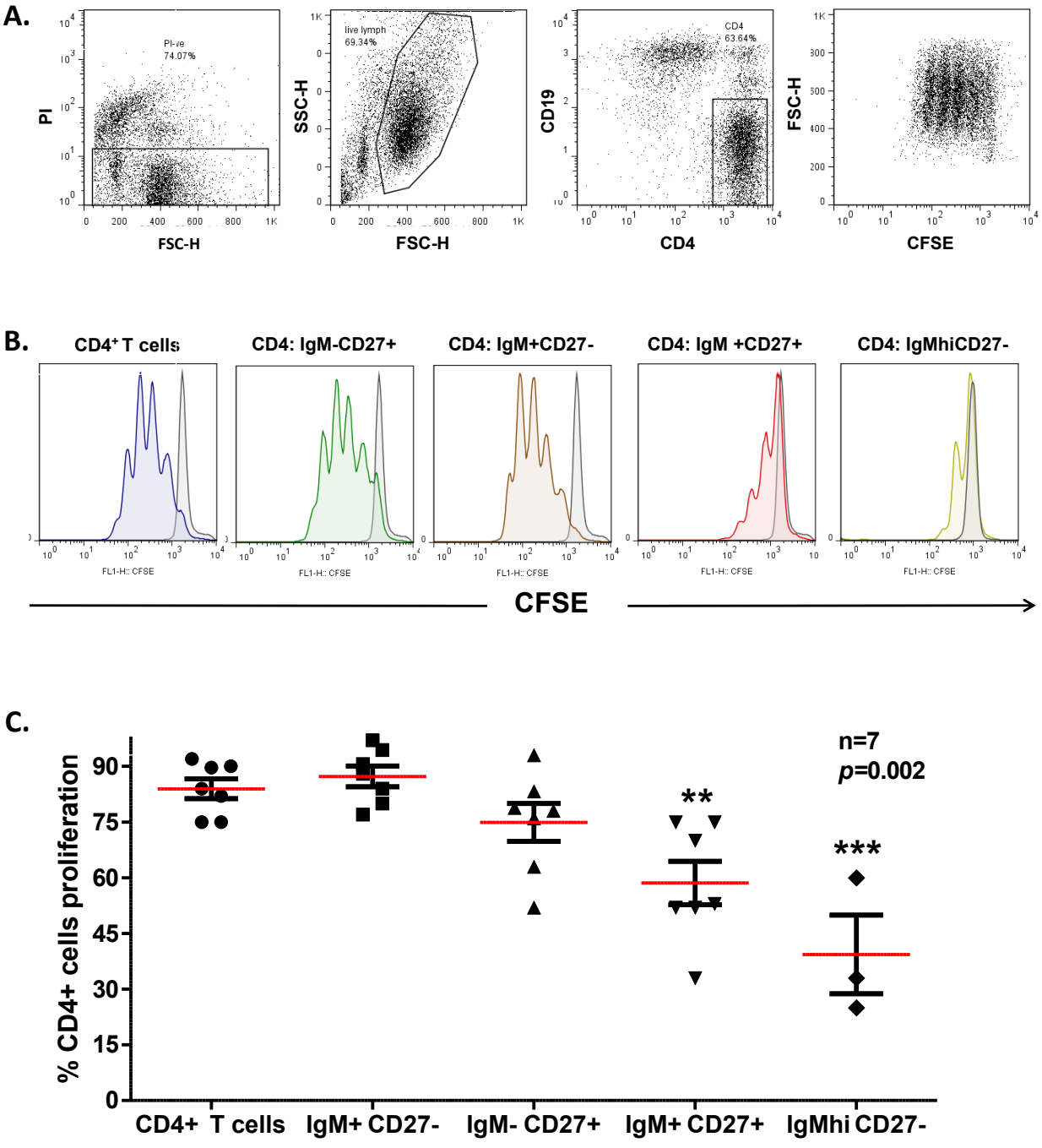
Gating strategy for B cell sorting. **A**. Gated PI^- live lymphocytes, after excluding doublets, were assessed for CD19 expression. $CD19^+$ B cells were subdivided according to IgM and CD27 expression into IgM^+CD27^- naïve, $IgM^{hi}CD27^-$ transitional, IgM^+CD27^+ nonswitched and IgM^-CD27^+ switched memory B cells. **B.** Post sort purity check.

IV.2.6.2 Suppression of $CD4^+$ T cell proliferation

CFSE stained selected $CD4^+$ T cells were cultured with each sorted B cell subsets at a ratio of 1:1 and stimulated with anti-CD3/ anti-CD28 beads. The percentage of divided $CD4^+$ cells was assessed by flow cytometry after 96 h of incubation. For each experiment a proliferation control (PC); stimulated $CD4^+$ T cells cultured without any B cells, and negative control; unstimulated selected $CD4^+$ T cells were included, **figure (IV-9A)**. I found from seven independent experiments using different healthy controls that unstimulated $CD4^+$ T cells did not proliferate at all by the end of the incubation. The percentage of dividing $CD4^+$ T cells from each condition was compared with that of PC and with each other. Eighty four per cent of positive control cells divided in the absence of B cells; [84%± 2.6%; (75%-92%]. There was no suppression of $CD4^+$ T cell proliferation when cultured with $CD27^-$ IgM^+ naïve B cells ; [88%± 2.8%; (77%-97%] and $CD27^+$ IgM^- switched memory B cells; [78%± 5.09%; (52%-93%]; p=0.4 and p=0.3 respectively.

However, IgM memory B cells significantly suppressed $CD4^+$ T cell proliferation when compared with PC, naïve and switched memory B cells; [53%± 5.8; (33%-75%)] p=0.002. Analysis of transitional B cells effects on $CD4^+$ T cell proliferation was possible in three experiments only due to the reduced viability of this subset in culture. I observed a suppressive effects of this subset [33%± 10.5% (25%-60%), n=3]; consistent with published data,(Blair et al., 2010) **figure (IV-9B and IV-9C)**. The reduced proliferation of $CD4^+$ T cells upon culturing with IgM memory or transitional B cells indicates that these

subsets possess inducible regulatory properties or contain a specialised regulatory B cell population.



D.

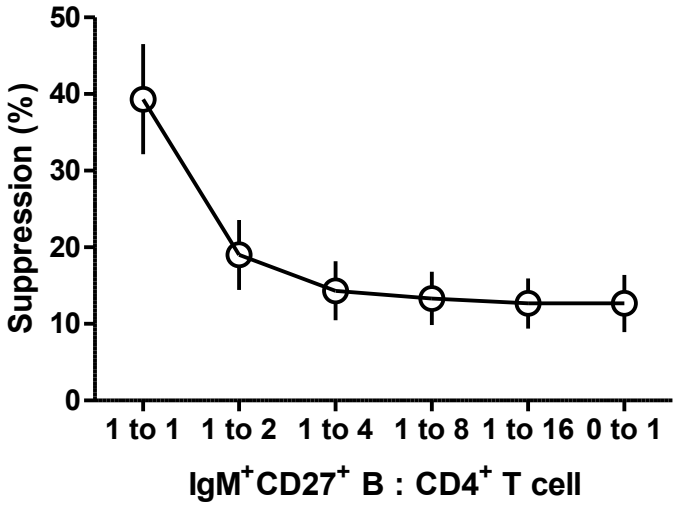


Figure IV-9: Proliferation suppression assay ($CD4^+$ T cells)

B cell subset suppression assay showing CFSE proliferation profiles of $CD4^+$ T cells cultured for 96 hours with anti-CD3/anti-CD28 beads and sorted B cell subsets at 1:1 ratio. Stimulated selected $CD4^+$ T cells labelled with CFSE (positive control) and unstimulated $CD4^+$ T cells (negative control) were included with each experimental run. At end of culture cells were stained with PI, CD19-PerCP and CD4-APC. **A.** Representative FACS plots of $CD4^+$ T cell suppression assay with gating strategy. Gated CD4 T cells from live PI^- lymphocyte population were analysed for CFSE dilution by flow cytometry after 4 days with sorted IgM^+ $CD27^-$ naïve B cells at 1 to 1 ratio. **B.** Proliferation of $CD4^+$ T cells cultured alone (blue), with IgM^-CD27^+ switched memory B cells (green), IgM^+CD27^- naïve B cells (brown), IgM^+CD27^+ nonswitched memory B cells (red) and $IgM^{hi}CD27^-$ transitional B cells (yellow) at a ratio of 1:1. Negative unstimulated control (grey) was included. IgM^+CD27^+ nonswitched memory B cell exhibited suppressive effect on CD4 T cells. **C.** *in vitro* suppression effect of different $CD19^+$ B cell subsets (1:1 ratio) was assayed using anti-CD3/anti-CD28-stimulated $CD4^+$T cells and determined by inhibition of CFSE-labelled cell proliferation (mean ± SEM of seven independent experiments). **D**. Suppression of $CD4^+$ T-cell proliferation in the presence of IgM^+CD27^+ nonswitched T-cell incubated at the indicated B to T cells ratios. Data are representative of 3 independent experiments**.**

IV.2.6.3 The suppressive property of IgM memory B cells is cell dose dependent

I showed that at a ratio of 1 to 1 (B to T), IgM memory B cells exhibit a suppressive effect on autologous $CD4^+$ T cells. I then asked the question of whether this effect is cell dose dependent. To address this question, I repeated the same experiment, as in IV.2.6.2, with selected IgM memory B cells from three healthy individuals in three independent runs. The ratio of (B to T) cell was sequentially halved starting from 1 to 1 down to 1 in 16. From these three experiments, the proliferation of $CD4^+$ T cells was reduced from 90± 3.7%; (80%-92%) in PC to 54%±7.17%; (53-75%) at the ratio of (1:1). Notably, the inhibition of $CD4^+$ T cell proliferation was reduced in a stepwise manner from 54% at 1:1 to 74% at 1:2 to 83% at 1:4 and fully abrogated afterwards; **figure (IV-9D)**. In view of the poor viability of transitional B cells in my in vitro system I was not able to carry out the same experiment with this subset. Overall, this observation suggests that the inhibitory effects of IgM memory B cells may require a minimum B: T cell ratio to be achieved.

IV.2.6.4 IgM memory B cells suppressed IFNγ but not TNFα production by CD4⁺ T cells

Selected total CD4⁺ T cells were cultured with each sorted B cell subsets at a ratio of 1:1 and stimulated with anti CD3/ anti CD28 beads. The percentage of IFNγ⁺ and TNFα⁺ CD4⁺ cells was assessed by intracellular staining after 48 h of incubation. For each experiment a positive control (PC); stimulated CD4⁺ T cells cultured without any B cells, and negative control; unstimulated selected CD4⁺ T cells were included as detailed in **II.9.2**. Furthermore, I used autologous regulatory T cells (Treg) cultured with total CD4⁺ T cells also at a ratio of 1 to 1 as a positive control according to published protocols.(Collison and Vignali, 2011) The relative frequency (*RF*) of each IFNγ and TNFα production resulting from culturing with a given B cell subset was calculated relative to each corresponding PC as follows:

$$RF(cytokine) = \frac{\%\ cytokine{+}CD4{+}\ T\ cells\ per\ condition}{\%\ IFN\gamma{+}CD4{+}\ T\ cells\ of\ PC}$$

For instance, 5.4% of CD4⁺ T cells stained positive for IFN⁺ upon culture with IgM memory B cells compared with 9.2% IFN⁺CD4⁺ T cells in the positive control tubes which would mean 58.6% (5.4%/9.2%) of the control.

The same equation was employed for TNFα experiments *RF (TNFα)*. Therefore, *RF* for PC condition was calculated as 1. *RF*s of each condition are presented as mean± SEM with (range) and statistically compared with *RF* of positive control using one way ANOVA and two tailed t test for comparing means and the p value was considered significant when <0.05.

IV.2.6.4.1 IgM memory B cells Suppress IFNγ$^+$ CD4$^+$ T cells

I found from five independent experiments using healthy controls that unstimulated T cells produced negligible amounts of IFNγ. As expected, CD4$^+$ T cells stimulated with anti CD3/CD28 produced IFNγ in the absence of B cells [mean IFNγ$^+$ CD4$^+$ T cells; 4.07%± 1.36 (1.02-9.2%)]. CD4$^+$ T cells cultured with all three sorted B cell subsets namely naïve, switched and IgM memory B cells produced less IFNγ compared with the positive control. The mean percentages of IFNγ$^+$ CD4$^+$ T cells for each condition were [3.2%± 1.01 (0.9%-7%); 2.99%±0.67 (1.07%-4.8%) and 2.5%±0.79 (0.6%-5.4%)] respectively. There was a trend to statistical difference when comparing the percentage of IFNγ$^+$ CD4$^+$ T cells among the groups (p=0.07). Lack of significance could be explained by the considerable variation of IFNγ$^+$ CD4$^+$ T cells' frequencies observed per condition between tested individuals. To eliminate the variation effect, I calculated *RF* for each culture condition relative to each individual's own positive control.

$$RF(INF\gamma) = \frac{\%\ IFN\gamma^{+}CD4^{+}\ T\ cells\ per\ condition}{\%\ IFN\gamma^{+}CD4^{+}\ T\ cells\ of\ PC}$$

Based on this analysis, the mean *RF (IFNγ)* of CD4$^+$ T cells cultured with naïve, switched and IgM memory B cells were [0.83± 0.02 (0.77-0.9); 0.95±0.16 (0.5-1.5) and 0.62±0.04 (0.53-0.78)] respectively, **figure (IV-10A)**. One way ANOVA test showed that the relative frequency of IFNγ$^+$ CD4$^+$ T cells cultured with IgM memory B cells at 1:1 was the only significantly lower frequency than the control p=0.021. **Figure (IV-10B)** shows the cumulative IFNγ percentage inhibition induced by co-culturing CD4$^+$T cells with B cell

subsets. Unfortunately, for the same reason explained above, we could not analyse the effect of transitional B cells on $CD4^+$ T cell production of IFNγ.

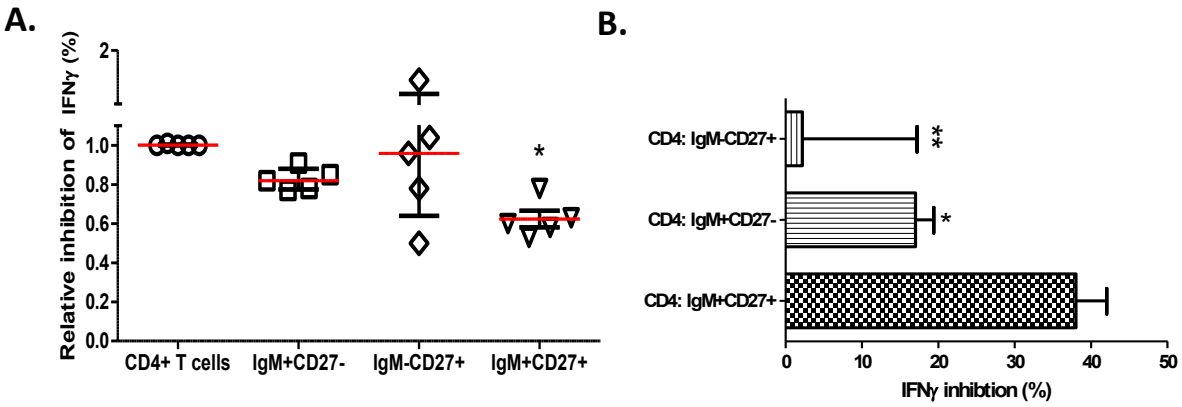


Figure IV-10: IFNγ suppression assay ($CD4^+$ T cells)

A. *in vitro* suppression effect of different $CD19^+$ B cell subsets was assayed using anti-CD3/anti-CD28-stimulated $CD4^+$T cells and determined by inhibition of $IFN\gamma^+CD4^+$ T cells cultured for 48 hours with sorted B cell subsets at 1:1 ratio. At the end of the culture cells were stained with PI, CD19-PerCP and CD4-APC and IFNγ-FITC. Unstimulated controls were used as negative control. Relative inhibition of IFNγ production was calculated relative to positive control, ($CD4^+$ T cells). The results were compared with ANOVA and two-tailed t test analysis (n=5). **B.** Cumulative results expressed as median ± interquartile range. The results were compared with nonparametric two-tailed t test analysis (CD4: IgM^+CD27^+ versus other groups), $^*p < 0.05$, $^{**}p < 0.01$

IV.2.6.4.2 IgM memory B cells do not suppress $TNF\alpha^+CD4^+$ T cells

We also analysed $TNF\alpha^+$ $CD4^+$ frequencies from the same controls as in **section IV.2.6.4.1.** The positive control cells produced TNFα in the absence of B cells [$TNF\alpha^+$ $CD4^+$ T cells; 16.4%± 1.3 (13-21%)]. $CD4^+$ T cells cultured with all three sorted B cell subsets naïve, switched and IgM memory B cells produced comparable TNFα to the positive control. The means of for each condition were [15.2%± 1.3 (10.7-23.1%); 19.4%± 1.2 (17.4-23.5%) and 14.92%± 1.47 (11.5-20%)] respectively, p=0.2. I also calculated *RF* for each culture condition relative to each individual's own positive control.

$$RF(TNF\alpha) = \frac{\%\ TNF\alpha^{+}CD4^{+}\ T\ cells\ per\ condition}{\%\ IFN\gamma^{+}CD4^{+}\ T\ cells\ of\ PC}$$

The means *RF* of for naïve, switched and IgM memory B cells were [0.91± 0.1 (0.7-1.2); 1.15± 0.06 (1.06-1.4) and 0.89± 0.05 (0.69- 0.97)] respectively, **figure (IV-11).** Similarly, ANOVA test and two tailed t-test for pairwise comparisons of each condition with the control did not reveal any significant difference.

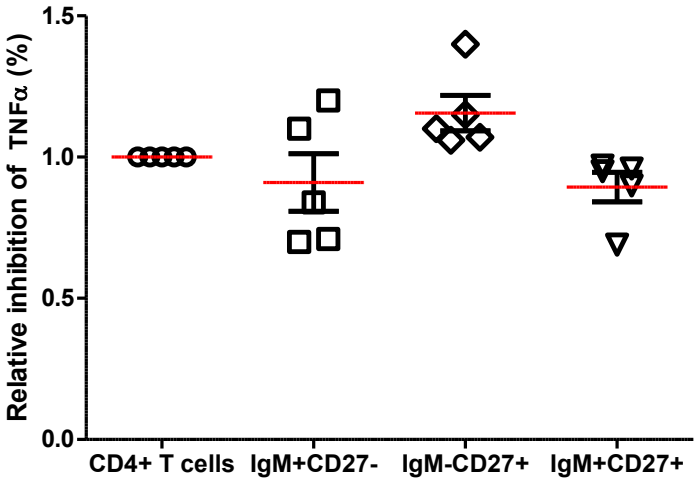


Figure IV-11: **TNFα suppression assay (CD4⁺ T cells)**

in vitro suppression effect of different CD19⁺ B cell subsets was assayed using anti-CD3/anti-CD28-stimulated CD4⁺T cells and determined by inhibition of TNFα⁺CD4⁺ T cells cultured for 48 hours with sorted B cell subsets at 1:1 ratio. At the end of the culture cells were stained with PI, CD19-PerCP and CD4-APC and TNFα-FITC. Unstimulated controls were used to determine TNFα⁺CD4⁺ T cell gate. Relative inhibition of TNFα production was calculated relative to positive control, (CD4⁺ T cells). The results were compared with ANOVA test for multiple comparison analysis (n=5).

IV.2.6.4.3 IgM memory B cell induced suppression is comparable to Treg

In order to estimate the inhibitory capacity of IgM memory B cells, I compared its suppressive effect on cytokine production of CD4⁺ T cell to that of regulatory T cells

(Treg); defined as $CD25^{hi}$ $CD127^{-}$ CD4 T cells. Selected Treg were co-cultured with autologous $CD4^{+}$ T cells at 1:1 ratio in 4 independent experiments. The inhibition of IFNγ and TNFα production induced by Treg was compared with that of IgM memory B cells and the positive control from the same run. Unlike IgM memory B cells, Treg suppressed both IFNγ and TNFα production by autologous CD4 T cells. The means *RF (IFNγ)* of Treg and IgM memory B cells were significantly lower than that of positive control [0.5± 0.08 (0.34-0.71); p=0.001 and 0.62± 0.04 (0.53-0.78); p=0.014] respectively, **figure (IV-12A and IV-12B)**. Interestingly, there was no significant difference between the inhibitory effect of both Treg and IgM memory B cells on IFNγ production by T cells (mean *RF* 0.5 vs. 0.62; p=1.0), figure (11C). However, the mean *RF (TNFα)*, on the other hand, was only significantly lower than that of positive control for Tregs [0.59± 0.08 (0.32-0.8); p=0.001]. The inhibitory capacity of IgM memory B cells on IFNγ production is therefore predicted, under our experimental conditions, to be comparable to Treg.

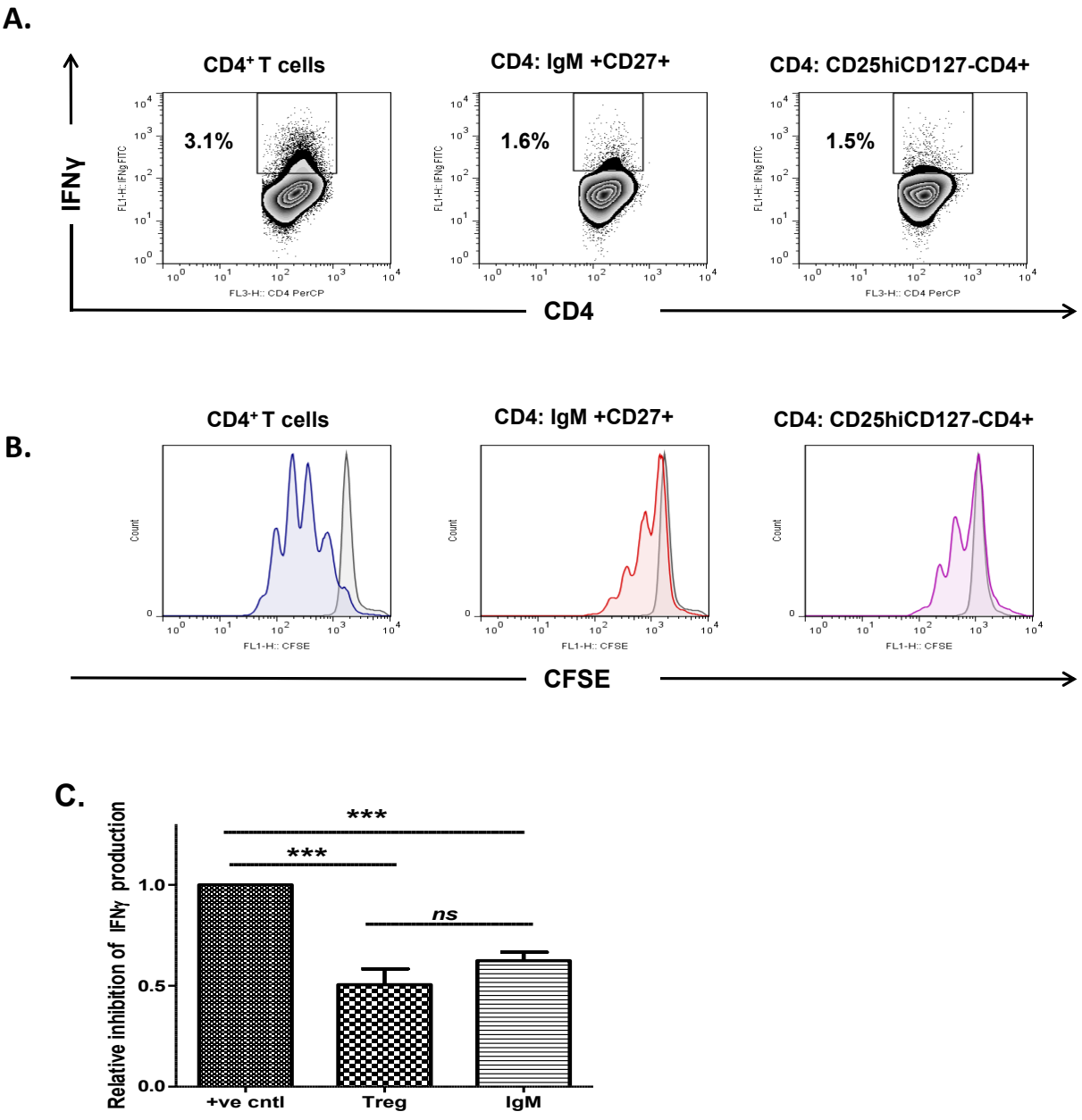


Figure IV-12: Breg suppression capacity is comparable to Treg

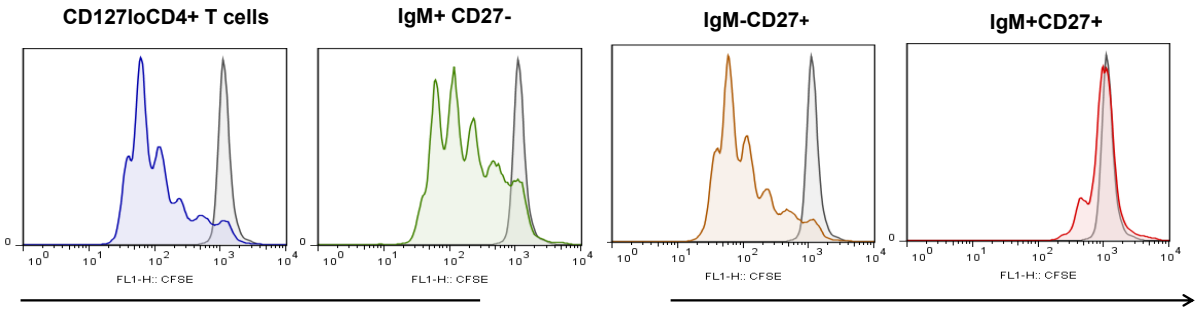
IgM^{+}CD27^{+} B cells suppressive effect on IFNγ production and CD4 T cell proliferation is comparable to Treg. **A**. Sorted IgM^{+}CD27^{-}CD19^{+}PI^{-} B cells and CD25^{hi}CD127^{-} Treg from healthy individuals were cultured 1:1 with autologous CD4^{+}T cells for 48 hours with anti-CD3/anti-CD28 beads. PMA, Ionomycin and BFA were added in the last 6 hours of culture and subsequently cells were surface stained for the expression of CD4 and intracellularly stained for IFNγ. Frequencies of IFNγ^{+} CD4^{+} were assessed relative to the corresponding unstimulated controls. Representative FACS plots for 1 out of 3 individuals, showing the frequencies of IFNγ^{+} CD4^{+}T cells. **B.** Proliferation of CD4^{+} T cells cultured alone (blue),

with IgM^+CD27^+ nonswitched memory B cells (red) or $CD25^{hi}CD127^-$ Treg (pink) at a ratio of 1:1. Negative unstimulated control is shown in grey. **C**. Bar graphs show percentage inhibition of the frequency of $CD4^+IFN\gamma^+$ T cells after co-culture with $IgM^+CD27^-CD19^+PI^-$ B cells and $CD25^{hi}CD127^-$ Treg; n=3.

IV.2.6.5 Direct $CD4^+$ T cells suppression by IgM memory B cells is not dependent on the presence of Tregs in the culture system

I also examined the effect of culturing B cell subsets with Treg depleted-$CD4^+$ T cells at a ratio of 1:1. For this purpose I used a commercially available Treg kit as shown in **section II.5.2.2.** The kit produces positively selected $CD127^-$ $CD25^{hi}$ $CD4^+$ regulatory T cells and negatively selected CD4 T cells. Similar to the effect on total CD4 T cells, IgM memory B cells were suppressive on the proliferation of Treg depleted CD4 compared with total $CD4^+$ T cells [(79%±10.9 (58-94%) vs. 6.5%±2.07 (2.8-9.7%) n=3, p=0.021)], figure **(IV-13A and IV- 13B)**.

A.



B.

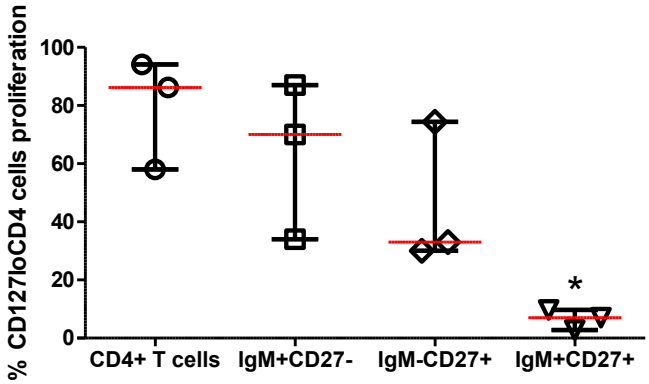


Figure IV-13: Proliferation suppression assay (Treg)

A. Proliferation of Treg-depleted CD4+ T cells cultured alone (blue), with $IgM^{-}CD27^{+}$ switched memory B cells (green), $IgM^{+}CD27^{-}$ naïve B cells (brown) or $IgM^{+}CD27^{+}$ nonswitched memory B cells (red) at a ratio of 1:1. Negative unstimulated control (grey) was included. $IgM^{+}CD27^{+}$ nonswitched memory B cell exhibited suppressive effect on $CD127^{lo}CD4^{+}$ T cells. **B**. *in vitro* suppression effect of different $CD19^{+}$ B cell subsets (1:1 ratio) was assayed using anti-CD3/anti-CD28-stimulated Treg-depleted $CD4^{+}$T cells and determined by inhibition of CFSE-labelled cell proliferation (median ± interquartile range); n=3; *<0.05.

IV.2.7 Suppressive effects is IL10 dependent and cell specific

I have shown that IL10$^+$ B cells are enriched within the CD27$^+$IgM$^+$ memory B cell subset. Sort-purified IgM memory cells displayed regulatory properties by suppressing the proliferation and cytokine production of autologous CD4$^+$ T cells. Furthermore, I showed that the suppressive ability of IgM memory B cells is comparable to that of Treg. To further understand the mechanism underlying the regulatory properties of IgM memory B cells, I repeated the experiments outlined in **section II. 9. 3** in the presence or absence of IL10 blocking antibodies.

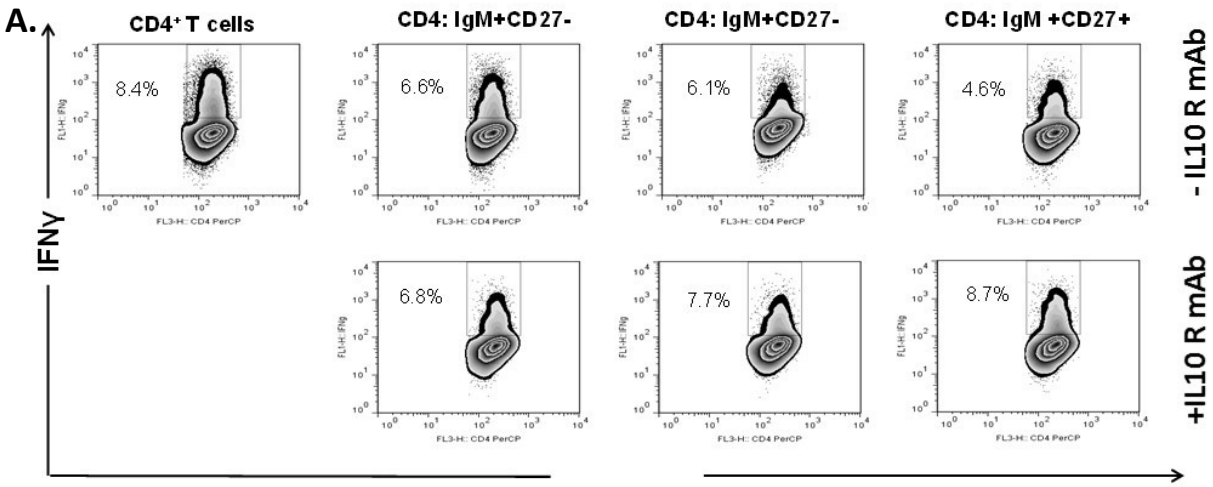
IV.2.7.1 IL10 blocking experiments:

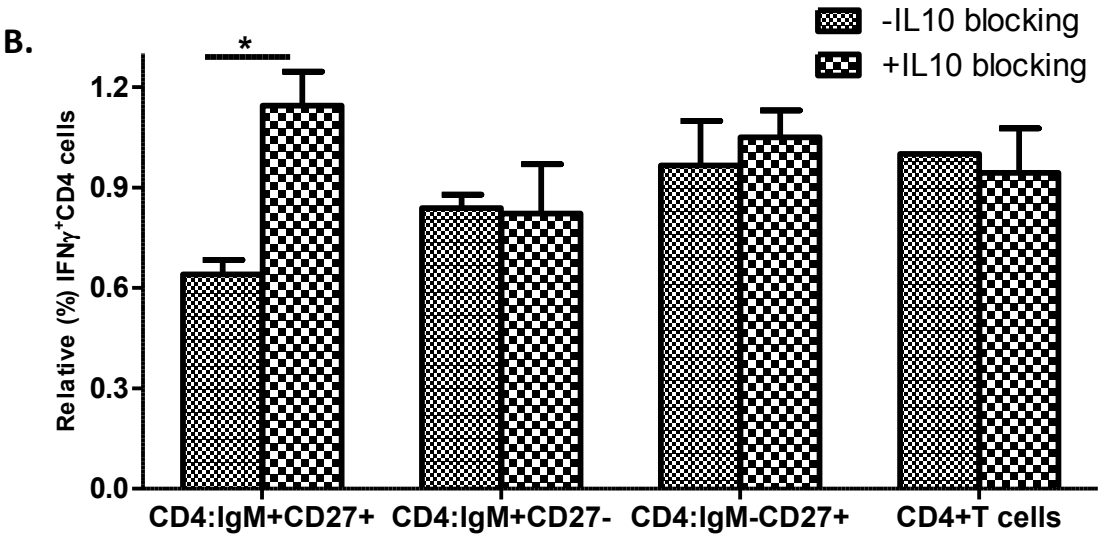
IV.2.7.1.1 Effect of IL10 blocking on IFNγ production by CD4$^+$ T cells

To address this question, I performed IL10 blocking experiments by adding anti-CD210 (IL10 R) blocking antibodies to the culture system in 4 independent experiments. Relative frequency *RF (IFNγ$^+$)* in CD4$^+$ T cells cultured with sort-purified B cell subsets in the presence or absence of IL10 blocking antibodies was calculated.

Blocking IL10 R in the positive control wells had no effect on IFNγ production (2.47%± 0.7 vs. 2.5%± 0.9, p=0. 8]. Similarly, there was no difference between the relative frequencies of IFNγ$^+$ CD4$^+$ T cells seen when CD4$^+$ T cells were cultured with naïve B cells with and without IL10 blocking; [83.8%± 0.4 (76-93%) vs. 86.5%± 1.6 (60-128%), p=0.9] and with switched memory B cells with and without IL10 blocking; [120%± 0.04 (106-160%) vs. 115%± 1.0 (101-139%), p=1] respectively. However, the addition of IL10R blocking Ab in wells containing stimulated CD4$^+$T cells + IgM memory B cells led to significant recovery of IFNγ production, [62.%± 0.4 (58-81%) vs. 115%± 1.0 (91-139%),

p=0.02], **figure (IV-14A and IV- 14B)**.





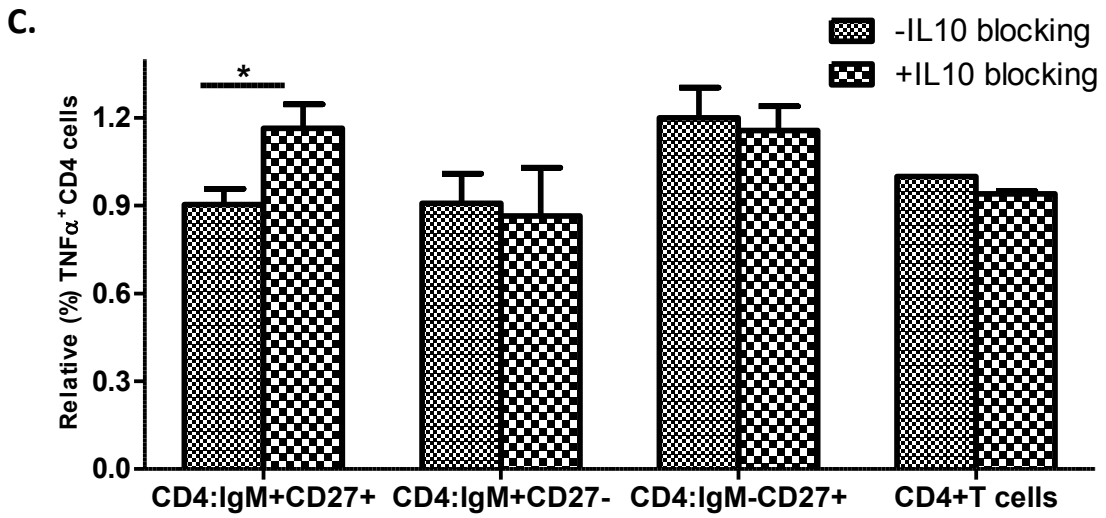


Figure IV-14: IgM$^+$CD27$^+$ CD19$^+$ B cell suppression is dependent on IL10.

A. Purified CD4$^+$ T cells were cultured with sort-purified B cell subsets: IgM$^+$CD27$^-$CD19$^+$, IgM$^+$CD27$^+$CD19$^+$, and IgM$^-$CD27$^+$CD19$^+$PI$^-$ cells and stimulated with anti-CD3/anti-CD28 beads in the presence or absence of blocking antibodies IL10R. PMA, Ionomycin and BFA were added for the last 6 hours of culture and subsequently cells were surface stained for the expression of CD4 and intracellularly stained for IFNγ. Flow cytometry plots of IFNγ expression by CD4$^+$T cells cultured alone (positive control) or in the presence of indicated B cell subsets with and without IL10 blocking antibodies. Frequencies of IFNγ$^+$ CD4$^+$T cells were assessed relative to the corresponding unstimulated controls. **B**. Bar charts compare the relative percentage RF of IFNγ$^+$ CD4$^+$ and **C**. TNFα$^+$ CD4$^+$ T with and without IL10 blocking. Cumulative data are presented as mean ± SEM of three independent experiments. The results were compared with two-tailed nonparametric t test analysis, $^*p < 0.05$.

Overall, these results indicate that the regulatory properties of IgM memory B cells are IL10 dependent; however, any contributory effects of cell to cell contact cannot be excluded based on these experiments.

IV.2.7.1.2 Effect of IL10 blocking on TNFα^{+} CD4^{+} T cell frequencies

As described in **section IV.2.6.4.2**, B cell subsets did not have significant suppressive effects on TNFα production by CD4 T cells.

Expectedly, blocking IL10 did not have a major impact on TNFα production by CD4^{+} T cell presented as frequencies or *RF (TNFα)*, **figure (IV-14C)**. Interestingly, blocking IL10 in the presence of IgM memory B cells resulted in a significant increase of *RF (TNFα)* compared to the "no IL10 blockade" [90%± 0.5 (69- 97%) vs. 116%± 0.8 (101-137%), p=0.019].

IV.3 Discussion

In a B cell activation model proposed by Duddy et al, B cells receiving bystander T cell help via CD154 (CD40L)-CD40 interaction produce large amounts of IL10. Whereas B cells engaged to an Ag in presence of T cell help produce more pro-inflammatory cytokines promoting GC formation (Duddy et al., 2004). In agreement with this paradigm, in my experiments, stimulation of purified B cells with L cells alone induced more IL10 secretion, as measured by ELISA. On the other hand, BCR engagement in addition to CD40 stimulation reduced IL10 production by purified B cells. I therefore propose that B cells receiving bystander stimulation in close vicinity to an inflammatory signal may regulate over spilling of the reaction via IL10 production.

The next step was to optimise the intracellular IL10 assay to allow phenotypic characterisation of IL10 producing B cells. Using this assay, I found that $IL10^{+}$ $CD19^{+}$ B cells are enriched within the $CD24^{hi}CD38^{lo}$ B cells. Almost 75% of $IL10^{+}$ $CD19^{+}$ B cells were positive for CD27 which further support a memory phenotype. In addition, $IL10^{+}$ $CD19^{+}$ B cells expressed IgD and high levels of IgM. Overall, this combination of markers is consistent with IgM memory B cell phenotype.

CD23 is a marker of follicular and naïve B cells which distinguishes them from $CD23^{-}$ splenic marginal zone B cells. Interestingly, marginal zone precursor B cells express CD23 and become $CD23^{-}$ as they mature towards MZB cells (Pillai and Cariappa, 2009).

In the PB, IgM memory B cells are the circulating counterpart of MZ B cells and therefore do not express CD23 (Weill et al., 2009). The fact that $IL10^{+}$ $CD19^{+}$ B cells did not express CD23 further supports their IgM memory B cell origin.

CD1d is a glycoprotein expressed by various antigen-presenting cells in humans. CD1d, related to the class I MHC molecules, presents lipid antigens to, and activates NKT cells. Murine B10 regulatory B cells express high levels of the functionally important CD1d and are positive for CD5. Except for CD5, the rest of the identified phenotype is shared with MZ B cells (Yanaba et al., 2008). I, therefore, assessed CD5 and CD1d expression on $IL10^{+}CD19^{+}$ B cells and found that $IL10^{+}$ B cells did not preferentially express CD5. However, they expressed higher CD1d levels in agreement with the murine studies. CD1d has been shown to be important for MZ B cells function in promoting self-tolerance via communication with NKT cells (Sonoda and Stein-Streilein, 2002, Allan et al., 2011).

CD25, both basal and induced expression, has been studied as a marker of B cells with regulatory properties (Tretter et al., 2008, Brisslert et al., 2006). I found that $IL10^{+}$ B cells were positive for CD25; however, the analysis was undertaken after 15 hours of activation. All $IL10^{+}$ B cells expressed CD69 which is also a general marker of cellular activation (Maino et al., 1995). In light of the available data, I cannot propose a functional role for CD25 expression in B cell regulatory function. The remainder of the $IL10^{+}$ B cell phenotype included analysis for expression of CD20, CD21, CD22, CD40, HLA DR and CD268 (BAFFR). My data suggest that $IL10^{+}$B cells are $CD24^{hi}$ $CD38^{lo}$ $CD27^{+}CD23^{-}$ IgD^{+} IgM^{hi} $CD1d^{hi}$ $CD19^{+}$ which are consistent with IgM memory B cell phenotype. To further test that the identified cell population in fact possesses regulatory properties, I carried out functional assessment of the sort-purified IgM memory B cell population, compared to naïve and switched memory B cells. IgM memory B cells ($CD27^{+}$ IgM^{+})

suppressed the proliferation of autologous CD4 T cells. Proliferation suppression revealed a cell dose dependent effect, analogous to Treg suppression (Venken et al., 2007). Furthermore, IgM memory B cells suppressed specifically cytoplasmic production of CD4 IFNγ but interestingly not TNFα. The suppressive effect of IgM memory B cells on $IFN\gamma^{+}$ CD4 T^{+} cells was comparable to that of Treg. These findings indicate that the identified $IL10^{+}$ B cells have immunoregulatory function.

My results are in keeping with Tedder et al's published data reporting enrichment of $IL10^{+}$ B cells in the memory B cell subset. The authors reported that $IL10^{+}$ B cells are enriched within $CD24^{hi}CD27^{+}$ B cells which include both IgM^{+} and switch memory B cells. Furthermore, $IL10^{+}$ B cells were reported to express CD148, a memory B cell marker. While sort-purified $CD24^{hi}CD27^{+}$ failed to suppress cytokine production by CD4 T cells, they suppressed both IFNγ and TNFα cytoplasmic production of $CD14^{+}$ monocytes. I did not assess the impact of IgM memory B cells on $CD14^{+}$ monocytes. Blair and colleagues, on the hand, described an $IL10^{+}$ B cell subset with suppressive effect that shared phenotypic similarities with $CD24^{hi}$ $CD38^{hi}$ transitional B cells(Blair et al., 2010). In their study, sort-purified $CD24^{hi}$ $CD38^{-}$ memory B cells did not suppress effector T cell function. $CD24^{hi}$ $CD38^{-}$ B cell population is more or less equivalent to $CD24^{hi}CD27^{+}$ B cells used in the previous study. In agreement with these two studies, I also found that naïve B cells ($CD27^{-}$ IgM^{+}) did not have any suppressive effects. Although, I attempted to purify transitional B cells defined as $CD27^{-}$ IgM^{hi} B cells, these studies were challenging due to the pro-apoptotic tendency of these cells. Despite discrepancies in the phenotypic

assignment of Bregs in the different published studies, my data suggest that $CD24^{hi}IgM^{hi}CD19^{+}$ B cells will likely be enriched in $IL10^{+}$ regulatory cells.

To investigate whether the regulatory function was mediated through IL10, I used IL10 blockade as prescribed previously (Blair et al., 2010). Blocking IL10 led to full reversal of IFNγ inhibition observed in the presence of IgM memory B cells. These findings suggest that the regulatory function of Bregs are at least partly IL10 dependent.

Chapter V. IL10 producing B cells in cGVHD

V.1 Introduction

Chronic GVHD resembles in many aspects systemic autoimmune and collagen diseases with prominent fibrotic features such as systemic lupus erythematosus (SLE), Sjögren syndrome, and scleroderma (Goerner et al., 2002, Lee et al., 2003). Multiple auto-antibodies associated with autoimmune diseases are frequently detected with cGVHD(Kapur et al., 2008). Direct causal relationship with some manifestations of cGVHD, however, has been rarely established (Svegliati et al., 2007). The frequent production of auto-antibodies in patients with cGVHD may reflect critical breakdown in peripheral B-cell tolerance and/or insufficient immune regulation after allogeneic HSCT. A number of studies in human regulatory T cells (Treg) post SCT reported both increased and decreased Treg numbers with overall impaired regulatory function (Clark et al., 2004, Zorn et al., 2005).

Regulatory B cell dysfunction has been observed in a number of autoimmune conditions, suggesting a role in disease pathogenesis (Mauri and Blair, 2010b). Virtually no data exist on the potential role of regulatory B cells in GVHD as an auto-allo-immune condition. Studying this subset may provide an explanation for the pathophysiology of cGVHD and may have implications for the treatment of this disease. Chronic GVHD is unique in that some therapeutic options, not possible in autoimmune conditions, can be implemented. For example, if a deficient subset was associated with cGVHD development or manifestation, replacement of this subset from the same donor, in the context of clinical trial, is an option.

I showed, in **Chapter IV**, that IL10^{+} CD19^{+} B cells enriched within the IgM memory B cell subset. Sort-purified IgM memory B cells; enriched for IL10- producing B cells, exhibit suppressive effects on CD4^{+} T cell proliferation. In addition, IgM memory B cells suppress IFNγ production by CD4^{+} T cells. Finally this attribute is likely to be partially IL10 dependent and is likely to target a specific effector cell subset. Based on these findings, I evaluated the role of IL10^{+} CD19^{+} B cells in cGVHD.

V.2 Results

The purpose of this chapter is to examine the role of the IgM-memory-like IL10$^+$ B cell population in a cohort of cGVHD patients compared to patients with “no cGVHD” and healthy controls. I examined whether reduced or hypo-functional IL10$^+$ B cells are implicated in the pathogenesis of cGVHD.

For IL10 intracellular detection, I stimulated PBMC with L cells alone; PBL, and L cells with anti BCR; PBXL and determined their frequencies out of a gated population of live CD19$^+$ B cells. For IFNγ detection, I stimulated PBMC with PMA and Ionomycin in vitro and assessed the frequencies of CD4$^+$ IFNγ$^+$ T cells.

V.2.1 Patients characteristics

Blood samples were available for intracellular (IC) cytokine detection from (25/33) patients described in **Chapter VI** and **table (VI-2)**. All patients were more than six months post SCT with no evidence of primary disease relapse. Patients receiving high-dose steroids (> 50 mg prednisone) at the time of sampling or had received rituximab for any indication were excluded. Chronic GVHD status at the time of sample collection was classified according to documented clinical and laboratory data using National Institutes of Health (NIH) cGVHD consensus criteria and modified Seattle criteria for limited versus extensive disease. Of the 23 assessable patients, eleven patients had “active cGVHD”. Twelve patients never developed cGVHD after HSCT. None of the “no cGVHD” patients were receiving immunosuppressant therapy at the time of sample collection.

The median time to samples collections was 384 days (100-2947) after transplantation for patients with cGVHD compared to 565.5 days (250-1112) for those without cGVHD (*P* = .53). I also studied six healthy controls.

V.2.2 Chronic GVHD is associated with reduced IL10$^+$ B cell population

I examined the frequencies and absolute counts of IL10 producing CD19$^+$ B cells using intracellular staining techniques in 13 cGVHD patients, 12 "no cGVHD" patients and 6 healthy controls. The absolute count of IL10$^+$ CD19$^+$ B cells were calculated by multiplying the percentage of IL10$^+$ CD19$^+$ B cell by the absolute number of CD19$^+$ B cells. Two cGVHD patients (2/13) were excluded from the analysis in view of the low CD19$^+$ events (<1000) within the live lymphocyte gate secondary to prior rituximab therapy.

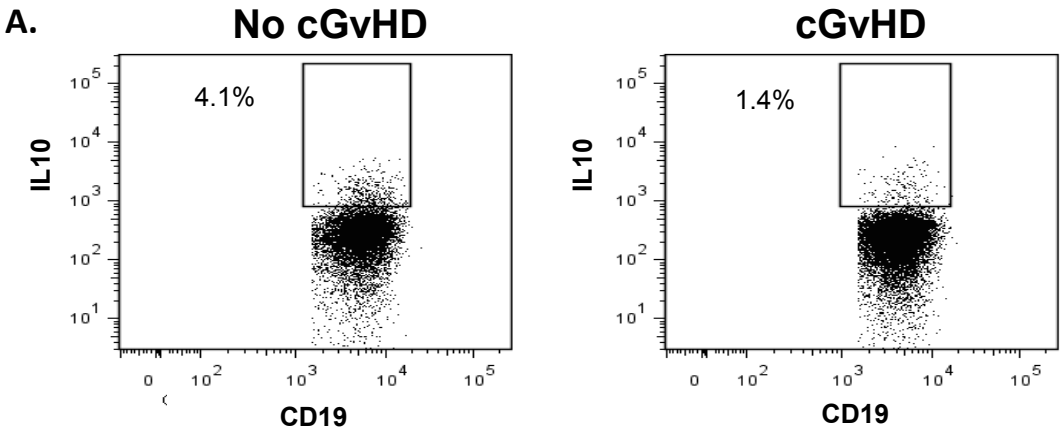
V.2.2.1 B cells from cGVHD patients have impaired in vitro IL10 responses to CD154 stimulation in comparison with "no GVHD" and HC groups

I demonstrated, in **Chapter IV**, that stimulation of PBMC with CD154-expressing L cells for 48 h yields the highest frequency of IL10 production by CD19$^+$ B cells. In contrast, stimulation with L cells and anti BCR led to significantly lower level of IL10 production by CD19$^+$ B cells in keeping with their proposed pro-inflammatory action.

Thawed PBMC were rested for 6 hours, followed by co-incubation with irradiated L cells at a ratio of 1 to 10 for 48 h (PBL condition). IC cytokine staining for IL10 production by CD19$^+$ B cells was performed as outlined in **section II.8.1**. Post-transplant patients without cGVHD had comparable frequencies of IL10$^+$ CD19$^+$ B cells to healthy controls [1.72%± 0.45 (0.8-5.52) vs. 2.16%± 0.36 (1.3-5.5), p=0.44]. However, in the cGVHD group, a significantly smaller fraction of gated CD19$^+$ B cells were IL10 positive,

compared to patients with “no cGVHD” and HC, [1.02%± 0.17 (0.22-2.26) vs.1.72%± 0.45 (0.8-5.52) vs. 2.16± 0.36 (1.3-5.5), p=0.001] respectively; **figure (V-1A and V-1B).**

When PBMC were stimulated with both L cells and anti-BCR for 48 h (PBXL), there was no difference in the frequencies of $IL10^{+}$ $CD19^{+}$ B cells among cGVHD, “no GVHD” and HC, [0.6± 0.18 (0.09-2.1) vs. 0.97± 0.29 (0.15- 3.01) vs. 0.75± 0.28 (0.5-1.98), p=0.4] respectively; figure (1C). However, cGVHD patients had significantly lower absolute numbers ($IL10^{+}$ $CD19^{+}$ B cell per µL) compared with “no GVHD” and HC, [0.32± 0.35 (0.00-3.01) vs. 3.04± 1.3 (0.6- 17.9) vs. 1.89± 1.9 (1.02- 11.4), p=0.007] respectively; **figure (V-1C)**. Absolute number of total $CD19^{+}$ B cells was calculated by multiplying the respective percentage obtained by flow cytometry by the absolute lymphocyte number (cell/µl) obtained from same day diagnostic FBC.



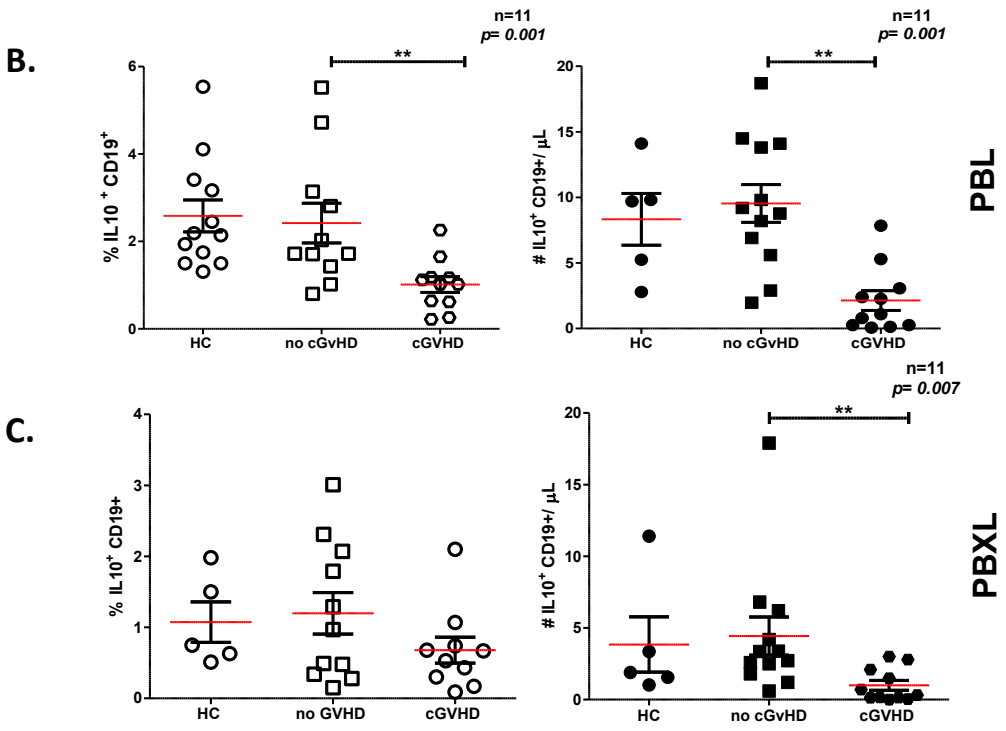


Figure V-1: cGVHD is associated with lower IL10 production

B cells from cGVHD patients produce less IL10 compared to patients without cGVHD and healthy controls. PBMC from cGVHD, no cGVHD and healthy controls (HC) were incubated with L cells alone or with L cells and anti-BCR for 48 hours. PMA, Ionomycin and BFA were added for the last 6 hours of culture and cells were analysed for the expression of CD19, CD3, and PI and intracellularly stained for IL10. **A**. Representative FACS plots of IL10 IC staining in gated $CD19^+$ B cells from cGVHD and no cGVHD patients. Frequencies of $IL10^+$ $CD19^+$ B cells were assessed relative to isotype controls. **B.** cGVHD patients had lower frequencies and absolute numbers of $IL10^+$ B cells when stimulated with L cells compared to patients without cGVHD patient and healthy controls. **C**. Stimulation with L cells and anti-BCR was associated with lower numbers of $IL10^+$B cells in cGVHD patients compared with patients without cGVHD and HC.

Therefore, this observation suggests that cGVHD is associated with impaired IL10 production by B cells. However, in the absence of a specific marker for Bregs, it is not clear whether this represents a numerical defect in Bregs or a functional deficit in IL10 production by B cells.

V.2.2.2 B cells from cGVHD patients produce Less IL10 in response to stimulation with CD40L

In addition to the overall reduced in vitro IL10 responses presented in **V.2.2.1**, cGVHD patients failed to exhibit the expected higher L10 production observed when B cells are stimulated with CD154 alone; PBL, compared to combined stimulation, PBXL. In cGVHD patients, the frequency and absolute cell numbers of $IL10^{+}$ $CD19^{+}$ B cells were not significantly lower in PBXL compared to PBL [0.6%± 0.18 (0.09- 2.01%) vs. 1.02%± 0.18 (0.22- 2.26%), p=0.14] and [$IL10^{+}$ $CD19^{+}$ B cell per µL, 0.32± 0.35 (0.0- 3.01) vs. 1.09± 0.75 (0.07-7.84), p=0.23] respectively . Conversely, B cells from patients without GVHD mounted a significant increase when stimulated with L cells alone compared to L cells in the presence of anti-BCR, in frequencies, [0.98%± 0.27 (0.15-3.01) vs. 1.87%± 0.43 (0.8-5.25), p=0.023] and cell counts [$IL10^{+}$ $CD19^{+}$ B cell per µL, 3.04± 1.3 (0.6-017.9) vs. 8.98± 1.44 (1.97- 18.7), p=0.009] respectively, comparable to that observed in HC; figure **(V-2A and V-2B).**

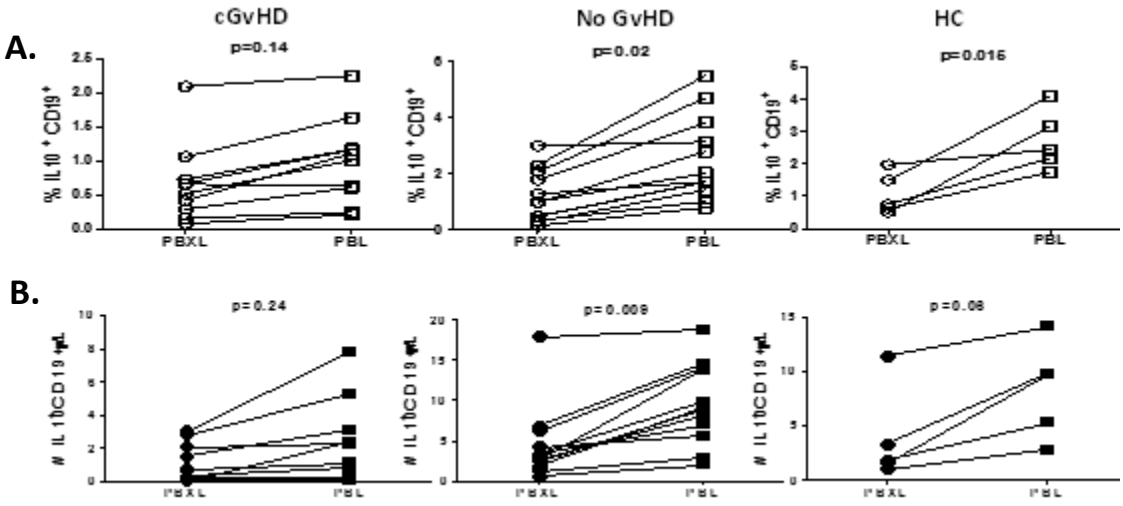


Figure V-2: B cells from cGVHD patients produce Less IL10 in response to stimulation with CD40L

A. Frequency **B**. absolute number of $IL10^+$ B cells significantly higher when stimulated with L cells alone compared with L cells and anti-BCR in no cGVHD patients and healthy controls. There was no significant difference in the frequencies or numbers of $IL10^+CD19^+$ B cells when stimulated with L cells alone compared with L cells and anti-BCR. All values are expressed as mean± SEM; n=11; the results were compared with two-tailed nonparametric t test analysis, $^{**}P<0.01$.

Failure to increment $IL10^+$ B cells upon stimulation with L cells alone in cGVHD samples suggests that either IL10 capable B cells are numerically or functionally reduced or are less responsive to CD154 stimulation analogous to SLE under-responsiveness to CD40 activation.

V.2.3 Chronic GVHD patients have comparable relative IgM memory B cell population

Since $IL10^+$ B cells are enriched within the IgM memory B cells, we analysed the frequencies of IgM memory B cells in post-transplant samples from patients with and

without cGVHD. As presented in **Chapter VI**, IgM memory B cell frequencies are lower in both post-HSCT patient groups compared with healthy individuals. The absolute number of IgM memory B cells was not significantly different in cGVHD vs. no cGVHD patients (median count, 11 vs. 12.6 cell/µl; p=0.3), although the former had a relatively higher proportion of IgM memory B cells (median percentage 5.1% vs. 3.1%; p=0.03). However, active cGVHD patients, on the other hand, had a smaller transitional B cell population compared with "no GVHD" patients (median 0.5 vs. 22.3 cell/µl; p=0.0003). This observation suggests that B cells with the capacity to produce IL10 may be numerically deficient in cGVHD; however, in view of the lack of a specific cell marker, it is not possible to rule out a specific deficiency of IL10 capable B cells. The other possibility, if we assume number sufficiency, is that these cells are functionally defective.

V.2.4 IFNγ$^+$ CD4$^+$ T cells are increased in post-transplant patients

As presented in **Chapter IV**, IL10$^+$ B cells specifically can suppress IFNγ production by CD4$^+$ T cells in vitro. To assess the relevance of this observation directly ex-vivo, PBMC from patients with or without cGVHD were stimulated in vitro and the frequencies of IFNγ$^+$ CD4$^+$ T cells assessed. As previously reported, the frequency of IFNγ$^+$ CD4$^+$ T cells was significantly higher in post-transplant patients compared to HC (Nakamura et al., 2005). However, patients with or without cGVHD had similar frequencies of IFNγ$^+$ CD4$^+$ T cells 22.3%± 4.13 (4.2- 44.13) vs. 24.9%± 4.6 (7.2-60.7) p=0.63, **figure (V-3A and V-3B).** Similarly, there was no statistical difference in the absolute number of IFNγ$^+$ CD4$^+$ T cells between the two post-transplant groups; p=0.16, **figure (3 C).**

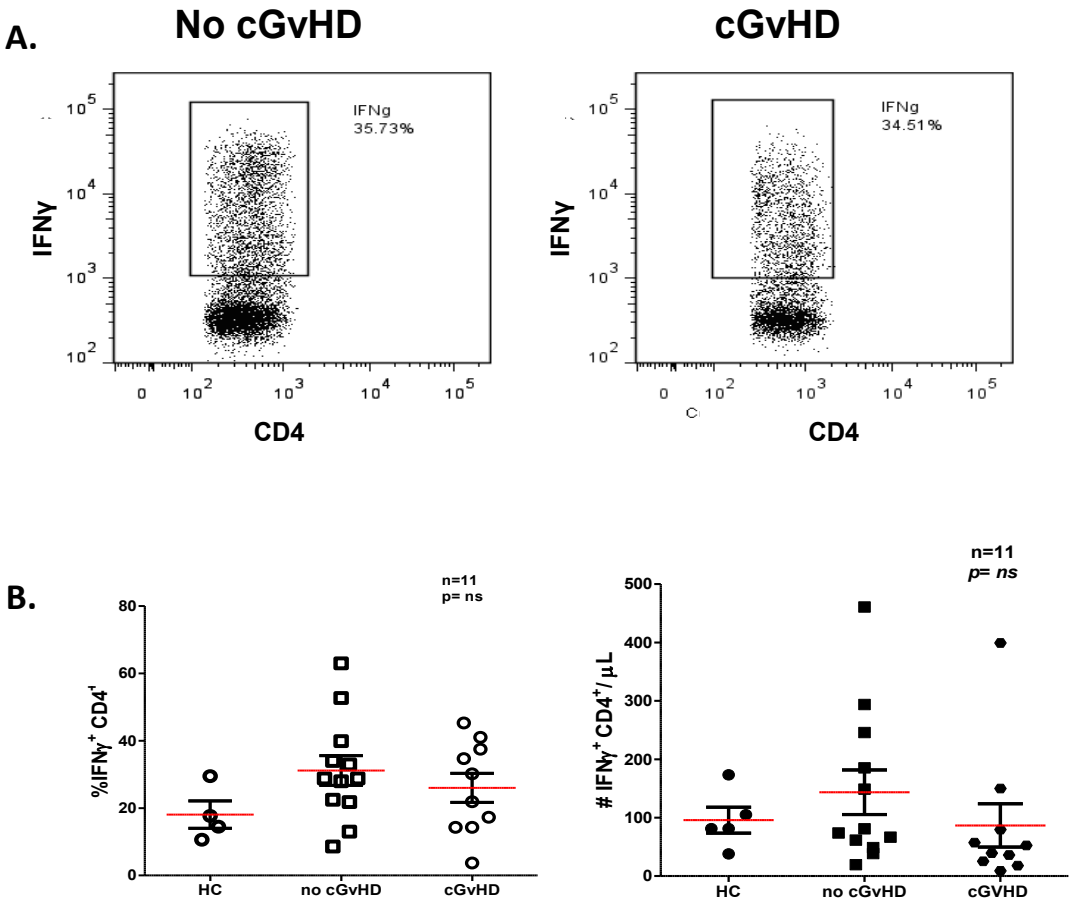


Figure V-3: IFNγ⁺ CD4⁺ T cells post SCT

CD4⁺ T cells from patients with and without cGVHD produce comparable amounts of IFNγ. PBMC from cGVHD, no cGVHD and healthy controls (HC) were stimulated with PMA and Ionomycin for 6 hours. Cells were surface stained for the expression of CD4, PI and intracellularly stained for IFNγ. **A**. Representative FACS plots of IFNγ IC staining in gated CD4⁺T cells from cGVHD and no cGVHD patients. Frequencies of IFNγ⁺ CD4⁺ T cells were assessed relative to unstimulated controls. **B**. CD4⁺ T cells from cGVHD produced comparable frequencies and absolute numbers of IFNγ⁺ CD4⁺ T cells compared with no cGVHD patients and healthy controls. All values are expressed as mean± SEM; n=11; the results were compared with two-tailed nonparametric t test analysis.

V.2.5 Active cGVHD is characterised by increased IFNγ$^+$ CD4$^+$ T cell / IL10$^+$ B cell ratio

The frequencies of IFNγ$^+$ CD4$^+$ T cells were not significantly different in patients with or without cGVHD. In view of the reduced IL10$^+$ CD19$^+$ B cell population observed in cGVHD patients, I expected a relative increase in IFNγ$^+$ effector CD4$^+$ T cell/IL10$^+$ B cell ratio. Therefore, I calculated the ratios of IFNγ$^+$ CD4$^+$ T cell/ IL10$^+$ B cell (IFNγ/IL10) frequencies and absolute numbers for each patient groups.

As anticipated, cGVHD patients had a significantly higher IFNγ/IL10 ratio (both frequencies and absolute counts), than "no GVHD" patients; [frequency ratio: 17.15±12.35 (9.8- 152.7) vs. 13.95± 2.6 (2.7- 35.5); p= 0.06 and cell/µL: 33.8± 137.1 (1.83- 1496) vs. 11.76± 11.3 (1.37- 143); p=0.046) respectively, **figure (V-4)**.

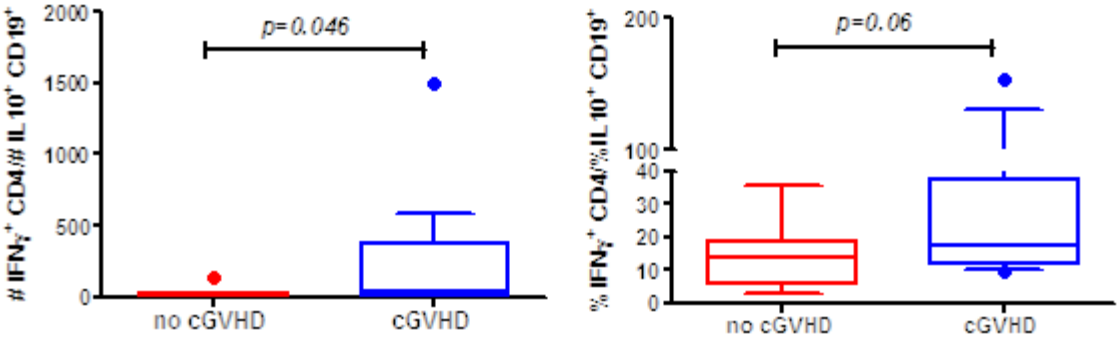


Figure V-4: IFNγ$^+$CD4$^+$/IL10$^+$ CD19$^+$ ratio in patients with and without cGVHD

Box and whiskers plots comparing the ratio of IFNγ$^+$CD4$^+$/IL10$^+$ CD19$^+$ absolute cell numbers (#) and IFNγ$^+$CD4$^+$/IL10$^+$CD19$^+$ frequencies. All values are expressed as mean± SEM; n=11; the results were compared with two-tailed nonparametric t test analysis.

These results imply that on average for every 12 IFNγ^{+} effector T cells one regulatory B cell is available in absence of cGVHD, whereas in patients with cGVHD only one regulatory B cell is available for every 34 effector CD4^{+} T cells. An imbalance between the regulatory and pro inflammatory networks is therefore present in cGVHD patients. This imbalance appears to be the result of a deficient regulatory network, as opposed to increased pro-inflammatory effector mechanisms.

V.3 Discussion

Chronic GVHD resembles autoimmunity both clinically and in terms of its pathogenesis. It has been shown that Tregs are numerically and functionally impaired in cGVHD, which in turn may contribute to impaired self- tolerance and survival of autoreactive effector T cells (Matsuoka et al., 2010).

Regulatory B cells have been recognised as powerful regulators of the immune response with a increasingly identified role in human autoimmune diseases (Mauri and Bosma, 2012). No data exist on the potential role of regulatory B cells in GVHD. Studying this subset may provide insight into the pathophysiology of cGVHD and may have implications for its treatment.

In my thesis, I have shown reduced frequencies and absolute numbers of IL10 producing B cells in the PB of patients with cGVHD compared with patients without GVHD and HCs. Furthermore, cGVHD patients failed to produce the expected increment, in both $IL10^{+}$ B cell frequencies and numbers, when stimulated with L cells alone over dual stimulation. These results suggest either a numerical deficiency in IL10 producing B cells or a defect in B cell response to CD154 stimulation. A suboptimal response to activation with L cells alone may indicate a deficit in CD40 signalling or reduced CD40 expression on B cells. It has been reported that regulatory B cells from SLE patients are refractory to CD40 engagement; i.e. B- T cell interaction, with reduced phosphorylation of STAT3 downstream of CD40. In SLE, alternative stimulation with CpG restored a normal IL10 response (Blair et al., 2010). I was not able to quantify CD40 expression on B cells or stimulate B cells from cGVHD patients with CpG in view of the limited patient sample

availability. There is some evidence that SCT recipients accumulate less Antigen-driven T cell-dependent somatic mutations. . Therefore, an intrinsic B cell deficit or impaired T-B cell cross talk, rather than a defect in T cell help may explain this observation (Glas et al., 2000).

The reduced $IL10^+$ B cells in cGVHD may represent quantitative or functional deficiencies. Low inducible $IL10^+$ B cell number in cGVHD could be the direct result of lower frequencies of IL10 producing B cells or their precursors. The fact that IgM memory B cells are normally maintained in the spleen (Weill et al., 2009) and that cGVHD worsens the splenic hypofunction post SCT may further support this notion (Cuthbert et al., 1995). To further investigate a quantitative explanation and since $IL10^+$ B cells share a similar phenotype to IgM memory B cells, I compared IgM memory B cell frequencies in patients with or without cGVHD and HC. In our cohort and as previously reported by Dana Farber group (Sarantopoulos et al., 2009), chronic GVHD patients had relatively higher frequencies of IgM memory B cells compared with patients without cGVHD. This finding might suggest that there is no numerical deficiency of $IL10^+$ B cell in cGVHD. However, this conclusion cannot be fully supported since, in the absence of specific markers for $IL10^+$ B cells, analogous to FoxP3 in Treg, one cannot exclude a specific deficit of an $IL10^+$ B cell subset with a maintained overall IgM memory B cell population. Other studies have reported reduced IgM memory B cells in cGVHD (D'Orsogna et al., 2009, Hilgendorf et al., 2012); nonetheless, in our setting, a reduction in IgM memory B cells cannot be used to propose a quantitative deficit for IL10 producing B cells in cGVHD. Transitional B cells have been shown to possess IL10 dependent immunoregulatory

function (Blair et al., 2010). We observed a smaller population of transitional B cells in cGVHD patients which may contribute to the overall lower B cell production of IL10. Alternatively, a functional deficit in IL10 production by B cells is another possibility. However, the lack of a specific marker renders exploring this proposition not possible at present.

I assessed IFNγ^{+} CD4^{+} T cells in the study groups as I detected a unique inhibitory effect of IgM memory B cells on these cells. In agreement with a previous report (Nakamura et al., 2005), I found no significant difference in IFNγ^{+} CD4^{+} T cells in cGVHD and "no GVHD" patients. However, the ratio of IFNγ^{+} CD4^{+} T cells to IL10^{+} B cells was significantly higher in cGVHD patients, which implies an imbalance in the regulatory and effector systems. The proposed imbalance adds to the recognised association of Treg imbalance with development of cGVHD. A smaller Treg population relative to total CD4 cells, which by default includes effector cells, predisposes SCT patients to cGVHD (Matsuoka et al., 2010). My finding supports a wider regulatory network imbalance, affecting both Breg and Tregs, and extends the current investigation to assessing factors that are common to both lineages such as post-transplant environment and cell reconstitution.

Reconstitution of Treg after myeloablative conditioning is characterised by reduced CD31^{+} thymic naïve Treg and predominance of peripherally expanded memory Treg (Matsuoka et al., 2010). CD4 lymphopaenia post SCT is associated with rapidly expanding Treg, with a subsequent decline attributable to their apoptosis. The post SCT environment, with damage inflicted on lymphoid generation sites, could explain this pattern of reconstitution which in some recipients may favour the preferential recovery

of pro-inflammatory effector cells. Likewise, B cell regeneration sites such as the bone marrow, spleen and lymph nodes are damaged by the SCT conditioning (Sale et al., 1992). Moreover, cGVHD further worsens splenic hypofunction seen in SCT patients (Cuthbert et al., 1995). There is experimental evidence to support that cGVHD-associated damage to bone marrow niche further impairs B cell lymphopoiesis (Shono et al., 2010).

I speculate that the reduction of IL10 capable B cells in cGVHD is the cumulative result of B cell regeneration in an environment favouring the disease. Early prophylactic intervention with adoptive immunotherapy might therefore tip the balance in favour of immune regulation and may therefore be beneficial. One way of achieving this could be through infusion of donor lymphocytes enriched with regulatory cells, for instance IgM memory cells, at an early stage which minimises damage and predisposition to cGVHD development. Obviously, this would need to be confirmed in an animal model and subsequently in the context of a clinical study.

Chapter VI. GVHD and B cells

VI.1 Introduction:

Chronic graft versus host disease (cGVHD) remains a major limiting factor of stem cell transplantation (SCT) success. Chronic GVHD occurs in 30 to 65% of allogeneic SCT recipients with a 5-year mortality rate of 30 to 50% (Blazar et al., 2012b). At present, the immunopathophysiology is not fully understood, which hinders the development of effective treatments. Traditionally GVHD has been approached as a T cell mediated process and the current therapies reflect this understanding (Inamoto and Flowers, 2011). Recently, increasing evidence has supported the involvement of B cells in cGVHD pathophysiology. The occurrence of GVHD in the settings of immune disparity and the identification of auto-antibodies implies either a direct breakdown in the B cell tolerance process or T to B cell cross-talk (Kapur et al., 2008, Glas et al., 2000). It has been shown that cGVHD patients have lower bone marrow B cell precursors compared with transplant patients without cGVHD (Storek et al., 2001, Sanchez-Garcia et al., 2006). Chronic GVHD appears to disturb the post-transplant B cell reconstitution in which it is characterized by blunt recapitulation with a lower circulating B cells count (Storek et al., 1993). Generally, the recovery of memory B cells following allogeneic SCT and building the immunoglobulin repertoire may need many years to reach normality (Storek et al., 1997, Avanzini et al., 2005). Multiple studies, however, have demonstrated that cGVHD further disrupts the memory population size. The total number of $CD27^+$ B cells was found to be significantly lower in cGVHD patients compared with their no cGVHD counterpart (D'Orsogna et al., 2009, Hilgendorf et al., 2012, Greinix et al., 2008, Corre et

al., 2010). In addition, memory B cells derived from the cGVHD inflammatory environment are expectedly activated and at a higher metabolic state (Allen et al., 2012). The high BAFF levels required during the reconstitution period, in the presence of a relatively smaller naïve B cell population, promotes the survival of autoreactive B cells (Sarantopoulos et al., 2009, Sarantopoulos et al., 2007). Increased $CD21^-$ B cells in the peripheral blood of active cGVHD patients were reported. This population was referred to as immature/ transitional B cells based the lack of CD21 expression (Greinix et al., 2008). Other groups, however, observed decreased transitional B cells in cGVHD patients (Hilgendorf et al., 2012, Storek et al., 1993).

Classical memory B cells are mainly characterised by the expression of CD27, a member of the TNF family, and possess somatically mutated V genes. However, $CD27^-$ isotype switched memory B cells have been described (Sanz et al., 2008). Of special interest is epithelial-associated lymphoid tissues residing FCR $L4^+$ expressing B cells with activated phenotype. Although this subset lacks CD27 expression, it is still classified as memory B cells by virtue of the class switch and somatic hypermutation. FCR $L4^+$ B cells respond to cytokines and CD40L ligation rather than BCR crosslinking, suggesting a potential regulatory function (Ehrhardt et al., 2005, Ehrhardt et al., 2008).

Recently, a similar $CD21^{-/lo}$ B cell population was described in the peripheral blood of chronic HIV viraemic patients and individuals living in malaria endemic areas. The subset had relatively higher expression of inhibitory and homing receptors compared with naïve and classical memory B cells (Moir et al., 2008, Weiss et al., 2009). Likewise, this population had lower proliferative potential and reduced ability to differentiate into

antibody secreting cells. The inhibitory profile was similar to that of exhausted $CD8^+$ T cells observed in chronic viral infections and therefore dubbed exhausted B cells (Moir et al., 2008). Interestingly, exhausted B cells were enriched within HIV specific B cells.

In my project, I studied the frequency and phenotype of $CD21^-$ B cells in patients with cGVHD. I found that patients with cGVHD had significantly higher frequencies of $CD21^-$ B cells and that the majority of circulating $CD21^-$ B cells in these patients is phenotypically and functionally similar to exhausted B cells

VI.2 Results:

VI.2.1 Patient Characteristics:

All patient samples were collected after written informed consent according to local policy guidelines at the Hammersmith Hospital, Imperial College NHS trust, and in accordance with the declaration of Helsinki.

Blood samples were obtained from 33 patients who had undergone allogeneic HSCT between 1998 and 2011 and were six months or more post HSCT. This cohort included patients who underwent reduced intensity or myeloablative conditioning SCT and receiving bone marrow or mobilised peripheral blood stem cell grafts. All patients were more than six months post SCT with no evidence of primary disease relapse. Patients receiving high-dose steroids (> 50 mg prednisone) at the time of sampling or had received rituximab for any indication were excluded. Chronic GVHD status at the time of sample collection was classified according to documented clinical and laboratory data using National Institutes of Health (NIH) cGVHD consensus criteria and modified Seattle

criteria for limited versus extensive disease. Of the 30 assessable patients, thirteen patients had “active cGVHD” and two had “inactive cGVHD”. Inactive cGVHD was defined as patients with a history of cGVHD who were off immune-suppressive therapy and had quiescent disease at the time of analysis. Fourteen patients never developed cGVHD after HSCT. None of the “no cGVHD” patients were receiving immunosuppressive therapy at the time of sample collection. A total of 16 patients were assigned to the “cGVHD” group 14 active and 2 with inactive cGVHD and 14 to the “no cGVHD.

Median time to cGVHD was 335 days (180-2947) after transplantation for patients with cGVHD compared with 481 days (349-4000) to sampling after transplantation for patients with no cGVHD (p= 0.24). Other clinical characteristics are shown in **table (VI-1).** I also studied 11 healthy controls. The rest of cGVHD patients’ characteristics are shown in **table (VI-2).**

Table VI-1: Patient characteristics

	All (N=33)	no cGVHD (N=14)	cGVHD (N=19)
Median age in years {range}	47 {25-68}	52.8 {25-65}	46.5 {37-68}
Sex			
Male	24 (73)	8 (57)	16 (84)
Female	9 (27)	6 (43)	3 (16)
Diagnosis			
Acute leukemia	11(33)	6 (42.8)	6 (31.5)
Chronic myeloid leukemia	11 (33)	5 (35.7)	5 (26.3)
Malignant lymphoma	3 (10)	1 (7)	2 (10.5)
Cutaneous T cel lymphoma	4 (12)	1 (7)	3 (15)
Others	4 (12)	1 (7)	3 (7)
Disease status at transplantation			
Standard risk	19 (58)	7 (40)	12 (63)
High risk	14 (42)	7 (50)	7 (37)
Conditioning regimen			
Myeloablative conditioning	17 (51.5)	7 (50)	10 (53)
Reduced intensity conditioning	16 (48.5)	7 (50)	9 (47)
Stem cell donors			
Related	16 (48.5)	3 (21.5)	13 (68)
Unrelated	17 (51.5)	11 (78.5)	6 (32)
HLA-identical	31 (94)	12 (86)	19 (100)
HLA-mismatched	2 (6)	2 (14)	0 (0)
Stem cell source			
Bone Marrow	6 (18)	4 (28.5)	2 (10.5)
Peripheral Blood Stem Cells	27 (82)	10 (71.5)	17 (89.5)
Median number of $CD34^+$ cells $x10^6$ /kg transplanted	8.6 {0.79-12.77}	8.7 {0.79-12.2}	8.7 {2.8-12.77}
Post-transplant immunosuppressive prophylaxis			
Cyclosporine-Methotrexate	14 (42)	6 (42)	8 (42)
Cyclosporine-Methotrexate- Campath	13 (39)	4 (28.5)	9 (47)
Cyclosporine-Methotrexate-ATG	4 (12)	4 (28.5)	0 (0)
other	2 (6)	0 (0)	2 (11)
Acute GVHD			
Grade 0	14 (42)	8 (58)	6 (31)
Grades I-II	13 (39)	5 (35)	8 (42)
Grades III-IV	6 (18)	1 (7)	5 (27)
Donor lymphocyte infusion (DLI)			
Received DLI	16 (48)	4 (28.5)	12 (63)
Received no DLI	17 (52)	10 (71.5)	7 (27)

Table VI-2: chronic GVHD characteristics

	cGVHD (N=19) number (percent)
Onset type of chronic GVHD	
De novo	9 (47.5)
Quiescent	8 (42)
Progressive	2 (10.5)
cGVHD status	
Resolved	2 (10.5)
Active	17 (89.5)
cGVHD organ involvement	
Skin	15 (78)
Eyes	6 (31.5)
Oral mucosa	4 (21)
Liver	5 (26)
Lungs	2 (10.5)
Musculoskeletal	1 (5)
1 or 2 organs	10 (52.5)
> 2 organs	9 (47.5)
Overall cGvHD grade	
Mild	5 (26.3)
Moderate	7 (36.8)
Severe	7 (36.8)
Immunosuppressive therapy	
None	3 (16)
MMF+ECP+/- steroids	11 (58)
Others	5 (26)

VI.2.2 B cell subsets in cGVHD:

VI.2.2.1 Chronic GVHD patients have lower number of circulating B cells:

Consistent with previous reports (Corre et al., 2010), cGVHD patients had significantly lower lymphocyte counts compared with patients without cGVHD and healthy controls (median 1300 vs. 2050 vs. 1700 cell/µl; p=0.008 and p= 0.01 respectively); **figure (1) A**. Nevertheless, not all cGVHD patients met the clinical diagnosis of lymphopenia i.e. count <1000/ µl.(Mitrovic et al., 2012) Similarly, cGVHD patients had lower numbers of $CD19^{+}$ B cells compared with no-GVHD counterparts (median 216 vs. 378 cell/µl; p=0.02); **figure (VI-1) B**. There was no difference between B cell numbers in cGVHD patients and healthy controls.

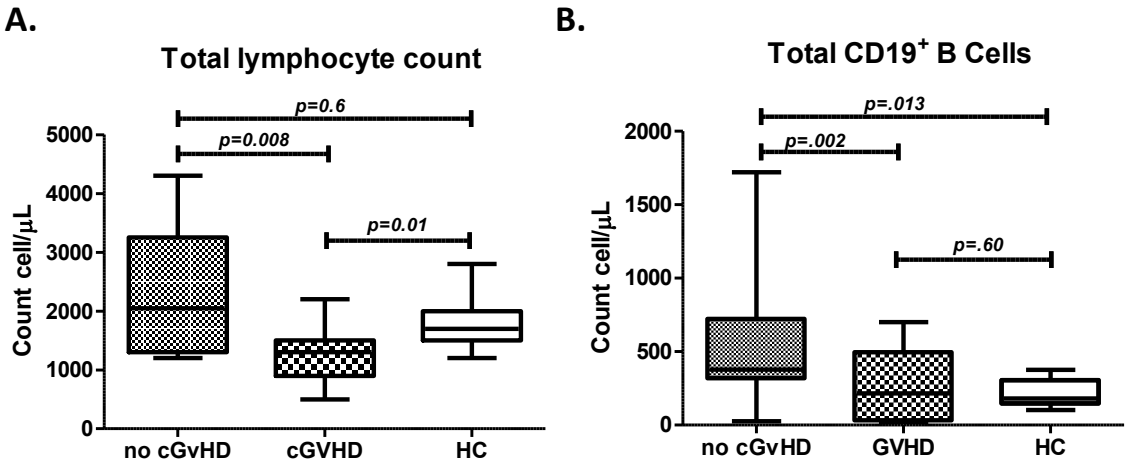


Figure VI-1: lymphocyte and $CD19^{+}$ B cell counts among patients with and without cGVHD and HC

Box plots, showing medians, 25th and 75th percentiles and ranges, comparing total lymphocyte count **(A)** and total $CD19^{+ve}$ B cells **(B)** between 16 cGVHD patients, 14 "no cGVHD" patients and 11 healthy controls.

VI.2.2.2 Chronic GVHD patients have lower number of naive B cells:

I then examined the frequencies of B cell subsets in the post-transplant groups. Naïve B cells were defined as ($CD19^{+}IgM^{+}CD27^{-}$) B cells, total $CD27^{+}$ memory B cells were defined as ($CD19^{+}$ $CD27^{+}$) and further subdivided into IgM memory $CD19^{+}$ $CD27^{+}$ IgM^{+} B cells and class-switched memory B cells ($CD19^{+}$ $CD27^{+}$ IgM^{-}). To quantify transitional B cells and confirm other B cell subsets frequencies, I also performed the analysis using the markers CD24 and CD38. Transitional B cells were defined as $CD19^{+}CD24^{hi}CD38^{hi}$, naïve cells $CD19^{+}CD24^{+}CD38^{+}$; total memory B cells $CD19^{+}CD24^{hi}CD38^{-}$ and finally circulating plasmablasts as $CD19^{+}CD24^{-}CD38^{hi}$. In agreement with the described reduction of circulating naïve B cells (Sarantopoulos et al., 2009), active cGVHD patients had lower absolute number and frequencies of naïve B cells compared with the “no cGVHD” patients (median 158 vs. 338 cell/µl; p=0.03 and 77.7% vs.88.5%; p=0.01) respectively. There was no significant difference in naïve B cell number between cGVHD patients and healthy controls (median 158 vs. 149.2 cell/µl; p=0.8). Likewise, active cGVHD patients had a smaller transitional B cell population compared with both “no GVHD” patients and healthy controls (median 0.5 vs. 22.3 vs. 4.75 cell/µl; p=0.0003 and p= 0.0001 respectively). **Table (VI-3)** and **figure (VI-2)**

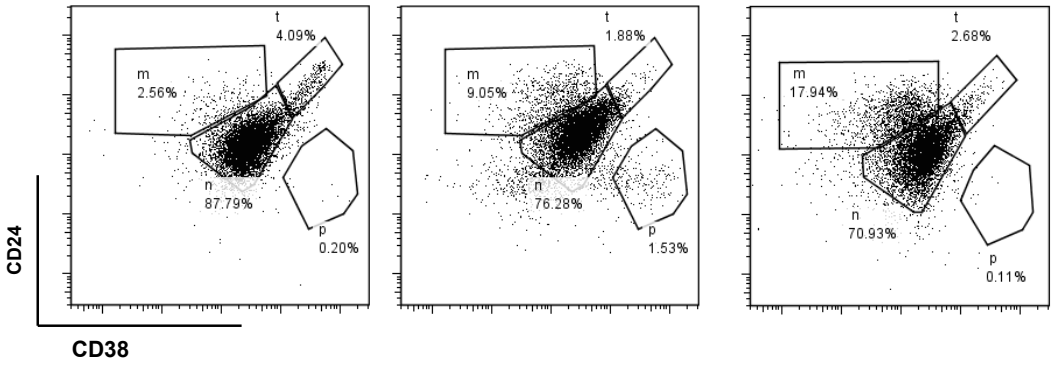


Figure VI-2: B cell subsets in "no GVHD", cGVHD and a HC

FACS plots demonstrating $CD19^+$ B cell in a representative "no GVHD" patient, cGVHD patient and a healthy control. Gated $CD19^+$ B cells were examined for CD24 and CD38 expression; transitional ($CD19^+CD24^{hi}CD38^{hi}$), naïve ($CD19^+CD24^+CD38^+$), memory ($CD19^+CD24^{hi}CD38^-$) and plasmablasts ($CD19^+CD24^-CD38^{hi}$).

VI.2.2.3 Chronic GVHD patients have higher percentage of $CD27^+$ B cells

cGVHD and "no GVHD" patients had a significantly lower absolute number and frequencies of total $CD27^+$ memory B cells compared with healthy individuals 22 vs. 33.5 vs. 63.6 cell/µl; p=0.012 and p=0.047 respectively and 15.6% vs. 6.6% vs. 25%, p=0.0004 and p=0.06 respectively. Although cGVHD patients had lower $CD27^+$ memory B cells compared with "no GVHD" patients , the difference was not significant median count 22 vs. 33.6 cell/µl; p=0.23. However, due to fewer $CD27^-$ B cells, cGVHD patients had a relatively higher proportion of circulating $CD27^+$ memory B cells 15.6% vs. 6.6%; p= 0.014. Further analysis of class-switched and IgM memory B cell subpopulations revealed that both B cell subgroups were also less frequent in each post-HSCT group compared with healthy individuals. Chronic GVHD patients tended to have lower number IgM^+ 11 vs. 12.6 cell/µl; p=0.3 and IgM^- 6.9 vs. 19.2 cell/µl; p= 0.23 respectively with relatively higher percentage IgM^+ 5.1% vs. 3.1%; p=0.03 and IgM^- 7.6% vs. 3.05%;

p=0.054 respectively of IgM^{-} $CD27^{+}$ B and IgM^{+} $CD27^{+}$ B memory cells compared with their "no GVHD" counterparts, **figure (VI-3) and table (3).**

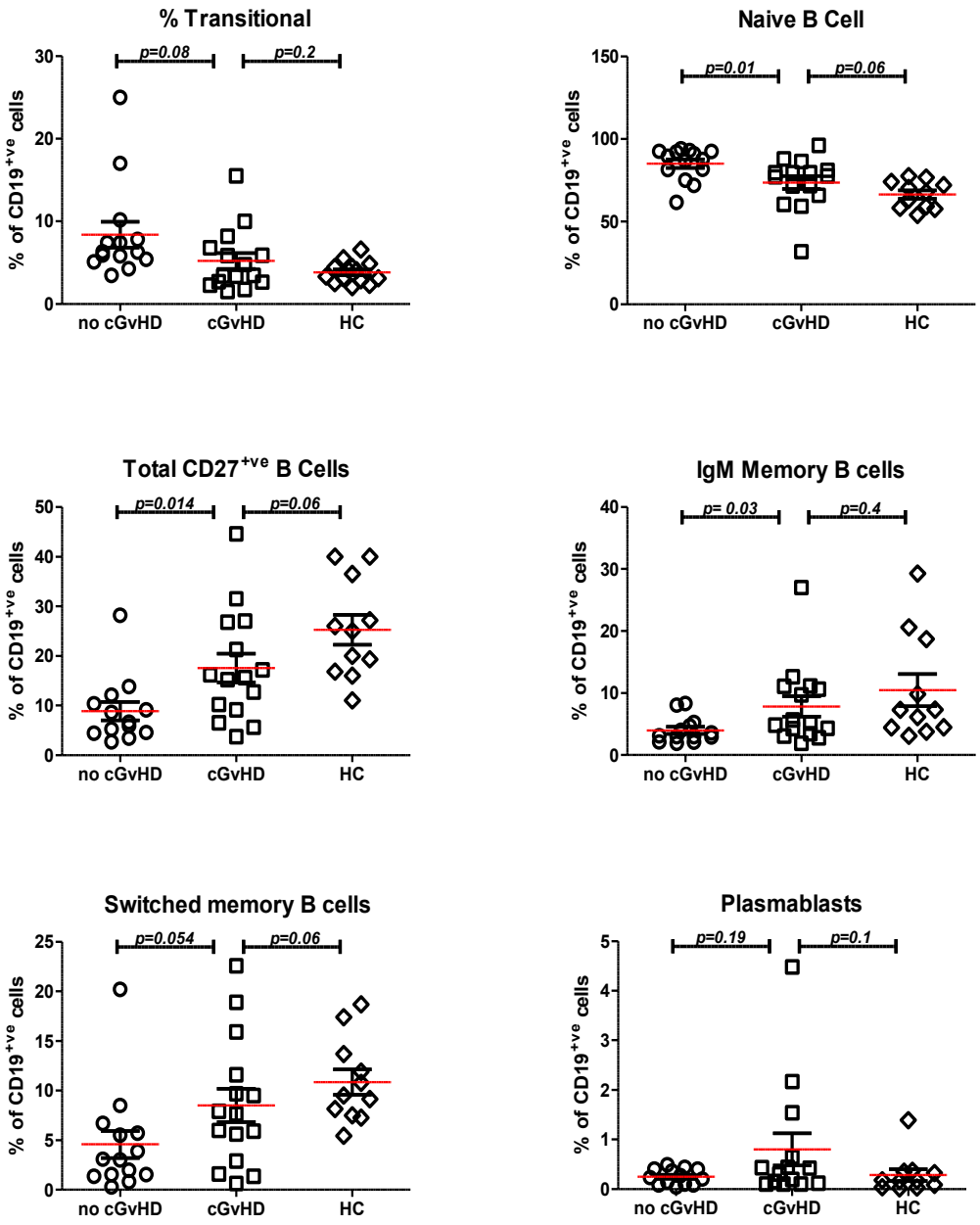


Figure VI-3: Comparison of B cell subset frequencies in "no GVHD" patients (n=14), cGVHD patients (n=16) and healthy controls (n=11)

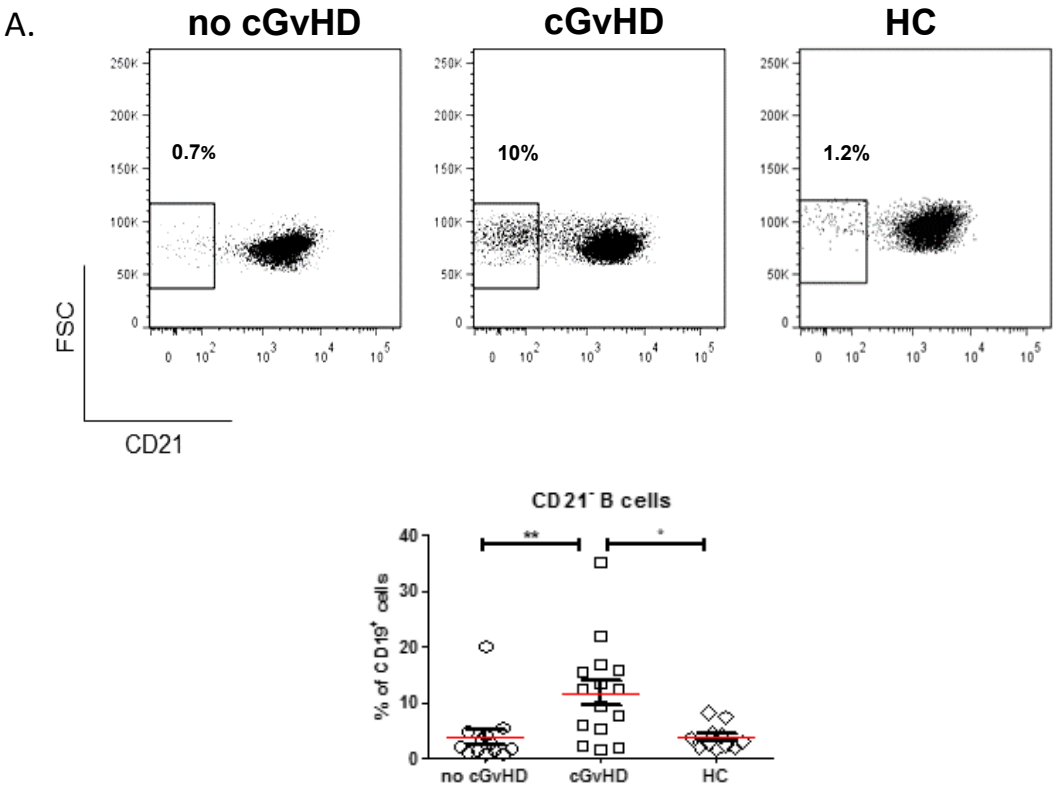
Table VI-3: Absolute counts of cell subsets in "no GVHD" patients (n=14), cGVHD patients (n=16) and healthy controls (n=11)

B cell subset (cell/μl)	No GVHD	cGVHD	Healthy Controls
Transitional	22.3	0.5	4.62
***P* value**	**0.0003**		**0.0001**
Naïve	338	158	149.2
P value	**0.01**		**0.03**
Total $CD27^+$	33.5	22	62.3
P value	**0.04**		**0.012**
IgM^+ $CD27^+$	12.59	11.08	14.41
P value	**0.31**		**0.32**
IgM^- $CD27^+$	19.22	6.97	26
P value	**0.023**		**0.03**
Plasmablasts	1	0.42	0.18
P value	**0.26**		**0.06**

VI.2.2.4 Chronic GVHD patients have higher frequencies of $CD21^-$ B cells:

An expanded population of $CD21^-$ B cells have been recently reported in active cGVHD patients (Greinix et al., 2008). We identified an expanded population of $CD19^+CD21^-$ B cells 12.2% (1.3%-34.9%) in patients with cGVHD. In contrast significantly fewer $CD19^+CD21^-$ B cells were present in the peripheral blood of patients without cGVHD [2.12%, (0.67%-20.1%); p=0.004] and healthy controls [3%, (1.5%- 8.15%); p=0.01]. Notably, the only patient without cGVHD with increased frequencies of $CD19^+CD21^-$ B cells had evidence of EBV reactivation at the time of analysis; **figure (4A).** The majority of $CD19^+CD21^-$ B cells in the peripheral blood of cGVHD patients expressed high levels

of CD20, lacked expression of CD10, CD27, CD24 and CD38 and expressed lower IgD and IgM compared with $CD21^{+}$ B cells and therefore, did not display the phenotype of transitional B cells, **figure (VI-2B and VI-2C)**; such a profile is similar to that of exhausted B cells recently described in HIV patients (Weiss et al., 2009).



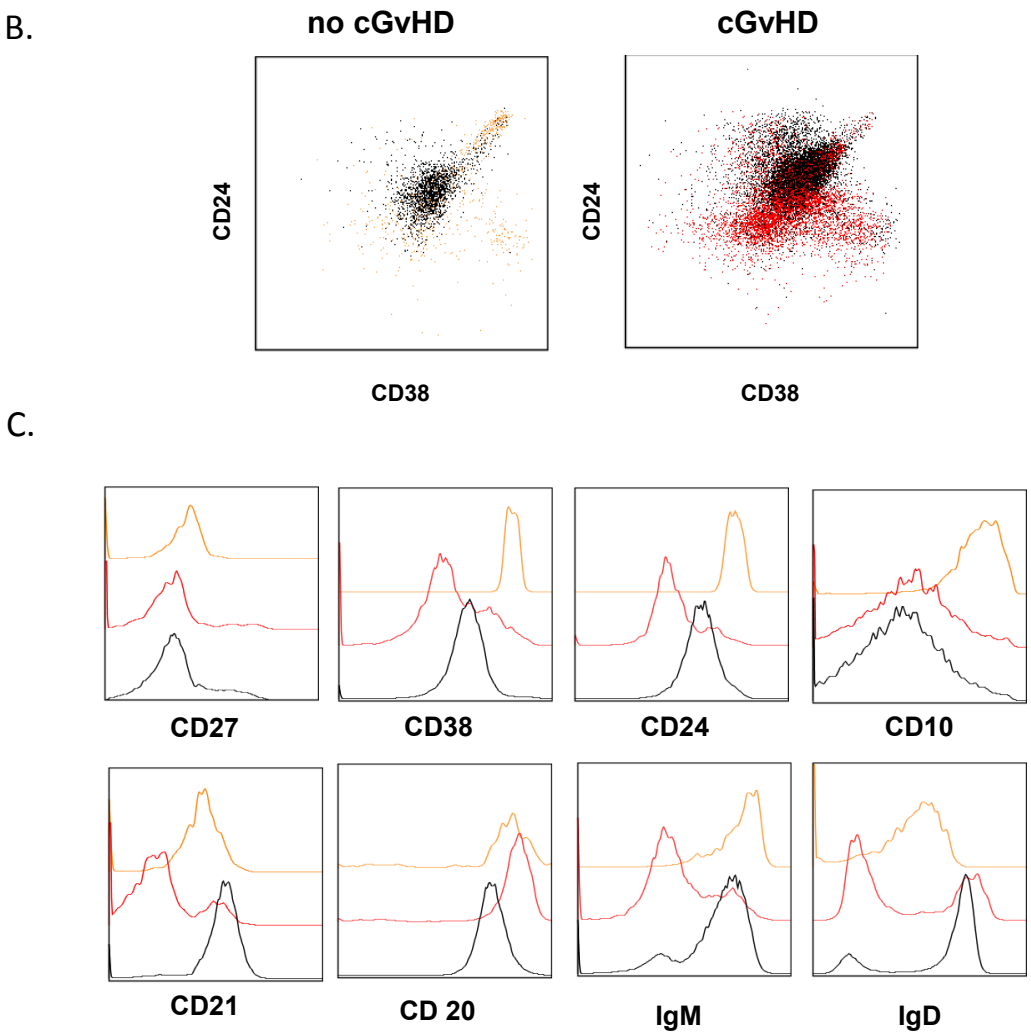


Figure VI-4: CD21 expression examination in cGVHD, no GVHD and HC

A. FACS plots of gated $CD19^+$ B-cell examining CD21 expression in a representative "no GVHD" patient, GVHD patient and a healthy control. Chronic GVHD patients (n=16) have significantly higher percentage of circulating $CD21^-$ B cells compared with "no GVHD" patients (n=14) and healthy controls (n=11) **B.** Colour-coded gating on $CD19^+$ B cells in representative "no GVHD" and active cGVHD patients; (Yellow) $CD19^+/CD21^-$ B cells of "no cGVHD" patient, (Red) $CD19^+/CD21^-$ B cells of "cGVHD" patient, (Black) total $CD21^+$ B cells. The majority of $CD21^-$ B cells (yellow) in "no cGVHD" fall in the $CD24^{hi}CD38^{hi}$ transitional B cell and $CD24^-CD38^{hi}$ plasmablast regions. Conversely, the majority of $CD21^-$ B cells in "cGVHD" case (Red) fall in $CD24^-CD38^-$ exhausted B cell and $CD24^-CD38^{hi}$ plasmablast regions. **C.** Phenotypic characterisation of $CD21^-$ B cells, transitional (Yellow) and exhausted B cells (Red) compared with $CD21^+$ B cells. Transitional B cells express high CD24, CD38 and IgM, positive for CD10, CD20 and IgD and negative for CD27. Exhausted B cells are negative for CD10 and CD27; express low CD24 and CD38 and variable IgM and IgD.

The remaining $CD21^-$ B cells in cGVHD patients were distributed among plasmablasts ($CD19^+$, $CD10^-$, $CD21^-$, $CD27^{hi}$), activated memory B cells ($CD19^+$, $CD10^-$, $CD21^-$, $CD27^+$) and immature/ early transitional B cells ($CD19^+$, $CD10^+$, $CD21^-$, $CD27^-$). Using CD10 expression as a marker of immature/ early transitional B cells within the $CD21^-$ population, cGVHD patients had fewer immature B cells compared to no GVHD patients (median percentage 3.15% vs. 9.34%; p=0.045), however the absolute counts were not statistically different in the two groups (median count 0.77 vs. 1.2 cell/µl; p=0.16).

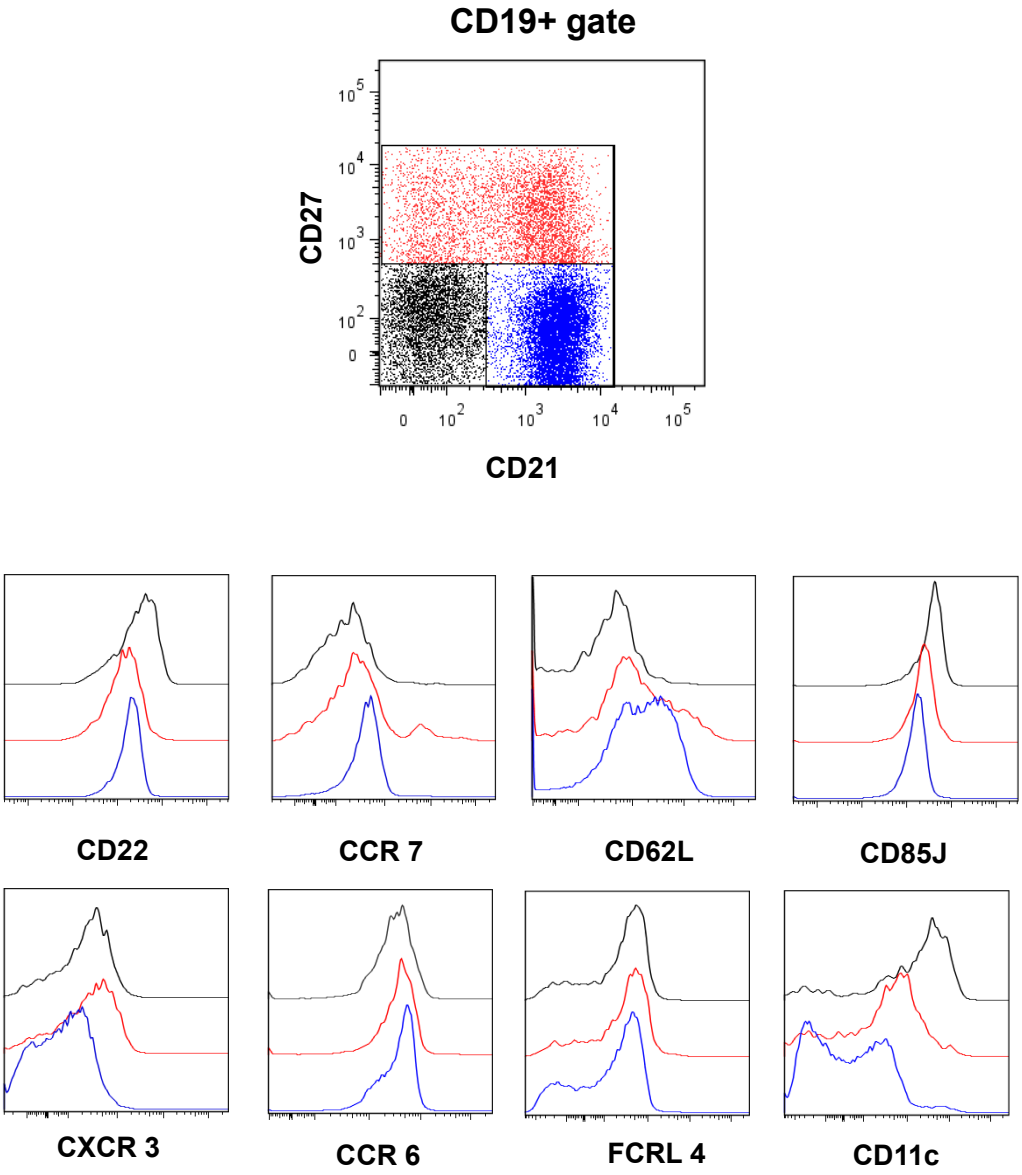
VI.2.2.5 Phenotypic characterisation of $CD21^-$ B cells in cGVHD patients:

Exhausted $CD21^-$ B cells have been shown to express an array of chemokine and inhibitory receptors a signature of exhaustion and tissue relocation (Weiss et al., 2009). Accordingly, I compared the expression of inhibitory receptors FCRL4, CD22 and CD85J and trafficking receptors CD11c, CXCR3, CCR6, CD62L and CCR7 on $CD19^+CD10^-CD21^-CD27^-$ B cell population with $CD19^+CD10^-CD21^+CD27^-$ naïve and $CD19^+CD10^-CD21^+CD27^+$ classical memory B cells.

$CD19^+CD10^-CD21^-CD27^-$ B cells of cGVHD patients expressed significantly higher levels of CD22 and CD85J compared with both naïve and classical memory B cells; **figure (VI-5)**. Although this subset also expressed a higher level of FCRL4 compared with naïve B cells, there was no significant difference in the expression of FCRL4 between the expanded $CD21^-$ B cells and classical memory B cells (p=0.02 and p=0.45 respectively). $CD21^-$ B cells in cGVHD patients expressed significantly higher CD11c and CXCR3

compared to naïve and classical memory B cells (p=0.008 and p=0.03 and p=0.007 and p=0.42 respectively). In contrast, the homing receptors CCR7 and CD62L were expressed at significantly lower levels on $CD21^-$ B cells compared with both classical memory and naive B cells (p=0.007 and p=0.008), indicating altered homing of this subset of B cells. Little difference was observed in the expression of CCR6 amongst the three B cell subsets; Figure 3 B. Overall, the inhibitory and homing profile of expanded $CD21^-$ B cells in cGVHD is similar to that described for the $^-$ exhausted B cells in HIV and in virus-specific $CD8^+$ T cell exhaustion in chronic viral infections (Moir et al., 2008, Moir et al., 2004, Weiss et al., 2009).

A.


CD19+ gate
CD27
CD21
CD22
CCR 7
CD62L
CD85J
CXCR 3
CCR 6
FCRL 4
CD11c

B.

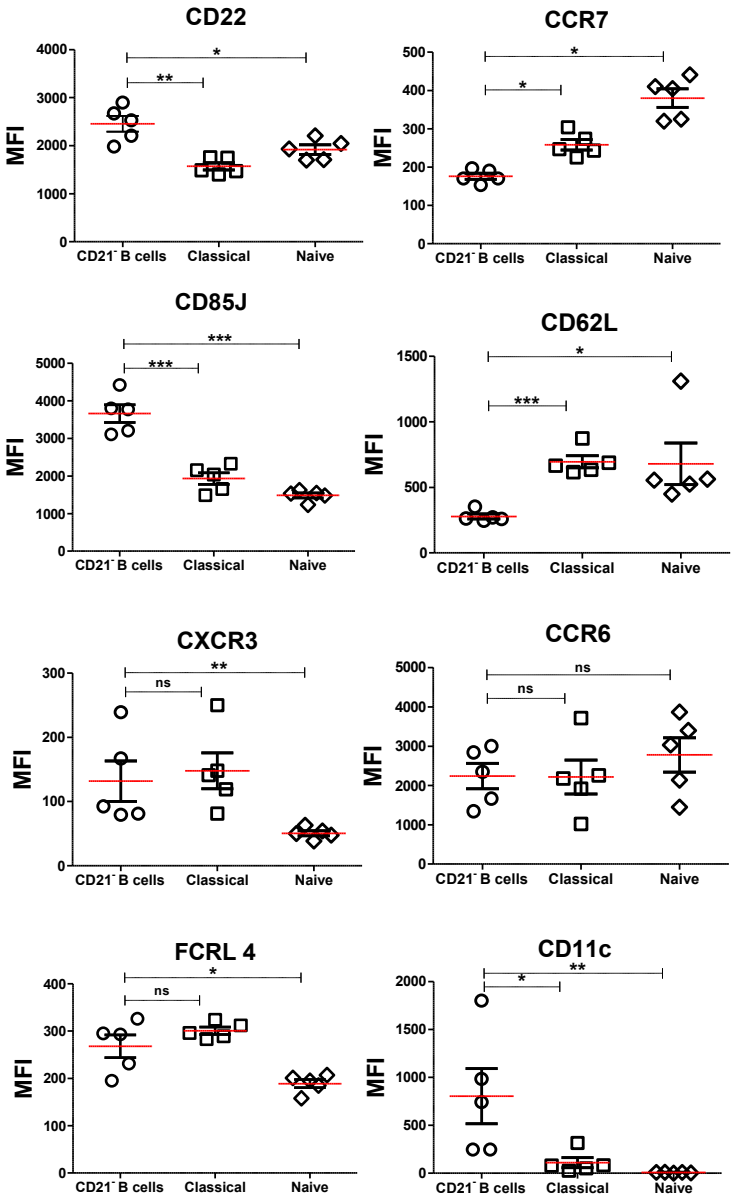


Figure VI-5: Inhibitory and chemokine receptor profile of naïve, classical memory and CD21$^-$ B cells

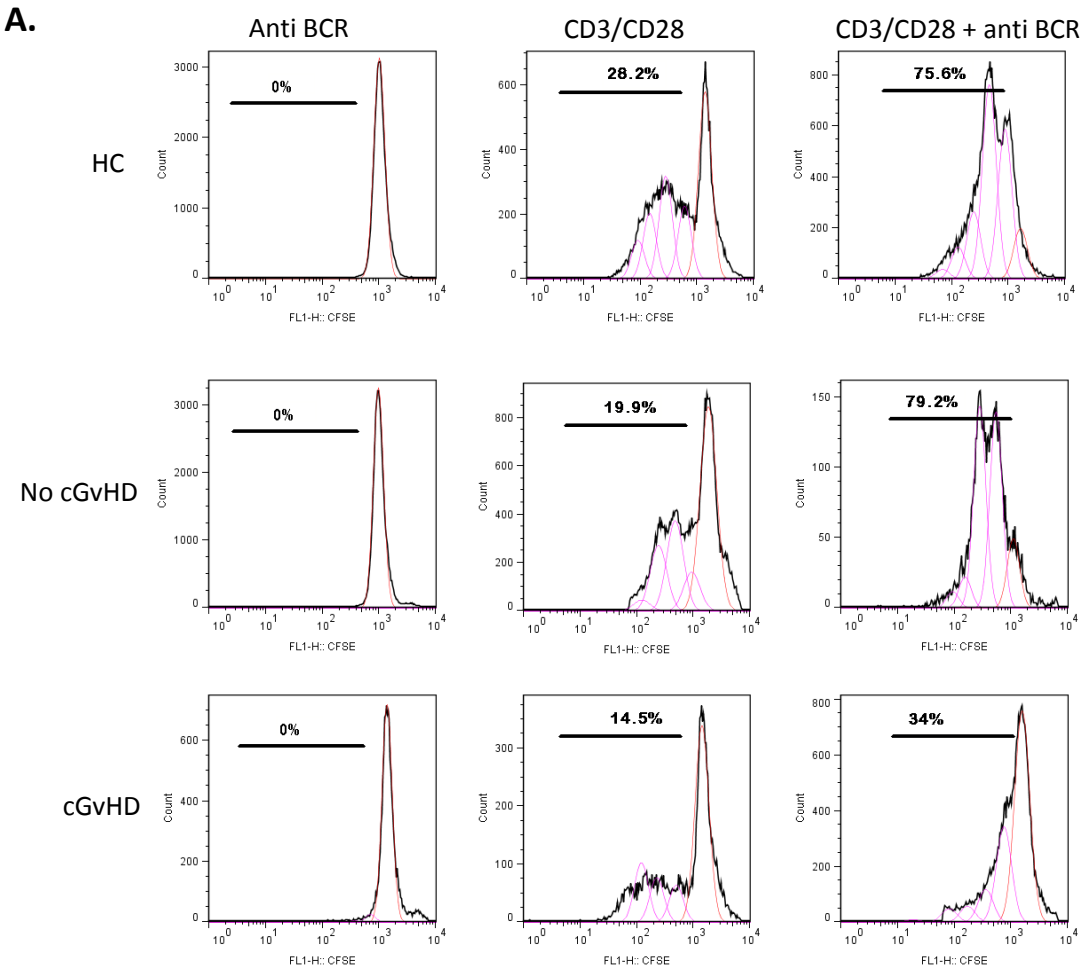
A. Colour-coded gating on CD27/CD21-stained B cells of a representative cGVHD patient used to examine the expression of inhibitory and chemokine receptors on naïve (blue), classical memory (red) and CD21$^-$ B cell (black). **B.** Inhibitory and homing receptor expression on CD21$^-$ B cells relative to naïve and classical CD27$^+$ memory B cells. Plots depict individual median fluorescence intensity (MFI) values for each cell subpopulation of 5 cGVHD patients.

VI.2.2.6 B cells from cGVHD patients have impaired proliferative potential:

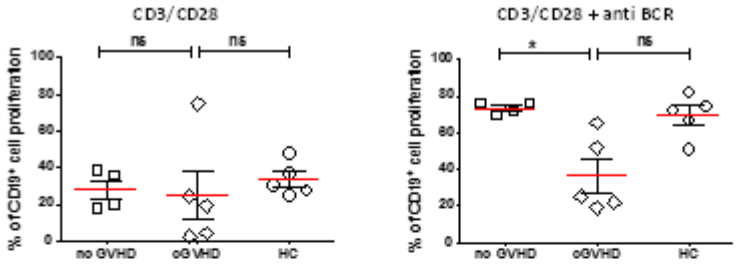
To further investigate the hypothesis that the expanded $CD19^{+}CD21^{-}$ B cells in cGVHD are exhausted, we compared the proliferative potential of $CD19^{+}$ B cells in patients with or without GVHD and healthy controls. The proliferative capacity of B cells was assessed in response to B cell receptor (BCR) and CD40 triggering. CFSE-stained PBMC from 5 cGVHD, 4 no GVHD patients and 5 healthy controls were stimulated with anti-CD3/anti-CD28 beads (CD40 triggering), anti IgM/anti IgG (BCR triggering) or the combination of anti-CD3/anti-CD28 and anti IgM/anti IgG triggering for 96h. Activated T cells upregulated CD154 receptor (CD40L) upon stimulation and in turn activate CD19+ B cells. The addition of anti-BCR in the presence of activated T cells provides dual stimulation of B cells (Blair et al., 2010).

B cells from patients with or without cGVHD and healthy controls stimulated with anti-BCR alone failed to proliferate at 96 hours, **figure (VI-6A)**. Analysis of CFSE-labelled B cells stimulated with anti-CD3/CD28 beads alone revealed poor proliferative response, with no significant difference in $CD19^{+}$ B cell proliferation in patients with or without cGVHD and healthy controls (median percentage of divided cells, 24.9% vs. 27.7% vs. 33.7% respectively; p=0.77); **figure (VI-6A and VI-6B).** The addition of BCR triggering to CD40 ligation resulted in significant enhancement in the proliferation of B cells from patients without GVHD and healthy controls (27.7% vs. 73.2%; p=0.0002 and 33.7% vs. 69.6% p=0.0006) respectively; Figure 4C. In contrast, the addition of CD40 ligation to BCR triggering failed to significantly enhance the proliferative capacity of B cells from cGVHD patients [24.9% (2.5-75) vs. 36.6% (19.2-65.3); p=0.47] and they proliferated at

significantly lower levels compared to patients without cGVHD and healthy controls (median percentage of divided cells, 36.6% vs. 73.2% vs. 69.6%; p= 0.02 respectively; p=0.006), **figure (VI-6C)**. These data indicate that B cells from cGVHD patients with a significantly expanded population of $CD19^{+}CD21^{-}$ B cells exhibit proliferative deficiencies when compared with B cells from patients without cGVHD or healthy controls.



B.



C.

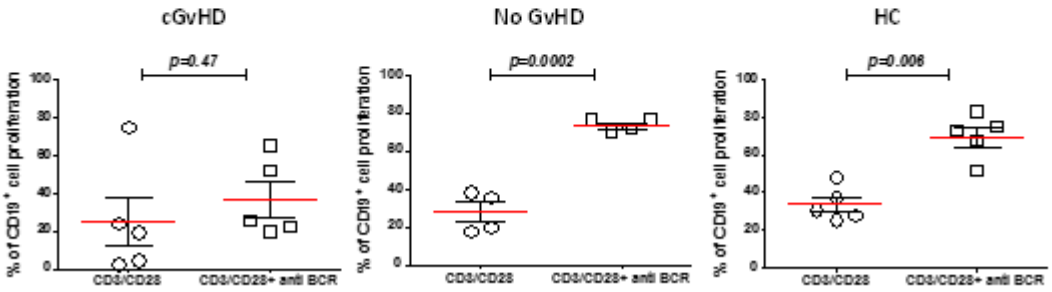


Figure VI-6: Proliferation assessment of B cells in no GVHD, GVHD and HC

Proliferation of $CD19^+$ B cell in a HC, no GVHD and cGVHD patients. CFSE stained PBMC were stimulated with anti BCR alone, anti CD3/ anti CD28 alone or both for 96h. **A.** representative CFSE proliferation histograms comparing gated $CD19^+$ B cells. Figures (B and C) Compare B cell proliferation of 5 cGVHD patients, 4 no GVHD patients and 5 healthy controls. **B.** Chronic GVHD patients had the lowest proliferative potential in response to dual stimulation compared with "no GVHD" patients and HC. **C.** Only B cells from cGVHD patients failed to proliferate when anti-BCR was added to CD40L. Percentage of proliferating B cells was calculated using proliferation platform of FlowJo analysis software.

VI.2.2.7 The frequency of $CD21^-$ B cells correlates with cGVHD severity:

SCT patients were classified according to the frequency of $CD19^+CD21^-$ B cells using a cut off of 5% (normal upper limit)(Moir et al., 2008) and $CD19^+CD21^-$ B cell frequencies were correlated with disease severity, **figure (VI-7) and table 4**. The Odds Ratios for severe, moderate and mild cGVHD were 26.9 ($p<0.001$), 19.5 ($p=0.001$) and 4.5 ($p=0.04$) respectively. The frequencies of $CD19^+CD21^-$ B cell were also predictive for the development and severity of cGVHD when considered as a continuous variable with an OR for severe, moderate and mild cGVHD of 1.31 ($p=0.001$), 1.22 ($p=0.005$) and 1.17 ($p=0.02$) respectively. We also examined the influence of other clinical variables including patient age; $p=0.47$, donor-recipient sex mismatch; $p=0.36$, conditioning regimen; $p=0.52$, cell dose; $p=0.12$, stem cell donor; $p=0.08$, GVHD prophylaxis; $p=0.11$ and history of acute GVHD; $p=0.09$ on the development and severity of cGVHD (Table 1). Variables found to be predictive at a level $p<0.1$ (history of aGVHD and stem cell donor), were included in a multivariate ordinal logistic regression model. Previous history of aGVHD ($p=0.01$) and $CD21^-$ B cell frequencies > 5% ($p=0.001$) were the only variables found to be independently associated with cGVHD.

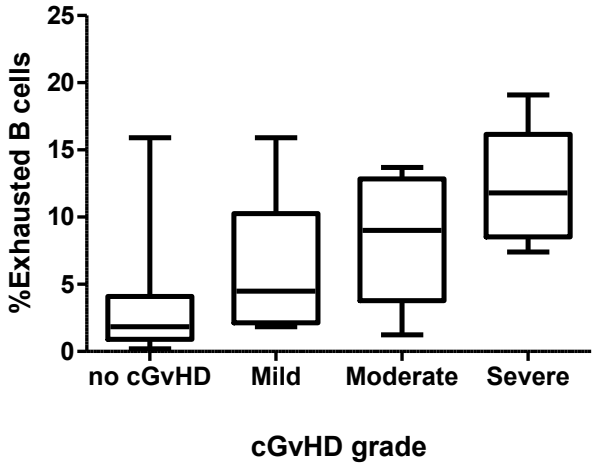


Figure VI-7: Box plot showing the frequency of exhausted B cells per cGVHD grades (mild to severe).

Table VI-4: NIH-defined cGVHD grade and exhausted B cells percentage, based on a cut off of 5%.

		cGvHD grade				
		No cGvHD	Mild	Moderate	Severe	Total
CD21- %	<5%	12	4	1	0	17
	>5%	2	1	4	5	12
Total		14	5	5	5	29

VI.2.3 Discussion:

I compared B cell subsets in the peripheral blood of patients with and without cGVHD and healthy controls. B cell reconstitution following allogeneic stem cell transplantation appears to recapitulate B cell ontogeny (Storek et al., 1993, Marie-Cardine et al., 2008). Transitional B cells appear first in the peripheral blood and progressively decrease, giving rise to naïve B cells (Marie-Cardine et al., 2008, Anolik et al., 2007). The balance of high BAFF levels required for the production of new B cells in relation to B cell number allows normal B cell recovery. High BAFF levels have been found to promote survival of auto-antigen reactive B cells (Lesley et al., 2004). Accordingly, the early period following allogeneic SCT is characterised by supranormal B cell numbers with high homeostatic levels of BAFF (Storek et al., 1993, Sarantopoulos et al., 2009). The majority of circulating B cells are antigen-naïve since the memory compartment remains significantly lower than normal up to one year post-transplant (Avanzini et al., 2005, Bemark et al., 2012, Cuss et al., 2006, Storek et al., 1997). The development of cGVHD has been found to further disrupt bone marrow B lymphopoiesis and consequently lower B cell count (Storek et al., 2001, Sanchez-Garcia et al., 2006). Consistent with the described lymphopenia associated with cGVHD, my cGVHD patients had significantly lower lymphocyte counts compared with both no GVHD patients and healthy controls (Corre et al., 2010). However, not all cGVHD patients in my study met the criteria for a diagnosis of lymphopenia of less than $1x10^3$ cells per µl (Mitrovic et al., 2012). In my study, although cGVHD patients had a comparable B cell count to that of healthy individuals, they would still be expected to have a higher BAFF to B cell ratio than normal.

Sarantopoulos et al has proposed that a high BAFF to B cell ratio in the setting of relative naïve B lymphopenia of cGVHD is a potential explanation for maintaining auto-reactive B cells. Although the absolute number of $CD27^+$ B cells was lower in cGVHD compared with no GVHD patients, they had relatively higher representation in the PB (Sarantopoulos et al., 2007, Sarantopoulos et al., 2009). Furthermore, cGVHD derived memory B cells were found to be chronically activated (Allen et al., 2012). Several studies have reported similar observations but with variable effects on the class-switched and non-switched $CD27^+$ memory B cells. In one study, memory B cell deficiency in cGVHD was limited to the IgM^+ memory B cell subset (D'Orsogna et al., 2009). In other studies both subpopulations were numerically and proportionally reduced in cGVHD compared with no GVHD counterpart (Hilgendorf et al., 2012, Greinix et al., 2008). My data are consistent with published literature. While my cGVHD patients had lower $CD27^+$ B cell population compared to no GVHD and HC, cGVHD patients had relatively higher $CD27^+$ B memory B cells as well as IgM and class-switch memory B cells in the peripheral blood. This observation resulted from the relatively lower $CD27^-$ (transitional and naïve) B cell numbers.

Interestingly, I found an extended circulating $CD21^-$ B cell population in the PB of cGVHD patients consistent with the previously published reports. Greinix el al referred to this subset as immature B cells, based on the sole lack of CD21 expression (Greinix et al., 2008). Using different transitional B cell markers such as the high expression of CD24, CD38 on $CD19^+$ B cells, or absence of CD27 and CD21 expression on $CD10^+$ $CD19^+$ B (Sims et al., 2005, Palanichamy et al., 2009, Suryani et al., 2010), I demonstrated that cGVHD

patients have fewer transitional B cells compared with patients without cGVHD. I herein investigated the expanded $CD21^-$ B cell population in cGVHD using an extended multicolour flow cytometry panel. The analysis revealed that the majority of $CD21^-$ B cells were $CD10^-$ $CD27^-$ $CD21^-$ $CD20^{hi}$, which is similar to the previously identified exhausted B cells described in patients with chronic HIV viraemia (Moir et al., 2008). I also found that the expanded $CD21^-$ B cell population in my cGVHD patients shared considerable phenotypic similarity with exhausted B cells. First, the expression of the inhibitory receptors CD22, CD85J and FCRL4 was higher compared with the naïve compartment. Second, another important feature was the higher expression of the chemokine receptors CD11c and CXCR3 and lower expression of the homing receptors CCR7 and CD62L. The profile of inhibitory and chemokine receptors favours relocation of this population to inflamed tissue.

Similar $CD21^-$ B cell populations have been observed in numerous conditions associated with chronic inflammation especially autoimmune and chronic viral infections e.g. rheumatoid arthritis (Isnardi et al., 2010), SLE (Wehr et al., 2004), HCV infection (Terrier et al., 2011, Charles, 2011), chronic HIV viraemia (Moir et al., 2008), chronic malarial infestation (Weiss et al., 2009), CVID (Rakhmanov et al., 2009), and cGVHD (Greinix et al., 2008). The expanded $CD21^-$ B cell population in these studies was shown to have impaired proliferative potential and Ca^{2+} responses. I therefore sought to examine the proliferation capacity of the expanded $CD21^-$ B cell population in patients with cGVHD. Given that $CD21^-$ B cells constituted a considerable proportion of PB B cells in cGVHD, and available samples were not enough to sort purify this population, I compared the

total B cell response to T cell induced stimulation in vitro. B cells from cGVHD patients mounted a lower proliferation response to BCR stimulation. Sorting this subset and studying the proliferation and other exhaustion assays in comparison to conventional B cells is therefore warranted.

Chronic GVHD provides a chronic inflammatory environment and in many aspects mimics autoimmune and fibrogenic conditions. B cells are constantly exposed to pathogenic antigens and are in a persistent activated state. It is conceivable that activated B cells compensate by dampening BCR mediated activation to prevent activation-induced self-destruction. Indeed higher expression of CD22, CD85J and FCRL4 and lower CD21 inhibit BCR mediated signalling and increase the activation threshold of B cells (Walker and Smith, 2008, Lamar et al., 2010, Barrington et al., 2005, Sohn et al., 2011) A number of investigators have shown the expansion of Ag driven clonal $CD21^{-/lo}$ B cells expressing autoreactive BCRs in the peripheral blood of HCV infected individuals with autoimmune and lymphoproliferative manifestations. These $CD21^{lo}$ B cells were unresponsive to BCR stimulation, with decreased proliferation and attenuated Ca response (Terrier et al., 2011, Charles et al., 2008). Similar findings were reported by Moir et al in chronic HIV viraemic patients where exhausted B cells were enriched within HIV specific B cells (Moir et al., 2008).

The question of whether exhaustion is a global process or an antigen specific phenomenon remains unanswered. Reversing B cell exhaustion in chronic infection may improve immunity against pathogenic organisms (Kardava et al., 2011). Using similar logic one could suggest that applying the same approach to autoimmune conditions or

cGVHD might augment the deleterious effects of Ag specific B cells. Further work is needed to study this subset in cGVHD patients and to ascertain its clonality and its origin with more in-depth molecular studies.

If exhausted B cells are clonal and Ag specific reversing their exhausted state may be detrimental, in which case eliminating host B cells (as with rituximab) and re-infusing normal donor B cells in the form of B cell DLI may be useful. However, if the exhaustion is a global phenomenon affecting the B cell repertoire, attempts at reversing it could be beneficial, especially if regulatory B cells are also affected. Nevertheless, in light of current available knowledge, $CD21^-$ B cells may serve as a useful biological marker of cGVHD.

Chapter VII. General Discussion and future plans

VII.1 General Discussion

HSCT is an important treatment modality for a number of haematological conditions. The outcome is severely affected by the associated complications especially graft-versus-host disease (GVHD). Chronic GVHD disease affects a considerable proportion of HSCT recipients with a high mortality rate (Blazar et al., 2012a). Alloreactive donor T cells play a pivotal role in the initiation and development of GVHD. Therefore, much research has been centred on the role of T cells in the pathogenesis of GVHD and clinical practice reflects this paradigm. T cell-directed immunosuppression forms the cornerstone of the prevention and treatment of GVHD (Ferrara et al., 2009); although, in some cases without significant success (Khaled et al., 2009).

Recently, B cells have been implicated in the pathogenesis of cGVHD in a number of pre-clinical and clinical studies. In keeping with a pathological role for B cells, around half of refractory cGVHD patients respond to rituximab, an anti-CD20 monoclonal antibody (Mohty et al., 2008). The fact that only half of cGVHD cases improve with B cell depletion indicates that B cells may not be pathogenic in certain circumstances. Moreover, B cell derangements are often observed during immune reconstitution following HSCT conditioning which further confound the understanding of the role of B cells in cGVHD. A number of descriptive studies have analysed the peripheral blood B cell subsets in cGVHD patients (Kuzmina et al., 2011, Corre et al., 2010). Interestingly, an expanded population of $CD19^{+}CD21^{-}$ B cells, identified as immature/transitional B cells, was

recently observed in cGVHD patients and was correlated with disease severity (Kuzmina et al., 2009, Kuzmina et al., 2013).

Further insight into the role of this subset in cGVHD is warranted especially since a number of studies have reported the presence of $CD21^-$ B cells with an exhausted phenotype in autoimmune disorders and chronic infections (Malaspina et al., 2006, Moir et al., 2008, Weiss et al., 2009, Charles et al., 2008, Terrier et al., 2011, Visentini et al., 2012). Chronic GVHD certainly shares considerable similarities with autoimmune diseases and is associated with a propensity to chronic infections.

Human IL10 producing regulatory B cells have been identified with more than one phenotype (Mauri and Blair, 2010b). Controversy with regards to their phenotype extends to their roles in human autoimmune diseases and thus far has not been resolved. Blair et al have defined these cells as $CD24^{hi}CD38^{hi}$ transitional B cells and demonstrated impaired function in SLE patients (Blair et al., 2010). Conversely, Tedder and colleagues identified $IL10^+$ B cells as $CD24^{hi}CD27^+$ memory B cells and importantly without quantitative impairment in a number of autoimmune diseases (Iwata et al., 2010). There is considerable evidence from mouse experiments to support both findings which suggest that more than one phenotype may actually exist. Further research into human $IL10^+$ B cells is therefore required to attempt reconciliation of the available data and harness their potential in clinical practice.

To date, no information is available on role of this subset of B cells in cGVHD. Chronic GVHD is a logical candidate for this study in view of its development in a disparate HSCT immune environment. It has been shown that regulatory T cells are reduced in cGVHD

(Zorn et al., 2005); it is likely that a wider regulatory dysfunction is implicated. Furthermore, HSCT allows the transfer of donor immune cells post HSCT which is not currently an option in other autoimmune disease settings.

In my PhD project, I aimed, first, to study B cell subsets in the peripheral blood of cGVHD patients with special focus on $CD21^-$ B cells. Secondly, I optimised the IL10 intracellular staining assay to phenotype $IL10^+$ B cells and ultimately, assess this population in cGVHD patients.

Flow cytometry provides a powerful tool to study cell populations through the use of specific staining and various gating techniques. I designed an extended panel that incorporates a constellation of markers used in a number of B cell classification schemes, in addition to CD21 and CD10 (Sanz et al., 2008). The panel was titrated and validated on healthy control samples before applying it to frozen samples from cGVHD patients. I correlated different B cell subsets according to different B cell phenotyping classifications. Interestingly, there was a considerable overlap among different classification systems, however, each had its own inherent limitation stemming mainly from the limited number of markers used. Furthermore, the panel allowed the assessment of rare B cell populations including $CD27^-$ memory B cells, transitional B cells, plasmablasts and $CD21^-$ B cells. I acknowledge that the analysis of $CD27^-$ memory subset in particular is based on the negative expression of IgM and IgD rather than positive expression of IgA or IgG. I also demonstrated that the CD27/IgM combination could be used as a sorting panel and is potentially superior to CD27/IgD. I also studied and compared B cell subsets in both genders as well as the effect of cryopreservation on the

expression of B cell specific surface markers. The latter is particularly important to validate studies on frozen products. Additional work is required to validate these findings on a larger sample cohort.

Next, I compared peripheral blood B cell subsets in a group of patients with and without cGVHD and healthy controls. Consistent with published data, cGVHD patients had a significantly lower lymphocyte count compared to patients without cGVHD and healthy controls (Corre et al., 2010). Although cGVHD patients had a comparable B cell count to that of healthy individuals, they would still be expected to have a higher BAFF to B cell ratio than normal. Sarantopoulos et al has proposed that a high BAFF to B cell ratio in the setting of relative naïve B lymphopenia of cGVHD is a potential explanation for maintaining auto-reactive B cells (Sarantopoulos et al., 2007, Sarantopoulos et al., 2009). Whilst my cGVHD patients had fewer $CD27^+$ B cells compared to the no GVHD group and HC, they had relatively higher frequencies of $CD27^+$ memory B cells (IgM and class-switch memory B cell proportions) in the peripheral blood, because of the relative naïve B cell lymphopenia (Hilgendorf et al., 2012, Greinix et al., 2008). Interestingly, I also identified an expanded $CD21^-$ B cell population in cGVHD patients, consistent with previous reports (Greinix et al., 2008). Further analysis revealed that the majority of $CD21^-$ B cells were $CD10^-CD27^-CD21^-CD20^{hi}$ and expressed a distinct profile of inhibitory and chemokine receptors, analogous to that of exhausted B cells described in patients with chronic HIV viraemia (Moir et al., 2008). Indeed, B cells from cGVHD patients mounted a lower proliferation response to BCR stimulation which further supports their exhausted phenotype.

I next demonstrated that IL10^{+} CD19^{+} B cells are enriched within the CD24^{hi}CD38^{lo} and expressed IgD and a high level of IgM. Those with an IL10^{+} B cell phenotype also expressed CD20, CD21, CD22, CD40, HLA DR and CD268 (BAFFR). These data therefore suggest that IL10^{+}B cells are CD24^{hi} CD38^{lo} CD27^{+}CD23^{-} IgD^{+} IgM^{hi} CD1d^{hi} CD19^{+} consistent with an IgM memory B cell phenotype. The functional assessment of sorted IgM memory B cells revealed that this population suppressed IFNγ production by CD4 T cells. In addition, IgM memory B cells suppressed CD4 T cell proliferation, to a similar extent to Treg. IL10 blocking experiments suggested that the regulatory function of IgM memory B cells is, at least partly, IL10 dependent. These results are in keeping with Tedder et al's report that IL10^{+} B cells belong to a memory subset, CD24^{hi}CD27^{+} B cells, and express CD148, another memory B cell marker. Interestingly, in their study, CD27^{+} B cells suppressed both IFNγ and TNFα production by CD14^{+} monocytes but not CD4^{+} T cells (Iwata et al., 2010). In my study, IL10^{+} B cells were further defined as CD27^{+}CD24^{hi} IgM memory B cells. Transitional B cells are defined as CD24^{hi} IgM^{hi}IgD^{+}CD1d^{hi}, in addition to being CD27^{-}, and can be both CD23^{-} and CD23^{+}. Taking all these findings into consideration, for clinical purposes, sort-purification based on a phenotype of CD24^{hi}IgM^{hi}CD19^{+} B cells is likely to yield an enriched population of IL10^{+} regulatory B cells.

It has been shown that Treg are numerically and functionally impaired in cGVHD, which in turn may contribute to impaired self- tolerance and survival of autoreactive effector T cells (Matsuoka et al., 2010). Regulatory B cells have been recognised as a powerful regulator of immune responses, with increasingly identified roles in human autoimmune

diseases (Mauri and Bosma, 2012). To date, no data exist on the potential role of regulatory B cells in GVHD. I found reduced frequencies and absolute $IL10^+$ $CD19^+$ B cell count in the peripheral blood samples from cGVHD patients compared to those with no GVHD and HC. Furthermore, cGVHD patients failed to produce the expected higher $IL10^+$ B cell frequencies and numbers, when stimulated with L cells alone compared to dual stimulation. These results suggest that either there is a numerical or functional deficiency of Breg in cGVHD or that these cells have a defective response to CD154 stimulation. Fewer $IL10^+$ B cells in cGVHD could simply mean fewer B cells capable of producing IL10or their precursors. In addition, hypofunction of IL10 capable B cells in the presence of normal numbers of IgM memory B cells is another possibility. However, the lack of a specific marker renders exploring this proposition challenging at present. It has been reported that regulatory B cells from SLE patients are refractory to CD40 engagement; i.e. B- T cell cross talk, with reduced phosphorylation of STAT3 downstream of CD40. In this study, alternative stimulation with CpG restored a normal IL10 response (Blair et al., 2010). There is some evidence that SCT recipients accumulate less Antigen-driven T cells consequent on an intrinsic B cell deficit or impaired T-B cross talk rather than on T cell help defect (Glas et al., 2000).

I also assessed $IFN\gamma^+$ $CD4^+$ T cell frequencies and function in patients with or without cGVHD and healthy controls. In agreement with a previous report (Nakamura et al., 2005), I found no significant difference in $IFN\gamma^+$ $CD4^+$ T cells between cGvHD and “no GvHD” patients. However, the ratio of $IFN\gamma^+$ $CD4^+$ T cells to $IL10^+$ B cells was significantly higher in cGVHD patients, which implies an imbalance between the regulatory and

effector systems. The proposed imbalance adds to the recognised association of Treg/Teff imbalance in cGVHD. A smaller Treg population relative to total CD4 cells, which by default include effector cells, predisposes SCT patients to cGVHD (Matsuoka et al., 2010). My findings support a wider deficit in regulatory networking, which appears to affect both Breg and Treg, and extends the current investigation to other factors that are common to both lineages such as post-transplant environment and cell reconstitution. B cell regeneration sites such as the bone marrow, spleen and lymph nodes are damaged by SCT conditioning (Sale et al., 1992). Chronic GVHD further worsens the splenic hypofunction seen in SCT patients (Cuthbert et al., 1995). Moreover, experimental evidence supports the notion that the cGVHD-associated damage to the bone marrow niche may further impair B cell lymphopoiesis (Shono et al., 2010).

Chronic GVHD provides an inflammatory environment in which B cells are exposed to self-antigens as well as pathogen-associated molecules. B cells are thereby constantly activated in a T cell dependent and independent manner. Furthermore, regulatory T cell deficits reported in cGVHD help maintain the pro-inflammatory environment of the disease. I additionally report that regulatory B cells are also defective in cGVHD in keeping with the pro-inflammatory nature of cGVHD.

A number of investigators have reported the presence of expanded populations of Ag-specific, clonal $CD19^{+}CD21^{-/lo}$ B cells in the peripheral blood of patients with chronic viraemia. These $CD19^{+}CD21^{lo}$ B cells were anergic, with decreased proliferation and attenuated Ca^{2+} response to BCR stimulation (Terrier et al., 2011, Charles et al., 2008, Moir et al., 2008). In addition, chronic non-specific T- independent activation of B cells

induced by HIV- associated molecules such as gp-120 in the presence of high BAFF levels was shown to cause functional exhaustion(He et al., 2006). I propose that the cGVHD environment may lead to B cell exhaustion, possibly via one or both of the activation pathways. Chronic GVHD is a unique condition, in that it mimics autoimmune disorders as well as being associated with chronic infections and colonisation.

It remains unclear whether the exhausted $CD19^{+}CD21^{-}$ B cells in cGVHD patients are directly involved in the pathogenesis of the disease, a consequence of constant activation, or the result of impaired B cell reconstitution. The question of whether exhaustion is a global process affecting all B cells or only antigen-specific B cells remains open. Without better tools to analyse Ag-specific B cells at the single-cell level, and a better understanding of antigens involved in the B cell-driven immune process in cGVHD, many of the concepts regarding the mechanisms of B cell exhaustion in cGVHD will remain speculative.

It is conceivable that B cell exhaustion confers protection from self-destruction and autoimmunity, in case the exhaustion was driven by specific self-antigens. On the other hand, global B cell exhaustion, in addition to self-protection, may contribute to immunodeficiency and susceptibility to severe infections. Interestingly, a recent study reported that downregulating the expression of inhibitory receptors on exhausted $CD21^{-}$ B cells using RNAi technology can effectively reverse B cell exhaustion in HIV and improve immunity against pathogenic organisms (Kardava et al., 2011). It is tempting to suggest that downregulating inhibitory receptors on $CD19^{+}CD21^{-}$ exhausted B cells in cGVHD may also improve their effector function and at least partially reverse the

immune impairment seen in these patients. Alternatively, if $CD21^-$ B cells are in fact antigen-specific and directly involved in the pathogenesis of GVHD, reversing their state of anergy may have deleterious consequences on disease severity.

Nevertheless, the findings that patients with more severe active cGVHD have higher $CD19^+CD21^-$ B cells in the peripheral blood by our group and others (Greinix et al., 2008, Kuzmina et al., 2013) supports a causal relationship between cGVHD and $CD19^+CD21^-$ B cells. At present, $CD19^+CD21^-$ B cells may serve as a potential biomarker to assess the severity of cGVHD and may provide a potential target for therapy. In addition, my data provide a possible explanation for the inadequacy of the antibody response against pathogens and the state of immunodeficiency observed in cGVHD (Hilgendorf et al., 2011).

Early studies in cGVHD with limited number of patients reported lower IL10 levels (Barak et al., 1995, Liem et al., 1999). In a small study, IL10 secretion by mononuclear cells derived from patients with chronic GVHD following in vitro stimulation was reduced (Korholz et al., 1997). Studies of donor and recipient IL10 polymorphisms yielded contradictory results (Lin et al., 2003, Rocha et al., 2002, Takahashi et al., 2000). Recently Hirayama and colleagues reported that IL10-producing $CD29^+$ monocytes were increased in cGVHD and that IL10 spot forming cells correlated with disease activity. They hypothesised that IL10-producing monocytes play a role in remodelling tissue damage in chronic GVHD (Hirayama et al., 2013). IL10 has not been used as an immunomodulatory agent for cGVHD treatment. However, recombinant IL10 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 IL10 was not associated with clinical improvement with the exception of psoriasis (Asadullah et al., 2003). Higher doses of systemically administered IL10 may have immunostimulatory properties with detrimental effect (Tilg et al., 2002). It is possible that IL10 the effect is dose dependent as well as cell specific. My results indicate that cellular contact may be required for IL10 to deliver immune regulation. Moreover, in agreement with a B cell activation model by Duddy et al (Duddy et al., 2004), I also propose that regulatory B cells receiving bystander stimulation via CD40 alone in close vicinity to a site of inflammation may control the inflammatory reaction. The fact that some cGVHD patients respond to rituximab suggests that B cells may have variable roles in the disease. B cell depletion is expected to improve cGVHD in cases where B cells play an effector role in the complex picture of the disease. Since IgM memory B cells, and in fact all described regulatory B cells, express CD20, rituximab will also deplete this subset. Targeting effector B cells, while maintaining regulatory B cells or delivering regulatory B cells is therefore required. The setting of allogeneic HSCT has the advantage that it allows the use of cellular therapy, such as donor lymphocyte infusions (DLI).

An early prophylactic intervention, such as infusion of regulatory subsets to tip the balance in favour of immune regulation, may prevent the development of cGVHD. One way of achieving this goal could be through adoptive transfer of donor lymphocytes enriched with regulatory cells, for instance IgM memory cells, at an early stage, which minimises damage and predisposition to cGvHD. This approach may also facilitate the transfer of passive immunity from the donor to recipient, such as B cell memory against

encapsulated bacteria. Applying the same approach i.e. IgM memory B cell DLI can be proposed for the treatment of established cGVHD. The validity of these approaches must first be confirmed in pre-clinical studies, using xenogenic models of GVHD and subsequently in the context of well-designed clinical studies.

VII.2 Conclusion

Based on these data, I propose that the cGVHD environment leads to B cell exhaustion, through chronic exposure to an inflammatory environment. Inflammation causes cell activation and expression of a phenotype favouring migration to GVHD sites. Deficiency of $IL10^+$ B cells, with increased ratio of $IFN\gamma^+$ $CD4^+$ T cells/$IL10^+$ B cells, in addition to a deficit in regulatory T cells in this context, supports the perpetuation of the inflammatory process. I propose that IgM^+CD27^+ B cell DLI could be a potential treatment modality for cGVHD. Another possibility is the reversal of B cell exhaustion, hence it is important to first establish that exhaustion is not driven by antigens involved in the pathogenesis of cGVHD.

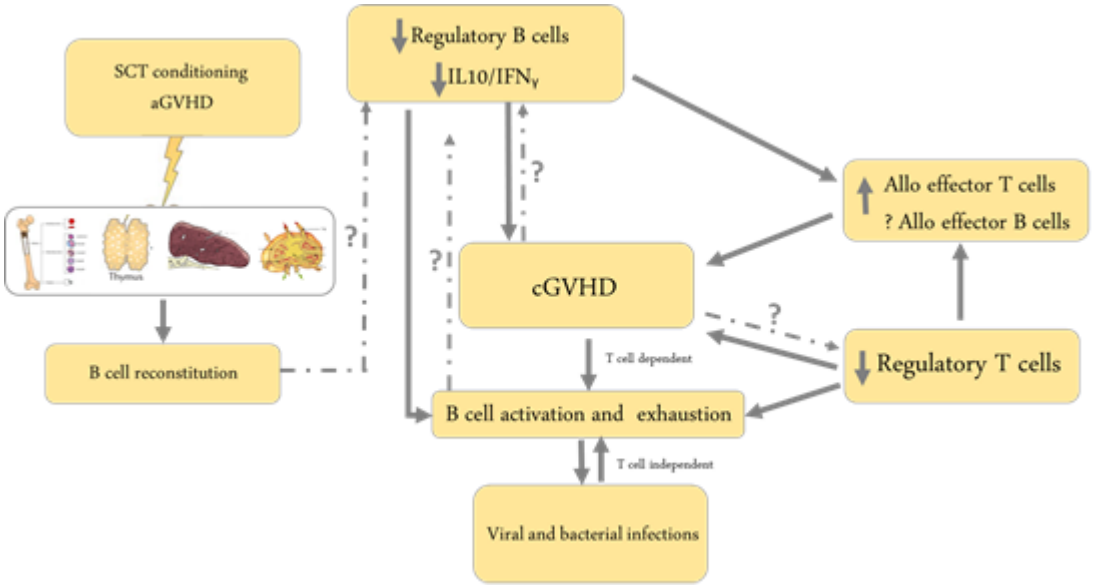


Figure VII-1: Schematic flow chart summarises the proposed mechanisms involved in B cell exhaustion in chronic GVHD.

VII.3 Future Plans

In my thesis, I have demonstrated that regulatory B cells are enriched within a subset of memory cells, in agreement with Tedder's study. Since they were able to show a suppressive effect on $CD14^+$ cells, I propose to explore the suppressive effects of IgM^+CD27^+ B cells on $CD14^+$ monocytes. In addition, $CD8^+$ T cells have a pathogenic role in aGVHD, therefore assessing the suppressive effect of regulatory B cells may help to understand their potential in acute GVHD. My data suggest that the immunoregulatory effect of IgM memory B cells is cell type specific and potentially conducted at a cellular level through IL10. To further assess whether cell-cell contact is required for regulatory B cells to deliver their suppressive property and to inhibit T cell effector cytokine and proliferation, I propose to perform trans-well experiments. Moreover, it has been reported that regulatory B cells enhance regulatory T cells and suppress Th 17 cells. I will therefore study the effect of IgM^+ $CD27^+$ memory B cells on $FoxP3^+$ T cells and $IL17^+$ T cells.

We have shown that $IL10^+$ B cells are reduced in cGVHD patients; therefore, to investigate the potential therapeutic role of IgM^+CD27^+ B cells in cGVHD, I am planning to study their suppressive ability on allogeneic CD4 T cells in an allogeneic mixed-lymphocyte reaction setting.

Since I found that different regulatory B cells share considerable phenotypic similarities, I would like to study the relationship between IgM memory B cells and the transitional cells. The question of whether $CD23^+$ or $CD23^-$ transitional B cells include the precursor for IgM memory B cells is an intriguing one. To investigate this possibility, I plan to

perform an in vitro study to evaluate the differentiation of transitional B cell into IgM memory cells. Assessment of the change in phenotype as transitional B cells differentiate in response to different stimuli and growth factors may provide the answer. I will plan to sort purify $IL10^{+}$ B cells, using IL10 cytokine capture (Miltenyi Biotec), to extract RNA and study the gene expression profile and ultimately identify a specific marker for this population (Yamada et al., 2010). Finally, to confirm that IgM memory B cells could prevent and suppress cGVHD, I plan to study the effect of donor IgM memory B cell infusion on the development of cGVHD as well as the treatment of established cGVHD in minor and major mismatched mouse models. Cell dose and time of infusion post transplantation can be determined for optimal clinical use.

With regards to the role of B cell exhaustion in cGVHD, I plan to sort purify $CD21^{-}$ $CD10^{-}$ $CD27^{-}$ and assess their proliferative capacity as well as their *in vitro* Ig production and compare it with the $CD21^{+}$ B cells in cGVHD, as well as non GVHD patients. It is hoped that this approach would answer the question of whether exhaustion is limited to $CD21^{-}$ B cells or is a global process. Furthermore, using T cell independent polyclonal stimulation protocols, such as CpG, I will assess whether exhaustion could be non-Ag specific, especially in the absence of known GVHD specific antigens. Transcriptional profiling is needed to better understand the origin of these cells and their role in the pathogenesis of cGVHD.

References:

ABOUELNASR, A., ROY, J., COHEN, S., KISS, T. & LACHANCE, S. 2013. Defining the role of sirolimus in the management of graft-versus-host disease: from prophylaxis to treatment. *Biol Blood Marrow Transplant,* 19**,** 12-21.

ABRAHAMSEN, I. W., SOMME, S., HELDAL, D., EGELAND, T., KVALE, D. & TJONNFJORD, G. E. 2005. Immune reconstitution after allogeneic stem cell transplantation: the impact of stem cell source and graft-versus-host disease. *Haematologica,* 90**,** 86-93.

AGEMATSU, K., NAGUMO, H., YANG, F. C., NAKAZAWA, T., FUKUSHIMA, K., ITO, S., SUGITA, K., MORI, T., KOBATA, T., MORIMOTO, C. & KOMIYAMA, A. 1997. B cell subpopulations separated by CD27 and crucial collaboration of CD27+ B cells and helper T cells in immunoglobulin production. *Eur J Immunol,* 27**,** 2073-9.

ALLAN, L. L., STAX, A. M., ZHENG, D. J., CHUNG, B. K., KOZAK, F. K., TAN, R. & VAN DEN ELZEN, P. 2011. CD1d and CD1c expression in human B cells is regulated by activation and retinoic acid receptor signaling. *J Immunol,* 186**,** 5261-72.

ALLEN, J. L., FORE, M. S., WOOTEN, J., ROEHRS, P. A., BHUIYA, N. S., HOFFERT, T., SHARF, A., DEAL, A. M., ARMISTEAD, P., COGHILL, J., GABRIEL, D. A., IRONS, R., ESSENMACHER, A., SHEA, T. C., RICHARDS, K., CUTLER, C., RITZ, J., SERODY, J., BALDWIN, A. S. & SARANTOPOULOS, S. 2012. B cells from patients with chronic GVHD are activated and primed for survival via BAFF-mediated pathways. *Blood,* 120**,** 2529-36.

ALLENDE, M. L., TUYMETOVA, G., LEE, B. G., BONIFACINO, E., WU, Y. P. & PROIA, R. L. 2010. S1P1 receptor directs the release of immature B cells from bone marrow into blood. *J Exp Med,* 207**,** 1113-24.

ALLMAN, D., LINDSLEY, R. C., DEMUTH, W., RUDD, K., SHINTON, S. A. & HARDY, R. R. 2001. Resolution of three nonproliferative immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation. *J Immunol,* 167**,** 6834-40.

ALLMAN, D. & PILLAI, S. 2008. Peripheral B cell subsets. *Curr Opin Immunol,* 20**,** 149-57.

ALOUSI, A. & DE LIMA, M. 2007. Reduced-intensity conditioning allogeneic hematopoietic stem cell transplantation. *Clin Adv Hematol Oncol,* 5**,** 560-70.

ANDERSON, S. M., TOMAYKO, M. M., AHUJA, A., HABERMAN, A. M. & SHLOMCHIK, M. J. 2007. New markers for murine memory B cells that define mutated and unmutated subsets. *J Exp Med,* 204**,** 2103-14.

ANOLIK, J. H., FRIEDBERG, J. W., ZHENG, B., BARNARD, J., OWEN, T., CUSHING, E., KELLY, J., MILNER, E. C., FISHER, R. I. & SANZ, I. 2007. B cell reconstitution after rituximab treatment of lymphoma recapitulates B cell ontogeny. *Clin Immunol,* 122**,** 139-45.

ANSELL, S. M., NOVAK, A. J., ZIESMER, S., PRICE-TROSKA, T., LAPLANT, B., DILLON, S. R. & WITZIG, T. E. 2009. Serum BLyS levels increase after rituximab as initial therapy in patients with follicular Grade 1 non-Hodgkin lymphoma. *Am J Hematol,* 84**,** 71-3.

ASADULLAH, K., STERRY, W. & VOLK, H. D. 2003. Interleukin-10 therapy--review of a new approach. *Pharmacol Rev,* 55**,** 241-69.

ASOKAN, R., BANDA, N. K., SZAKONYI, G., CHEN, X. S. & HOLERS, V. M. 2013. Human complement receptor 2 (CR2/CD21) as a receptor for DNA: Implications for its roles in the immune response and the pathogenesis of systemic lupus erythematosus (SLE). *Mol Immunol,* 53**,** 99-110.

ASOKAN, R., HUA, J., YOUNG, K. A., GOULD, H. J., HANNAN, J. P., KRAUS, D. M., SZAKONYI, G., GRUNDY, G. J., CHEN, X. S., CROW, M. K. & HOLERS, V. M. 2006. Characterization of human complement receptor type 2 (CR2/CD21) as a receptor for IFN-alpha: a potential role in systemic lupus erythematosus. *J Immunol,* 177**,** 383-94.

ATKINSON, K., HOROWITZ, M. M., GALE, R. P., VAN BEKKUM, D. W., GLUCKMAN, E., GOOD, R. A., JACOBSEN, N., KOLB, H. J., RIMM, A. A., RINGDEN, O. & ET AL. 1990. Risk factors for chronic graft-versus-host disease after HLA-identical sibling bone marrow transplantation. *Blood,* 75**,** 2459-64.

ATTANAVANICH, K. & KEARNEY, J. F. 2004. Marginal zone, but not follicular B cells, are potent activators of naive CD4 T cells. *J Immunol,* 172**,** 803-11.

AVANZINI, M. A., LOCATELLI, F., DOS SANTOS, C., MACCARIO, R., LENTA, E., OLIVERI, M., GIEBEL, S., DE STEFANO, P., ROSSI, F., GIORGIANI, G., AMENDOLA, G., TELLI, S. & MARCONI, M. 2005. B lymphocyte reconstitution after hematopoietic stem cell transplantation: functional immaturity and slow recovery of memory CD27+ B cells. *Exp Hematol,* 33**,** 480-6.

AVERY, D. T., ELLYARD, J. I., MACKAY, F., CORCORAN, L. M., HODGKIN, P. D. & TANGYE, S. G. 2005. Increased expression of CD27 on activated human memory B cells correlates with their commitment to the plasma cell lineage. *J Immunol,* 174**,** 4034-42.

BACH, F. H., ALBERTINI, R. J., JOO, P., ANDERSON, J. L. & BORTIN, M. M. 1968. Bone-marrow transplantation in a patient with the Wiskott-Aldrich syndrome. *Lancet,* 2**,** 1364-6.

BARAK, V., LEVI-SCHAFFER, F., NISMAN, B. & NAGLER, A. 1995. Cytokine dysregulation in chronic graft versus host disease. *Leuk Lymphoma,* 17**,** 169-73.

BARNES, D. W. & LOUTIT, J. F. 1957. Treatment of murine leukaemia with x-rays and homologous bone marrow. II. *Br J Haematol,* 3**,** 241-52.

BARR, T. A., BROWN, S., RYAN, G., ZHAO, J. & GRAY, D. 2007. TLR-mediated stimulation of APC: Distinct cytokine responses of B cells and dendritic cells. *Eur J Immunol,* 37**,** 3040-53.

BARRETT, J. 2006. Improving outcome of allogeneic stem cell transplantation by immunomodulation of the early post-transplant environment. *Curr Opin Immunol,* 18**,** 592-8.

BARRINGTON, R. A., ZHANG, M., ZHONG, X., JONSSON, H., HOLODICK, N., CHERUKURI, A., PIERCE, S. K., ROTHSTEIN, T. L. & CARROLL, M. C. 2005. CD21/CD19 coreceptor signaling promotes B cell survival during primary immune responses. *J Immunol,* 175**,** 2859-67.

BAUMGARTH, N. & ROEDERER, M. 2000. A practical approach to multicolor flow cytometry for immunophenotyping. *J Immunol Methods,* 243**,** 77-97.

BEMARK, M., HOLMQVIST, J., ABRAHAMSSON, J. & MELLGREN, K. 2012. Translational Mini-Review Series on B cell subsets in disease. Reconstitution after haematopoietic stem cell transplantation - revelation of B cell developmental pathways and lineage phenotypes. *Clin Exp Immunol,* 167**,** 15-25.

BERKOWSKA, M. A., DRIESSEN, G. J., BIKOS, V., GROSSERICHTER-WAGENER, C., STAMATOPOULOS, K., CERUTTI, A., HE, B., BIERMANN, K., LANGE, J. F., VAN DER BURG, M., VAN DONGEN, J. J. & VAN ZELM, M. C. 2011. Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. *Blood,* 118**,** 2150-8.

BERNASCONI, N. L., TRAGGIAI, E. & LANZAVECCHIA, A. 2002. Maintenance of serological memory by polyclonal activation of human memory B cells. *Science,* 298**,** 2199-202.

BILLINGHAM, R. E. 1966. The biology of graft-versus-host reactions. *Harvey Lect,* 62**,** 21-78.

BIRJANDI, S. Z., IPPOLITO, J. A., RAMADORAI, A. K. & WITTE, P. L. 2011. Alterations in marginal zone macrophages and marginal zone B cells in old mice. *J Immunol,* 186**,** 3441-51.

BLAIR, P. A., CHAVEZ-RUEDA, K. A., EVANS, J. G., SHLOMCHIK, M. J., EDDAOUDI, A., ISENBERG, D. A., EHRENSTEIN, M. R. & MAURI, C. 2009. Selective targeting of B cells with agonistic anti-CD40 is an efficacious strategy for the generation of induced regulatory T2-like B cells and for the suppression of lupus in MRL/lpr mice. *J Immunol,* 182**,** 3492-502.

BLAIR, P. A., NORENA, L. Y., FLORES-BORJA, F., RAWLINGS, D. J., ISENBERG, D. A., EHRENSTEIN, M. R. & MAURI, C. 2010. CD19(+)CD24(hi)CD38(hi) B Cells Exhibit Regulatory Capacity in Healthy Individuals but Are Functionally Impaired in Systemic Lupus Erythematosus Patients. *Immunity,* 32**,** 129-140.

BLAZAR, B., WHITE, E. S. & COURIEL, D. 2012a. Understanding chronic GVHD from different angles. *Biol Blood Marrow Transplant,* 18**,** S184-8.

BLAZAR, B. R., MURPHY, W. J. & ABEDI, M. 2012b. Advances in graft-versus-host disease biology and therapy. *Nat Rev Immunol,* 12**,** 443-58.

BOHNHORST, J. O., BJORGAN, M. B., THOEN, J. E., NATVIG, J. B. & THOMPSON, K. M. 2001. Bm1-Bm5 classification of peripheral blood B cells reveals circulating germinal center founder cells in healthy individuals and disturbance in the B cell subpopulations in patients with primary Sjogren's syndrome. *J Immunol,* 167**,** 3610-8.

BOUAZIZ, J. D., CALBO, S., MAHO-VAILLANT, M., SAUSSINE, A., BAGOT, M., BENSUSSAN, A. & MUSETTE, P. 2010. IL-10 produced by activated human B cells regulates CD4(+) T-cell activation in vitro. *Eur J Immunol,* 40**,** 2686-91.

BOUAZIZ, J. D., YANABA, K. & TEDDER, T. F. 2008. Regulatory B cells as inhibitors of immune responses and inflammation. *Immunol Rev,* 224**,** 201-14.

BRISSLERT, M., BOKAREWA, M., LARSSON, P., WING, K., COLLINS, L. V. & TARKOWSKI, A. 2006. Phenotypic and functional characterization of human CD25+ B cells. *Immunology,* 117**,** 548-57.

CAMBIER, J. C., GAULD, S. B., MERRELL, K. T. & VILEN, B. J. 2007. B-cell anergy: from transgenic models to naturally occurring anergic B cells? *Nat Rev Immunol,* 7**,** 633-43.

CAMERON, P. U., JONES, P., GORNIAK, M., DUNSTER, K., PAUL, E., LEWIN, S., WOOLLEY, I. & SPELMAN, D. 2011. Splenectomy Associated Changes in IgM Memory B Cells in an Adult Spleen Registry Cohort. *PLoS One,* 6**,** e23164.

CANTAERT, T., DOORENSPLEET, M. E., FRANCOSALINAS, G., PARAMARTA, J. E., KLARENBEEK, P. L., TIERSMA, Y., VAN DER LOOS, C. M., DE VRIES, N., TAK, P. P. & BAETEN, D. L. 2012. Increased numbers of CD5+ B lymphocytes with a regulatory phenotype in spondylarthritis. *Arthritis Rheum,* 64**,** 1859-68.

CAPOLUNGHI, F., CASCIOLI, S., GIORDA, E., ROSADO, M. M., PLEBANI, A., AURITI, C., SEGANTI, G., ZUNTINI, R., FERRARI, S., CAGLIUSO, M., QUINTI, I. & CARSETTI, R. 2008. CpG drives human transitional B cells to terminal differentiation and production of natural antibodies. *J Immunol,* 180**,** 800-8.

CARROLL, M. 1999. Role of complement receptors CD21/CD35 in B lymphocyte activation and survival. *Curr Top Microbiol Immunol,* 246**,** 63-8; discussion 69.

CARROLL, M. C. 1998. CD21/CD35 in B cell activation. *Semin Immunol,* 10**,** 279-86.

CARSETTI, R., ROSADO, M. M., DONNANNO, S., GUAZZI, V., SORESINA, A., MEINI, A., PLEBANI, A., AIUTI, F. & QUINTI, I. 2005. The loss of IgM memory B cells correlates with clinical disease in common variable immunodeficiency. *J Allergy Clin Immunol,* 115**,** 412-7.

CARSETTI, R., ROSADO, M. M. & WARDMANN, H. 2004. Peripheral development of B cells in mouse and man. *Immunol Rev,* 197**,** 179-91.

CARTER, N. A., ROSSER, E. C. & MAURI, C. 2012. Interleukin-10 produced by B cells is crucial for the suppression of Th17/Th1 responses, induction of T regulatory type 1 cells and reduction of collagen-induced arthritis. *Arthritis Res Ther,* 14**,** R32.

CARTER, N. A., VASCONCELLOS, R., ROSSER, E. C., TULONE, C., MUNOZ-SUANO, A., KAMANAKA, M., EHRENSTEIN, M. R., FLAVELL, R. A. & MAURI, C. 2011. Mice lacking endogenous IL-10-producing regulatory B cells develop exacerbated disease and present with an increased frequency of Th1/Th17 but a decrease in regulatory T cells. *J Immunol,* 186**,** 5569-79.

CHARLES, E. D., BRUNETTI, C., MARUKIAN, S., RITOLA, K. D., TALAL, A. H., MARKS, K., JACOBSON, I. M., RICE, C. M. & DUSTIN, L. B. 2011. Clonal B cells in patients with hepatitis C virus-associated mixed cryoglobulinemia contain an expanded anergic CD21low B-cell subset. *Blood,* 117**,** 5425-37.

CHARLES, E. D., GREEN, R. M., MARUKIAN, S., TALAL, A. H., LAKE-BAKAAR, G. V., JACOBSON, I. M., RICE, C. M. & DUSTIN, L. B. 2008. Clonal expansion of immunoglobulin M+CD27+ B cells in HCV-associated mixed cryoglobulinemia. *Blood,* 111**,** 1344-56.

CHEN, Y., KHANNA, S., GOODYEAR, C. S., PARK, Y. B., RAZ, E., THIEL, S., GRONWALL, C., VAS, J., BOYLE, D. L., CORR, M., KONO, D. H. & SILVERMAN, G. J. 2009. Regulation of dendritic cells and macrophages by an anti-apoptotic cell natural antibody that suppresses TLR responses and inhibits inflammatory arthritis. *J Immunol,* 183**,** 1346-59.

CHERUKURI, A., CHENG, P. C., SOHN, H. W. & PIERCE, S. K. 2001. The CD19/CD21 complex functions to prolong B cell antigen receptor signaling from lipid rafts. *Immunity,* 14**,** 169-79.

CHRISTOPEIT, M., SCHUTTE, V., THEURICH, S., WEBER, T., GROTHE, W. & BEHRE, G. 2009. Rituximab reduces the incidence of acute graft-versus-host disease. *Blood,* 113**,** 3130-1.

CLARK, F. J., GREGG, R., PIPER, K., DUNNION, D., FREEMAN, L., GRIFFITHS, M., BEGUM, G., MAHENDRA, P., CRADDOCK, C., MOSS, P. & CHAKRAVERTY, R. 2004. Chronic graft-versus-host disease is associated with increased numbers of peripheral blood CD4+CD25high regulatory T cells. *Blood,* 103**,** 2410-6.

COLLISON, L. W. & VIGNALI, D. A. 2011. In vitro Treg suppression assays. *Methods Mol Biol,* 707**,** 21-37.

CORRE, E., CARMAGNAT, M., BUSSON, M., DE LATOUR, R. P., ROBIN, M., RIBAUD, P., TOUBERT, A., RABIAN, C. & SOCIE, G. 2010. Long-term immune deficiency after allogeneic stem cell transplantation: B-cell deficiency is associated with late infections. *Haematologica,* 95**,** 1025-9.

CORREALE, J., FAREZ, M. & RAZZITTE, G. 2008. Helminth infections associated with multiple sclerosis induce regulatory B cells. *Ann Neurol,* 64**,** 187-99.

CUSS, A. K., AVERY, D. T., CANNONS, J. L., YU, L. J., NICHOLS, K. E., SHAW, P. J. & TANGYE, S. G. 2006. Expansion of functionally immature transitional B cells is associated with human-immunodeficient states characterized by impaired humoral immunity. *J Immunol,* 176**,** 1506-16.

CUTHBERT, R. J., IQBAL, A., GATES, A., TOGHILL, P. J. & RUSSELL, N. H. 1995. Functional hyposplenism following allogeneic bone marrow transplantation. *J Clin Pathol,* 48**,** 257-9.

D'COSTA, S., SLOBOD, K. S., BENAIM, E., BOWMAN, L., CUNNINGHAM, J., HOLLADAY, M., HOWLETT, N., SRIVASTAVA, D. K. & HURWITZ, J. L. 2001. Effect of extended immunosuppressive drug treatment on B cell vs T cell reconstitution in pediatric bone marrow transplant recipients. *Bone Marrow Transplant,* 28**,** 573-80.

D'ORAZIO, T. J. & NIEDERKORN, J. Y. 1998. Splenic B cells are required for tolerogenic antigen presentation in the induction of anterior chamber-associated immune deviation (ACAID). *Immunology,* 95**,** 47-55.

D'ORSOGNA, L. J., KRUEGER, R. G., MCKINNON, E. J. & FRENCH, M. A. 2007. Circulating memory B-cell subpopulations are affected differently by HIV infection and antiretroviral therapy. *AIDS,* 21**,** 1747-52.

D'ORSOGNA, L. J., WRIGHT, M. P., KRUEGER, R. G., MCKINNON, E. J., BUFFERY, S. I., WITT, C. S., STAPLES, N., LOH, R., CANNELL, P. K., CHRISTIANSEN, F. T. & FRENCH, M. A. 2009. Allogeneic hematopoietic stem cell transplantation recipients have defects of both switched and igm memory B cells. *Biol Blood Marrow Transplant,* 15**,** 795-803.

DANIELE, N., SCERPA, M. C., CANIGLIA, M., BERNARDO, M. E., ROSSI, C., CIAMMETTI, C., PALUMBO, G., LOCATELLI, F., ISACCHI, G. & ZINNO, F. 2012. Transplantation in the onco-hematology field: focus on the manipulation of alphabeta and gammadelta T cells. *Pathol Res Pract,* 208**,** 67-73.

DAS, A., ELLIS, G., PALLANT, C., LOPES, A. R., KHANNA, P., PEPPA, D., CHEN, A., BLAIR, P., DUSHEIKO, G., GILL, U., KENNEDY, P. T., BRUNETTO, M., LAMPERTICO, P., MAURI, C. & MAINI, M. K. 2012. IL-10-Producing Regulatory B Cells in the Pathogenesis of Chronic Hepatitis B Virus Infection. *J Immunol,* 189**,** 3925-35.

DENG, S., MOORE, D. J., HUANG, X., LIAN, M. M., MOHIUDDIN, M., VELEDEDEOGLU, E., LEE, M. K. T., SONAWANE, S., KIM, J., WANG, J., CHEN, H., CORFE, S. A., PAIGE, C., SHLOMCHIK, M., CATON, A. & MARKMANN, J. F. 2007. Cutting edge: transplant tolerance induced by anti-CD45RB requires B lymphocytes. *J Immunol,* 178**,** 6028-32.

DEOL, A. & LUM, L. G. 2010. Role of donor lymphocyte infusions in relapsed hematological malignancies after stem cell transplantation revisited. *Cancer Treat Rev,* 36**,** 528-38.

DI SABATINO, A., CARSETTI, R., ROSADO, M. M., CICCOCIOPPO, R., CAZZOLA, P., MORERA, R., TINOZZI, F. P., TINOZZI, S. & CORAZZA, G. R. 2004. Immunoglobulin M memory B cell decrease in inflammatory bowel disease. *Eur Rev Med Pharmacol Sci,* 8**,** 199-203.

DI SABATINO, A., ROSADO, M. M., CAZZOLA, P., BIANCHERI, P., TINOZZI, F. P., LAERA, M. R., CANTORO, L., VANOLI, A., CARSETTI, R. & CORAZZA, G. R. 2008. Splenic function and IgM-memory B cells in Crohn's disease patients treated with infliximab. *Inflamm Bowel Dis,* 14**,** 591-6.

DI SABATINO, A., ROSADO, M. M., CICCOCIOPPO, R., CAZZOLA, P., MORERA, R., CORAZZA, G. R. & CARSETTI, R. 2005. Depletion of immunoglobulin M memory B cells is associated with splenic hypofunction in inflammatory bowel disease. *Am J Gastroenterol,* 100**,** 1788-95.

DICKINSON, A. M. & CHARRON, D. 2005. Non-HLA immunogenetics in hematopoietic stem cell transplantation. *Curr Opin Immunol,* 17**,** 517-25.

DUDDY, M., NIINO, M., ADATIA, F., HEBERT, S., FREEDMAN, M., ATKINS, H., KIM, H. J. & BAR-OR, A. 2007. Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis. *J Immunol,* 178**,** 6092-9.

DUDDY, M. E., ALTER, A. & BAR-OR, A. 2004. Distinct profiles of human B cell effector cytokines: a role in immune regulation? *J Immunol,* 172**,** 3422-7.

DUNN-WALTERS, D. K., ISAACSON, P. G. & SPENCER, J. 1995. Analysis of mutations in immunoglobulin heavy chain variable region genes of microdissected marginal zone (MGZ) B cells suggests that the MGZ of human spleen is a reservoir of memory B cells. *J Exp Med,* 182**,** 559-66.

DUTY, J. A., SZODORAY, P., ZHENG, N. Y., KOELSCH, K. A., ZHANG, Q., SWIATKOWSKI, M., MATHIAS, M., GARMAN, L., HELMS, C., NAKKEN, B., SMITH, K., FARRIS, A. D. & WILSON, P. C. 2009. Functional anergy in a subpopulation of naive B cells from healthy humans that express autoreactive immunoglobulin receptors. *J Exp Med,* 206**,** 139-51.

EDRY, E. & MELAMED, D. 2004. Receptor editing in positive and negative selection of B lymphopoiesis. *J Immunol,* 173**,** 4265-71.

EHRENSTEIN, M. R. & NOTLEY, C. A. 2010. The importance of natural IgM: scavenger, protector and regulator. *Nat Rev Immunol,* 10**,** 778-86.

EHRHARDT, G. R., HIJIKATA, A., KITAMURA, H., OHARA, O., WANG, J. Y. & COOPER, M. D. 2008. Discriminating gene expression profiles of memory B cell subpopulations. *J Exp Med,* 205**,** 1807-17.

EHRHARDT, G. R., HSU, J. T., GARTLAND, L., LEU, C. M., ZHANG, S., DAVIS, R. S. & COOPER, M. D. 2005. Expression of the immunoregulatory molecule FcRH4 defines a distinctive tissue-based population of memory B cells. *J Exp Med,* 202**,** 783-91.

ESH-EBMT 2008. Haematopoietic Stem Cell Transplantation, The EBMT Handbook, 5th Edition.

EVANS, J. G., CHAVEZ-RUEDA, K. A., EDDAOUDI, A., MEYER-BAHLBURG, A., RAWLINGS, D. J., EHRENSTEIN, M. R. & MAURI, C. 2007. Novel suppressive function of transitional 2 B cells in experimental arthritis. *J Immunol,* 178**,** 7868-78.

FEARON, D. T. & CARROLL, M. C. 2000. Regulation of B lymphocyte responses to foreign and self-antigens by the CD19/CD21 complex. *Annu Rev Immunol,* 18**,** 393-422.

FECTEAU, J. F., COTE, G. & NERON, S. 2006. A new memory CD27-IgG+ B cell population in peripheral blood expressing VH genes with low frequency of somatic mutation. *J Immunol,* 177**,** 3728-36.

FEDORIW, Y., SAMULSKI, T. D., DEAL, A. M., DUNPHY, C. H., SHARF, A., SHEA, T. C., SERODY, J. S. & SARANTOPOULOS, S. 2012. Bone marrow B cell precursor number after allogeneic stem cell transplantation and GVHD development. *Biol Blood Marrow Transplant,* 18**,** 968-73.

FERGUSON, A. R., YOUD, M. E. & CORLEY, R. B. 2004. Marginal zone B cells transport and deposit IgM-containing immune complexes onto follicular dendritic cells. *Int Immunol,* 16**,** 1411-22.

FERRARA, J. L., LEVINE, J. E., REDDY, P. & HOLLER, E. 2009. Graft-versus-host disease. *Lancet,* 373**,** 1550-61.

FERRARA, J. L. & REDDY, P. 2006. Pathophysiology of graft-versus-host disease. *Semin Hematol,* 43**,** 3-10.

FILIPOVICH, A. H., WEISDORF, D., PAVLETIC, S., SOCIE, G., WINGARD, J. R., LEE, S. J., MARTIN, P., CHIEN, J., PRZEPIORKA, D., COURIEL, D., COWEN, E. W., DINNDORF, P., FARRELL, A., HARTZMAN, R., HENSLEE-DOWNEY, J., JACOBSOHN, D., MCDONALD, G., MITTLEMAN, B., RIZZO, J. D., ROBINSON, M., SCHUBERT, M., SCHULTZ, K., SHULMAN, H., TURNER, M., VOGELSANG, G. & FLOWERS, M. E. 2005. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: I. Diagnosis and staging working group report. *Biol Blood Marrow Transplant,* 11**,** 945-56.

FILLATREAU, S., SWEENIE, C. H., MCGEACHY, M. J., GRAY, D. & ANDERTON, S. M. 2002. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol,* 3**,** 944-50.

FISCHER, E. M., MOUHOUB, A., MAILLET, F., FREMEAUX-BACCHI, V., KRIEF, C., GOULD, H., BERRIH-AKNIN, S. & KAZATCHKINE, M. D. 1999. Expression of CD21 is developmentally regulated during thymic maturation of human T lymphocytes. *Int Immunol,* 11**,** 1841-9.

FISCHER, M. B., GOERG, S., SHEN, L., PRODEUS, A. P., GOODNOW, C. C., KELSOE, G. & CARROLL, M. C. 1998. Dependence of germinal center B cells on expression of CD21/CD35 for survival. *Science,* 280**,** 582-5.

FLOWERS, M. E., INAMOTO, Y., CARPENTER, P. A., LEE, S. J., KIEM, H. P., PETERSDORF, E. W., PEREIRA, S. E., NASH, R. A., MIELCAREK, M., FERO, M. L., WARREN, E. H., SANDERS, J. E., STORB, R. F., APPELBAUM, F. R., STORER, B. E. & MARTIN, P. J. 2011. Comparative analysis of risk factors for acute graft-versus-host disease and for chronic graft-versus-host disease according to National Institutes of Health consensus criteria. *Blood,* 117**,** 3214-9.

FORSTER, I. & RAJEWSKY, K. 1990. The bulk of the peripheral B-cell pool in mice is stable and not rapidly renewed from the bone marrow. *Proc Natl Acad Sci U S A,* 87**,** 4781-4.

GANTNER, F., HERMANN, P., NAKASHIMA, K., MATSUKAWA, S., SAKAI, K. & BACON, K. B. 2003. CD40-dependent and -independent activation of human tonsil B cells by CpG oligodeoxynucleotides. *Eur J Immunol,* 33**,** 1576-85.

GATTI, R. A., MEUWISSEN, H. J., ALLEN, H. D., HONG, R. & GOOD, R. A. 1968. Immunological reconstitution of sex-linked lymphopenic immunological deficiency. *Lancet,* 2**,** 1366-9.

GEORGES, G. E. & STORB, R. 2003. Review of "minitransplantation": nonmyeloablative allogeneic hematopoietic stem cell transplantation. *Int J Hematol,* 77**,** 3-14.

GLAS, A. M., NOTTENBURG, C. & MILNER, E. C. 1997. Analysis of rearranged immunoglobulin heavy chain variable region genes obtained from a bone marrow transplant (BMT) recipient. *Clin Exp Immunol,* 107**,** 372-80.

GLAS, A. M., VAN MONTFORT, E. H., STOREK, J., GREEN, E. G., DRISSEN, R. P., BECHTOLD, V. J., REILLY, J. Z., DAWSON, M. A. & MILNER, E. C. 2000. B-cell-autonomous somatic mutation deficit following bone marrow transplant. *Blood,* 96**,** 1064-9.

GLASS, B. H., J. GÖRLITZ, A. DREGER,P. SCHUBERT, J. WULF, G. NICKELSEN, M. TRUEMPER, L. SCHMITZ, N. 2008. Rituximab for Graft-Versus-Host-Disease-Prophylaxis after Allogeneic Stem Cell Transplantation Given as Treatment of High Risk Relapse of Aggressive Lymphoma: Results of a Randomized Phase II Study. *Blood (ASH Annual Meeting Abstracts),* 112.

GOERNER, M., GOOLEY, T., FLOWERS, M. E., SULLIVAN, K. M., KIEM, H. P., SANDERS, J. E., MARTIN, P. J. & STORB, R. 2002. Morbidity and mortality of chronic GVHD after hematopoietic stem cell transplantation from HLA-identical siblings for patients with aplastic or refractory anemias. *Biol Blood Marrow Transplant,* 8**,** 47-56.

GONNELLA, P. A., WALDNER, H. P. & WEINER, H. L. 2001. B cell-deficient (mu MT) mice have alterations in the cytokine microenvironment of the gut-associated lymphoid tissue (GALT) and a defect in the low dose mechanism of oral tolerance. *J Immunol,* 166**,** 4456-64.

GOOD-JACOBSON, K. L. & SHLOMCHIK, M. J. 2010. Plasticity and heterogeneity in the generation of memory B cells and long-lived plasma cells: the influence of germinal center interactions and dynamics. *J Immunol,* 185**,** 3117-25.

GRANHOLM, N. A. & CAVALLO, T. 1992. Autoimmunity, polyclonal B-cell activation and infection. *Lupus,* 1**,** 63-74.

GRATWOHL, A. 2007. Risk assessment in haematopoietic stem cell transplantation. *Best Pract Res Clin Haematol,* 20**,** 119-24.

GRATWOHL, A., BRAND, R., FRASSONI, F., ROCHA, V., NIEDERWIESER, D., REUSSER, P., EINSELE, H. & CORDONNIER, C. 2005. Cause of death after allogeneic haematopoietic stem cell transplantation (HSCT) in early leukaemias: an EBMT analysis of lethal infectious complications and changes over calendar time. *Bone Marrow Transplant,* 36**,** 757-69.

GRAY, M., MILES, K., SALTER, D., GRAY, D. & SAVILL, J. 2007. Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells. *Proc Natl Acad Sci U S A,* 104**,** 14080-5.

GREINIX, H. T., POHLREICH, D., KOUBA, M., KORMOCZI, U., LOHMANN, I., FELDMANN, K., ZIELINSKI, C. & PICKL, W. F. 2008. Elevated numbers of immature/transitional CD21- B lymphocytes and deficiency of memory CD27+ B cells identify patients with active chronic graft-versus-host disease. *Biol Blood Marrow Transplant,* 14**,** 208-19.

GUINAMARD, R., OKIGAKI, M., SCHLESSINGER, J. & RAVETCH, J. V. 2000. Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. *Nat Immunol,* 1**,** 31-6.

GUO, L., ZHANG, X., ZHENG, B. & HAN, S. 2008. IgM-mediated signaling is required for the development of a normal B cell memory response. *Mol Immunol,* 45**,** 1071-7.

HAHN, T., MCCARTHY, P. L., JR., ZHANG, M. J., WANG, D., ARORA, M., FRANGOUL, H., GALE, R. P., HALE, G. A., HORAN, J., ISOLA, L., MAZIARZ, R. T., VAN ROOD, J. J., GUPTA, V., HALTER, J., REDDY, V., TIBERGHIEN, P., LITZOW, M., ANASETTI, C., PAVLETIC, S. & RINGDEN, O. 2008. Risk factors for acute graft-versus-host disease after human leukocyte antigen-identical sibling transplants for adults with leukemia. *J Clin Oncol,* 26**,** 5728-34.

HARDY, R. R., CARMACK, C. E., SHINTON, S. A., KEMP, J. D. & HAYAKAWA, K. 1991. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. *J Exp Med,* 173**,** 1213-25.

HART, M., STEEL, A., CLARK, S. A., MOYLE, G., NELSON, M., HENDERSON, D. C., WILSON, R., GOTCH, F., GAZZARD, B. & KELLEHER, P. 2007. Loss of discrete memory B cell subsets is associated with impaired immunization responses in HIV-1 infection and may be a risk factor for invasive pneumococcal disease. *J Immunol,* 178**,** 8212-20.

HARTLEY, S. B., CROSBIE, J., BRINK, R., KANTOR, A. B., BASTEN, A. & GOODNOW, C. C. 1991. Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens. *Nature,* 353**,** 765-9.

HAWKINS, E. D., HOMMEL, M., TURNER, M. L., BATTYE, F. L., MARKHAM, J. F. & HODGKIN, P. D. 2007. Measuring lymphocyte proliferation, survival and differentiation using CFSE time-series data. *Nat Protoc,* 2**,** 2057-67.

HE, B., QIAO, X., KLASSE, P. J., CHIU, A., CHADBURN, A., KNOWLES, D. M., MOORE, J. P. & CERUTTI, A. 2006. HIV-1 envelope triggers polyclonal Ig class switch recombination through a CD40-independent mechanism involving BAFF and C-type lectin receptors. *J Immunol,* 176**,** 3931-41.

HELG, C., STAROBINSKI, M., JEANNET, M. & CHAPUIS, B. 1998. Donor lymphocyte infusion for the treatment of relapse after allogeneic hematopoietic stem cell transplantation. *Leuk Lymphoma,* 29**,** 301-13.

HENDRICKS, J., VISSER, A., DAMMERS, P. M., BURGERHOF, J. G., BOS, N. A. & KROESE, F. G. 2011. Class-switched marginal zone B cells in spleen have relatively low numbers of somatic mutations. *Mol Immunol,* 48**,** 874-82.

HILGENDORF, I., FREUND, M., JILG, W., EINSELE, H., GEA-BANACLOCHE, J., GREINIX, H., HALTER, J., LAWITSCHKA, A., WOLFF, D. & MEISEL, R. 2011. Vaccination of allogeneic haematopoietic stem cell transplant recipients: report from the international consensus conference on clinical practice in chronic GVHD. *Vaccine,* 29**,** 2825-33.

HILGENDORF, I., MUELLER-HILKE, B., KUNDT, G., HOLLER, E., HOFFMANN, P., EDINGER, M., FREUND, M. & WOLFF, D. 2012. The lack of memory B cells including T cell independent IgM+ IgD+ memory B cells in chronic graft-versus host disease is associated with susceptibility to infection. *Transpl Int,* 25**,** 87-96.

HIRAYAMA, M., AZUMA, E., NAKAGAWA-NAKAZAWA, A., KUMAMOTO, T., IWAMOTO, S., AMANO, K., TAMAKI, S., USUI, E. & KOMADA, Y. 2013. Interleukin-10 spot-forming cells as a novel biomarker of chronic graft-versus-host disease. *Haematologica,* 98**,** 41-9.

HULSPAS, R., O'GORMAN, M. R., WOOD, B. L., GRATAMA, J. W. & SUTHERLAND, D. R. 2009. Considerations for the control of background fluorescence in clinical flow cytometry. *Cytometry B Clin Cytom,* 76**,** 355-64.

HUSSAARTS, L., VAN DER VLUGT, L. E., YAZDANBAKHSH, M. & SMITS, H. H. 2011. Regulatory B-cell induction by helminths: implications for allergic disease. *J Allergy Clin Immunol,* 128**,** 733-9.

HYMES, S. R., ALOUSI, A. M. & COWEN, E. W. 2012. Graft-versus-host disease: part II. Management of cutaneous graft-versus-host disease. *J Am Acad Dermatol,* 66**,** 535 e1-16; quiz 551-2.

ILLGES, H., BRAUN, M., PETER, H. H. & MELCHERS, I. 2000. Reduced expression of the complement receptor type 2 (CR2, CD21) by synovial fluid B and T lymphocytes. *Clin Exp Immunol,* 122**,** 270-6.

INAMINE, A., TAKAHASHI, Y., BABA, N., MIYAKE, K., TOKUHISA, T., TAKEMORI, T. & ABE, R. 2005. Two waves of memory B-cell generation in the primary immune response. *Int Immunol,* 17**,** 581-9.

INAMOTO, Y. & FLOWERS, M. E. 2011. Treatment of chronic graft-versus-host disease in 2011. *Curr Opin Hematol,* 18**,** 414-20.

IORI, A. P., TORELLI, G. F., DE PROPRIS, M. S., MILANO, F., PUPELLA, S., GOZZER, M., MANCINI, F., MILANI, M. L., INTOPPA, S., CERRETTI, R., LUCARELLI, B., VALLE, V., MALANDRUCCOLO, L., IANNELLA, E., ARLEO, E., GUARINI, A. & FOA, R. 2008. B-cell concentration in the apheretic product predicts acute graft-versus-host disease and treatment-related mortality of allogeneic peripheral blood stem cell transplantation. *Transplantation,* 85**,** 386-90.

ISNARDI, I., NG, Y. S., MENARD, L., MEYERS, G., SAADOUN, D., SRDANOVIC, I., SAMUELS, J., BERMAN, J., BUCKNER, J. H., CUNNINGHAM-RUNDLES, C. & MEFFRE, E. 2010. Complement receptor 2/CD21- human naive B cells contain mostly autoreactive unresponsive clones. *Blood,* 115**,** 5026-36.

ITO, Y., KAWABE, S., KOJIMA, S., NAKAMURA, F., NISHIYAMA, Y., KANEKO, K., KIUCHI, T., ANDO, H. & KIMURA, H. 2011. Identification of Epstein-Barr virus-infected CD27+ memory B-cells in liver or stem cell transplant patients. *J Gen Virol,* 92**,** 2590-5.

IWATA, Y., MATSUSHITA, T., HORIKAWA, M., DILILLO, D. J., YANABA, K., VENTURI, G. M., SZABOLCS, P. M., BERNSTEIN, S. H., MAGRO, C. M., WILLIAMS, A. D., HALL, R. P., ST CLAIR, E. W. & TEDDER, T. F. 2011. Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. *Blood,* 117**,** 530-41.

JEGO, G., ROBILLARD, N., PUTHIER, D., AMIOT, M., ACCARD, F., PINEAU, D., HAROUSSEAU, J. L., BATAILLE, R. & PELLAT-DECEUNYNCK, C. 1999. Reactive plasmacytoses are expansions of plasmablasts retaining the capacity to differentiate into plasma cells. *Blood,* 94**,** 701-12.

JENTSCH-ULLRICH, K., KOENIGSMANN, M., MOHREN, M. & FRANKE, A. 2005. Lymphocyte subsets' reference ranges in an age- and gender-balanced population of 100 healthy adults--a monocentric German study. *Clin Immunol,* 116**,** 192-7.

JEONG, Y. I., HONG, S. H., CHO, S. H., LEE, W. J. & LEE, S. E. 2012. Induction of IL-10-Producing CD1d(high)CD5(+) Regulatory B Cells following Babesia microti-Infection. *PLoS One,* 7**,** e46553.

KAMBLE, R., OHOLENDT, M. & CARRUM, G. 2006. Rituximab responsive refractory acute graft-versus-host disease. *Biol Blood Marrow Transplant,* 12**,** 1201-2.

KAPUR, R., EBELING, S. & HAGENBEEK, A. 2008. B-cell involvement in chronic graft-versus-host disease. *Haematologica,* 93**,** 1702-11.

KARDAVA, L., MOIR, S., WANG, W., HO, J., BUCKNER, C. M., POSADA, J. G., O'SHEA, M. A., ROBY, G., CHEN, J., SOHN, H. W., CHUN, T. W., PIERCE, S. K. & FAUCI, A. S. 2011. Attenuation of HIV-associated human B cell exhaustion by siRNA downregulation of inhibitory receptors. *J Clin Invest,* 121**,** 2614-24.

KATZ, S. I., PARKER, D. & TURK, J. L. 1974. B-cell suppression of delayed hypersensitivity reactions. *Nature,* 251**,** 550-1.

KHALED, Y., REDDY, P. & KRIJANOVSKI, O. 2009. Emerging drugs for acute graft-versus-host disease. *Expert Opin Emerg Drugs,* 14**,** 219-32.

KIM, S. J., LEE, J. W., JUNG, C. W., MIN, C. K., CHO, B., SHIN, H. J., CHUNG, J. S., KIM, H., LEE, W. S., JOO, Y. D., YANG, D. H., KOOK, H., KANG, H. J., AHN, H. S., YOON, S. S., SOHN, S. K., MIN, Y. H., MIN, W. S., PARK, H. S. & WON, J. H. 2010. Weekly rituximab followed by monthly rituximab treatment for steroid-refractory chronic graft-versus-host disease: results from a prospective, multicenter, phase II study. *Haematologica,* 95**,** 1935-42.

KLEIN, U., KUPPERS, R. & RAJEWSKY, K. 1997. Evidence for a large compartment of IgM-expressing memory B cells in humans. *Blood,* 89**,** 1288-98.

KLEIN, U., RAJEWSKY, K. & KUPPERS, R. 1998. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *J Exp Med,* 188**,** 1679-89.

KNIPPENBERG, S., PEELEN, E., SMOLDERS, J., THEWISSEN, M., MENHEERE, P., COHEN TERVAERT, J. W., HUPPERTS, R. & DAMOISEAUX, J. 2011. Reduction in IL-10 producing B cells (Breg) in multiple sclerosis is accompanied by a reduced naive/memory Breg ratio during a relapse but not in remission. *J Neuroimmunol,* 239**,** 80-6.

KNOECHEL, B., LOHR, J., KAHN, E. & ABBAS, A. K. 2005. The link between lymphocyte deficiency and autoimmunity: roles of endogenous T and B lymphocytes in tolerance. *J Immunol,* 175**,** 21-6.

KOELSCH, K., ZHENG, N. Y., ZHANG, Q., DUTY, A., HELMS, C., MATHIAS, M. D., JARED, M., SMITH, K., CAPRA, J. D. & WILSON, P. C. 2007. Mature B cells class switched to IgD are autoreactive in healthy individuals. *J Clin Invest,* 117**,** 1558-65.

KOLB, H. J., SCHATTENBERG, A., GOLDMAN, J. M., HERTENSTEIN, B., JACOBSEN, N., ARCESE, W., LJUNGMAN, P., FERRANT, A., VERDONCK, L., NIEDERWIESER, D., VAN RHEE, F., MITTERMUELLER, J., DE WITTE, T., HOLLER, E. & ANSARI, H. 1995. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. *Blood,* 86**,** 2041-50.

KOPPEL, E. A., WIELAND, C. W., VAN DEN BERG, V. C., LITJENS, M., FLORQUIN, S., VAN KOOYK, Y., VAN DER POLL, T. & GEIJTENBEEK, T. B. 2005. Specific ICAM-3 grabbing nonintegrin-related 1 (SIGNR1) expressed by marginal zone macrophages is essential for defense against pulmonary Streptococcus pneumoniae infection. *Eur J Immunol,* 35**,** 2962-9.

KORETH, J., STEVENSON, K. E., KIM, H. T., MCDONOUGH, S. M., BINDRA, B., ARMAND, P., HO, V. T., CUTLER, C., BLAZAR, B. R., ANTIN, J. H., SOIFFER, R. J., RITZ, J. & ALYEA, E. P., 3RD 2012. Bortezomib-Based Graft-Versus-Host Disease Prophylaxis in HLA-Mismatched Unrelated Donor Transplantation. *J Clin Oncol,* 30**,** 3202-8.

KORHOLZ, D., KUNST, D., HEMPEL, L., SOHNGEN, D., HEYLL, A., BONIG, H., GOBEL, U., ZINTL, F. & BURDACH, S. 1997. Decreased interleukin 10 and increased interferon-gamma production in patients with chronic graft-versus-host disease after allogeneic bone marrow transplantation. *Bone Marrow Transplant,* 19**,** 691-5.

KRUETZMANN, S., ROSADO, M. M., WEBER, H., GERMING, U., TOURNILHAC, O., PETER, H. H., BERNER, R., PETERS, A., BOEHM, T., PLEBANI, A., QUINTI, I. & CARSETTI, R. 2003. Human immunoglobulin M memory B cells controlling Streptococcus pneumoniae infections are generated in the spleen. *J Exp Med,* 197**,** 939-45.

KUROSAKI, T., AIBA, Y., KOMETANI, K., MORIYAMA, S. & TAKAHASHI, Y. 2010. Unique properties of memory B cells of different isotypes. *Immunol Rev,* 237**,** 104-16.

KUZMINA, Z., GREINIX, H. T., KNOBLER, R., WOREL, N., KOUBA, M., WEIGL, R., KORMOCZI, U., ROTTAL, A., POHLREICH, D., ZIELINSKI, C. & PICKL, W. F. 2009. Proportions of immature CD19+CD21- B lymphocytes predict the response to extracorporeal photopheresis in patients with chronic graft-versus-host disease. *Blood,* 114**,** 744-6.

KUZMINA, Z., GREINIX, H. T., WEIGL, R., KORMOCZI, U., ROTTAL, A., FRANTAL, S., EDER, S. & PICKL, W. F. 2011. Significant differences in B-cell subpopulations characterize patients with chronic graft-versus-host disease-associated dysgammaglobulinemia. *Blood,* 117**,** 2265-74.

KUZMINA, Z., KRENN, K., PETKOV, V., KORMOCZI, U., WEIGL, R., ROTTAL, A., KALHS, P., MITTERBAUER, M., PONHOLD, L., DEKAN, G., GREINIX, H. T. & PICKL, W. F. 2013. CD19(+)CD21(low) B cells and patients at risk for NIH-defined chronic graft-versus-host disease with bronchiolitis obliterans syndrome. *Blood,* 121**,** 1886-95.

LAMAR, D. L., WEYAND, C. M. & GORONZY, J. J. 2010. Promoter choice and translational repression determine cell type-specific cell surface density of the inhibitory receptor CD85j expressed on different hematopoietic lineages. *Blood,* 115**,** 3278-86.

LEE, S. J., VOGELSANG, G. & FLOWERS, M. E. 2003. Chronic graft-versus-host disease. *Biol Blood Marrow Transplant,* 9**,** 215-33.

LENERT, P., BRUMMEL, R., FIELD, E. H. & ASHMAN, R. F. 2005. TLR-9 activation of marginal zone B cells in lupus mice regulates immunity through increased IL-10 production. *J Clin Immunol,* 25**,** 29-40.

LESLEY, R., XU, Y., KALLED, S. L., HESS, D. M., SCHWAB, S. R., SHU, H. B. & CYSTER, J. G. 2004. Reduced competitiveness of autoantigen-engaged B cells due to increased dependence on BAFF. *Immunity,* 20**,** 441-53.

LEVINE, J. E., PACZESNY, S. & SARANTOPOULOS, S. 2012. Clinical applications for biomarkers of acute and chronic graft-versus-host disease. *Biol Blood Marrow Transplant,* 18**,** S116-24.

LI, H., DEMETRIS, A. J., MCNIFF, J., MATTE-MARTONE, C., TAN, H. S., ROTHSTEIN, D. M., LAKKIS, F. G. & SHLOMCHIK, W. D. 2012a. Profound depletion of host conventional dendritic cells, plasmacytoid dendritic cells, and B cells does not prevent graft-versus-host disease induction. *J Immunol,* 188**,** 3804-11.

LI, X., ZHONG, H., BAO, W., BOULAD, N., EVANGELISTA, J., HAIDER, M. A., BUSSEL, J. & YAZDANBAKHSH, K. 2012b. Defective regulatory B-cell compartment in patients with immune thrombocytopenia. *Blood,* 120**,** 3318-25.

LI, Y., LI, H. & WEIGERT, M. 2002. Autoreactive B cells in the marginal zone that express dual receptors. *J Exp Med,* 195**,** 181-8.

LIEM, L. M., FIBBE, W. E., VAN HOUWELINGEN, H. C. & GOULMY, E. 1999. Serum transforming growth factor-beta1 levels in bone marrow transplant recipients correlate with blood cell counts and chronic graft-versus-host disease. *Transplantation,* 67**,** 59-65.

LIN, M. T., STORER, B., MARTIN, P. J., TSENG, L. H., GOOLEY, T., CHEN, P. J. & HANSEN, J. A. 2003. Relation of an interleukin-10 promoter polymorphism to graft-versus-host disease and survival after hematopoietic-cell transplantation. *N Engl J Med,* 349**,** 2201-10.

LIU, Y. J., OLDFIELD, S. & MACLENNAN, I. C. 1988. Memory B cells in T cell-dependent antibody responses colonize the splenic marginal zones. *Eur J Immunol,* 18**,** 355-62.

LO-MAN, R. 2011. Regulatory B cells control dendritic cell functions. *Immunotherapy,* 3**,** 19-20.

LODER, F., MUTSCHLER, B., RAY, R. J., PAIGE, C. J., SIDERAS, P., TORRES, R., LAMERS, M. C. & CARSETTI, R. 1999. B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. *J Exp Med,* 190**,** 75-89.

LOPES-CARVALHO, T. & KEARNEY, J. F. 2004. Development and selection of marginal zone B cells. *Immunol Rev,* 197**,** 192-205.

LUCAS, A. H. 2004. IgM memory B cells: origin, mutation, and mobilization by polysaccharide vaccination *Blood,* 104**,** 3417-3418.

LUNDY, S. K. 2009. Killer B lymphocytes: the evidence and the potential. *Inflamm Res,* 58**,** 345-57.

LYNCH, H. E., GOLDBERG, G. L., CHIDGEY, A., VAN DEN BRINK, M. R., BOYD, R. & SEMPOWSKI, G. D. 2009. Thymic involution and immune reconstitution. *Trends Immunol,* 30**,** 366-73.

MADAN, R., DEMIRCIK, F., SURIANARAYANAN, S., ALLEN, J. L., DIVANOVIC, S., TROMPETTE, A., YOGEV, N., GU, Y., KHODOUN, M., HILDEMAN, D., BOESPFLUG, N., FOGOLIN, M. B., GROBE, L., GREWELING, M., FINKELMAN, F. D., CARDIN, R., MOHRS, M., MULLER, W., WAISMAN, A., ROERS, A. & KARP, C. L. 2009. Nonredundant roles for B cell-derived IL-10 in immune counter-regulation. *J Immunol,* 183**,** 2312-20.

MADDALY, R., PAI, G., BALAJI, S., SIVARAMAKRISHNAN, P., SRINIVASAN, L., SUNDER, S. S. & PAUL, S. F. 2010. Receptors and signaling mechanisms for B-lymphocyte activation, proliferation and differentiation--insights from both in vivo and in vitro approaches. *FEBS Lett,* 584**,** 4883-94.

MAECKER, H. T., J. 2009. Selecting reagents for multicolor flow cytometry with BD™ LSR II and BD FACSCanto™ systems. *nature methods* 5.

MAINO, V. C., SUNI, M. A. & RUITENBERG, J. J. 1995. Rapid flow cytometric method for measuring lymphocyte subset activation. *Cytometry,* 20**,** 127-33.

MALASPINA, A., MOIR, S., HO, J., WANG, W., HOWELL, M. L., O'SHEA, M. A., ROBY, G. A., REHM, C. A., MICAN, J. M., CHUN, T. W. & FAUCI, A. S. 2006. Appearance of immature/transitional B cells in HIV-infected individuals with advanced disease: correlation with increased IL-7. *Proc Natl Acad Sci U S A,* 103**,** 2262-7.

MARIE-CARDINE, A., DIVAY, F., DUTOT, I., GREEN, A., PERDRIX, A., BOYER, O., CONTENTIN, N., TILLY, H., TRON, F., VANNIER, J. P. & JACQUOT, S. 2008. Transitional B cells in humans: characterization and insight from B lymphocyte reconstitution after hematopoietic stem cell transplantation. *Clin Immunol,* 127**,** 14-25.

MARIS, M., BOECKH, M., STORER, B., DAWSON, M., WHITE, K., KENG, M., SANDMAIER, B., MALONEY, D., STORB, R. & STOREK, J. 2003. Immunologic recovery after hematopoietic cell transplantation with nonmyeloablative conditioning. *Exp Hematol,* 31**,** 941-52.

MARKUS, H. S. & TOGHILL, P. J. 1991. Impaired splenic function in elderly people. *Age Ageing,* 20**,** 287-90.

MARTIN, F., OLIVER, A. M. & KEARNEY, J. F. 2001. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity,* 14**,** 617-29.

MARTIN, P. J., SCHOCH, G., FISHER, L., BYERS, V., ANASETTI, C., APPELBAUM, F. R., BEATTY, P. G., DONEY, K., MCDONALD, G. B., SANDERS, J. E. & ET AL. 1990. A retrospective analysis of therapy for acute graft-versus-host disease: initial treatment. *Blood,* 76**,** 1464-72.

MASEDA, D., SMITH, S. H., DILILLO, D. J., BRYANT, J. M., CANDANDO, K. M., WEAVER, C. T. & TEDDER, T. F. 2012. Regulatory B10 cells differentiate into antibody-secreting cells after transient IL-10 production in vivo. *J Immunol,* 188**,** 1036-48.

MASILAMANI, M., KASSAHN, D., MIKKAT, S., GLOCKER, M. O. & ILLGES, H. 2003. B cell activation leads to shedding of complement receptor type II (CR2/CD21). *Eur J Immunol,* 33**,** 2391-7.

MATSUOKA, K., KIM, H. T., MCDONOUGH, S., BASCUG, G., WARSHAUER, B., KORETH, J., CUTLER, C., HO, V. T., ALYEA, E. P., ANTIN, J. H., SOIFFER, R. J. & RITZ, J. 2010. Altered regulatory T cell homeostasis in patients with CD4+ lymphopenia following allogeneic hematopoietic stem cell transplantation. *J Clin Invest,* 120**,** 1479-93.

MATSUSHITA, T., YANABA, K., BOUAZIZ, J. D., FUJIMOTO, M. & TEDDER, T. F. 2008. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. *J Clin Invest,* 118**,** 3420-30.

MATTE-MARTONE, C., WANG, X., ANDERSON, B., JAIN, D., DEMETRIS, A. J., MCNIFF, J., SHLOMCHIK, M. J. & SHLOMCHIK, W. D. 2010. Recipient B cells are not required for graft-versus-host disease induction. *Biol Blood Marrow Transplant,* 16**,** 1222-30.

MAURER, D., FISCHER, G. F., FAE, I., MAJDIC, O., STUHLMEIER, K., VON JENEY, N., HOLTER, W. & KNAPP, W. 1992. IgM and IgG but not cytokine secretion is restricted to the CD27+ B lymphocyte subset. *J Immunol,* 148**,** 3700-5.

MAURER, M. A., RAKOCEVIC, G., LEUNG, C. S., QUAST, I., LUKACISIN, M., GOEBELS, N., MUNZ, C., WARDEMANN, H., DALAKAS, M. & LUNEMANN, J. D. 2012. Rituximab induces sustained reduction of pathogenic B cells in patients with peripheral nervous system autoimmunity. *J Clin Invest,* 122**,** 1393-402.

MAURI, C. & BLAIR, P. A. 2010a. Regulatory B cells in autoimmunity: developments and controversies. *Nat Rev Rheumatol,* 6**,** 636-43.

MAURI, C. & BLAIR, P. A. 2010. Regulatory B cells in autoimmunity: developments and controversies. *Nat Rev Rheumatol,* 6**,** 636-43.

MAURI, C. & BOSMA, A. 2012. Immune regulatory function of B cells. *Annu Rev Immunol,* 30**,** 221-41.

MAURI, C., GRAY, D., MUSHTAQ, N. & LONDEI, M. 2003. Prevention of arthritis by interleukin 10-producing B cells. *J Exp Med,* 197**,** 489-501.

MEYER-BAHLBURG, A., ANDREWS, S. F., YU, K. O., PORCELLI, S. A. & RAWLINGS, D. J. 2008. Characterization of a late transitional B cell population highly sensitive to BAFF-mediated homeostatic proliferation. *J Exp Med,* 205**,** 155-68.

MICHONNEAU, D., PEFFAULT DE LATOUR, R., PORCHER, R., ROBIN, M., BENBUNAN, M., ROCHA, V., RIBAUD, P., FERRY, C., DEVERGIE, A., VANNEAUX, V., GLUCKMAN, E., MAROLLEAU,

J. P., SOCIE, G. & LARGHERO, J. 2009. Influence of bone marrow graft B lymphocyte subsets on outcome after HLA-identical sibling transplants. *Br J Haematol,* 145**,** 107-14.

MIKLOS, D. B., KIM, H. T., MILLER, K. H., GUO, L., ZORN, E., LEE, S. J., HOCHBERG, E. P., WU, C. J., ALYEA, E. P., CUTLER, C., HO, V., SOIFFER, R. J., ANTIN, J. H. & RITZ, J. 2005. Antibody responses to H-Y minor histocompatibility antigens correlate with chronic graft-versus-host disease and disease remission. *Blood,* 105**,** 2973-8.

MIKLOS, D. B., KIM, H. T., ZORN, E., HOCHBERG, E. P., GUO, L., MATTES-RITZ, A., VIATTE, S., SOIFFER, R. J., ANTIN, J. H. & RITZ, J. 2004. Antibody response to DBY minor histocompatibility antigen is induced after allogeneic stem cell transplantation and in healthy female donors. *Blood,* 103**,** 353-9.

MILES, K., HEANEY, J., SIBINSKA, Z., SALTER, D., SAVILL, J., GRAY, D. & GRAY, M. 2012. A tolerogenic role for Toll-like receptor 9 is revealed by B-cell interaction with DNA complexes expressed on apoptotic cells. *Proc Natl Acad Sci U S A,* 109**,** 887-92.

MITROVIC, Z., PERRY, A. M., SUZUMIYA, J., ARMITAGE, J. O., AU, W. Y., COIFFIER, B., HOLTE, H., JAFFE, E. S., MONSERRAT, E., RAJAN, S. K., SAVAGE, K. J., TOBINAI, K., VOSE, J. M. & WEISENBURGER, D. D. 2012. The prognostic significance of lymphopenia in peripheral T-cell and natural killer/T-cell lymphomas: a study of 826 cases from the International Peripheral T-cell Lymphoma Project. *Am J Hematol,* 87**,** 790-4.

MIZOGUCHI, A. & BHAN, A. K. 2006. A case for regulatory B cells. *J Immunol,* 176**,** 705-10.
MIZOGUCHI, A., MIZOGUCHI, E., TAKEDATSU, H., BLUMBERG, R. S. & BHAN, A. K. 2002. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity,* 16**,** 219-30.

MIZOGUCHI, E., MIZOGUCHI, A., PREFFER, F. I. & BHAN, A. K. 2000. Regulatory role of mature B cells in a murine model of inflammatory bowel disease. *Int Immunol,* 12**,** 597-605.

MOENS, L., WUYTS, M., MEYTS, I., DE BOECK, K. & BOSSUYT, X. 2008. Human memory B lymphocyte subsets fulfill distinct roles in the anti-polysaccharide and anti-protein immune response. *J Immunol,* 181**,** 5306-12.

MOHTY, M., MARCHETTI, N., EL-CHEIKH, J., FAUCHER, C., FURST, S. & BLAISE, D. 2008. Rituximab as salvage therapy for refractory chronic GVHD. *Bone Marrow Transplant,* 41**,** 909-11.

MOIR, S., HO, J., MALASPINA, A., WANG, W., DIPOTO, A. C., O'SHEA, M. A., ROBY, G., KOTTILIL, S., ARTHOS, J., PROSCHAN, M. A., CHUN, T. W. & FAUCI, A. S. 2008. Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. *J Exp Med,* 205**,** 1797-805.

MOIR, S., MALASPINA, A., PICKERAL, O. K., DONOGHUE, E. T., VASQUEZ, J., MILLER, N. J., KRISHNAN, S. R., PLANTA, M. A., TURNEY, J. F., JUSTEMENT, J. S., KOTTILIL, S., DYBUL, M., MICAN, J. M., KOVACS, C., CHUN, T. W., BIRSE, C. E. & FAUCI, A. S. 2004. Decreased survival of B cells of HIV-viremic patients mediated by altered expression of receptors of the TNF superfamily. *J Exp Med,* 200**,** 587-99.

MOLRINE, D. C., SIBER, G. R., SAMRA, Y., SHEVY, D. S., MACDONALD, K., CIERI, R. & AMBROSINO, D. M. 1999. Normal IgG and impaired IgM responses to polysaccharide vaccines in asplenic patients. *J Infect Dis,* 179**,** 513-7.

MUTIS, T., BRAND, R., GALLARDO, D., VAN BIEZEN, A., NIEDERWIESER, D. & GOULMY, E. 2010. Graft-versus-host driven graft-versus-leukemia effect of minor histocompatibility antigen HA-1 in chronic myeloid leukemia patients. *Leukemia,* 24**,** 1388-92.

MUTIS, T. & GOULMY, E. 2002. Hematopoietic system-specific antigens as targets for cellular immunotherapy of hematological malignancies. *Semin Hematol,* 39**,** 23-31.

NAGASAWA, T. 2006. Microenvironmental niches in the bone marrow required for B-cell development. *Nat Rev Immunol,* 6**,** 107-16.

NAGASE, H., AGEMATSU, K., KITANO, K., TAKAMOTO, M., OKUBO, Y., KOMIYAMA, A. & SUGANE, K. 2001. Mechanism of hypergammaglobulinemia by HIV infection: circulating memory B-cell reduction with plasmacytosis. *Clin Immunol,* 100**,** 250-9.

NAKAMURA, K., AMAKAWA, R., TAKEBAYASHI, M., SON, Y., MIYAJI, M., TAJIMA, K., NAKAI, K., ITO, T., MATSUMOTO, N., ZEN, K., KISHIMOTO, Y. & FUKUHARA, S. 2005. IL-4-producing CD8(+) T cells may be an immunological hallmark of chronic GVHD. *Bone Marrow Transplant,* 36**,** 639-47.

NETA, R. & SALVIN, S. B. 1974. Specific suppression of delayed hypersensitivity: the possible presence of a suppressor B cell in the regulation of delayed hypersensitivity. *J Immunol,* 113**,** 1716-25.

NOVITZKY, N. & DAVISON, G. M. 2001. Immune reconstitution following hematopoietic stem-cell transplantation. *Cytotherapy,* 3**,** 211-20.

OBUKHANYCH, T. V. & NUSSENZWEIG, M. C. 2006. T-independent type II immune responses generate memory B cells. *J Exp Med,* 203**,** 305-10.

ODENDAHL, M., JACOBI, A., HANSEN, A., FEIST, E., HIEPE, F., BURMESTER, G. R., LIPSKY, P. E., RADBRUCH, A. & DORNER, T. 2000. Disturbed peripheral B lymphocyte homeostasis in systemic lupus erythematosus. *J Immunol,* 165**,** 5970-9.

OLIVIERI, J., COLUZZI, S., ATTOLICO, I. & OLIVIERI, A. 2011. Tirosin kinase inhibitors in chronic graft versus host disease: from bench to bedside. *ScientificWorldJournal,* 11**,** 1908-31.

PALANICHAMY, A., BARNARD, J., ZHENG, B., OWEN, T., QUACH, T., WEI, C., LOONEY, R. J., SANZ, I. & ANOLIK, J. H. 2009. Novel human transitional B cell populations revealed by B cell depletion therapy. *J Immunol,* 182**,** 5982-93.

PALLERA, A. M. & SCHWARTZBERG, L. S. 2004. Managing the toxicity of hematopoietic stem cell transplant. *J Support Oncol,* 2**,** 223-37; discussion 237-8, 241, 246-7.

PAREKH, V. V., PRASAD, D. V., BANERJEE, P. P., JOSHI, B. N., KUMAR, A. & MISHRA, G. C. 2003. B cells activated by lipopolysaccharide, but not by anti-Ig and anti-CD40 antibody, induce anergy in CD8+ T cells: role of TGF-beta 1. *J Immunol,* 170**,** 5897-911.

PASCUAL, V., LIU, Y. J., MAGALSKI, A., DE BOUTEILLER, O., BANCHEREAU, J. & CAPRA, J. D. 1994. Analysis of somatic mutation in five B cell subsets of human tonsil. *J Exp Med,* 180**,** 329-39.

PESTKA, S., KRAUSE, C. D., SARKAR, D., WALTER, M. R., SHI, Y. & FISHER, P. B. 2004. Interleukin-10 and related cytokines and receptors. *Annu Rev Immunol,* 22**,** 929-79.

PIATOSA, B., WOLSKA-KUSNIERZ, B., PAC, M., SIEWIERA, K., GALKOWSKA, E. & BERNATOWSKA, E. 2010b. B cell subsets in healthy children: reference values for evaluation of B cell maturation process in peripheral blood. *Cytometry B Clin Cytom,* 78**,** 372-81.

PIERCE, S. K. & LIU, W. 2010. The tipping points in the initiation of B cell signalling: how small changes make big differences. *Nat Rev Immunol,* 10**,** 767-77.

PILLAI, S. & CARIAPPA, A. 2009. The follicular versus marginal zone B lymphocyte cell fate decision. *Nat Rev Immunol,* 9**,** 767-77.

PUGA, I., COLS, M., BARRA, C. M., HE, B., CASSIS, L., GENTILE, M., COMERMA, L., CHORNY, A., SHAN, M., XU, W., MAGRI, G., KNOWLES, D. M., TAM, W., CHIU, A., BUSSEL, J. B., SERRANO, S., LORENTE, J. A., BELLOSILLO, B., LLORETA, J., JUANPERE, N., ALAMEDA, F., BARO, T., DE HEREDIA, C. D., TORAN, N., CATALA, A., TORREBADELL, M., FORTUNY, C., CUSI, V., CARRERAS, C., DIAZ, G. A., BLANDER, J. M., FARBER, C. M., SILVESTRI, G., CUNNINGHAM-RUNDLES, C., CALVILLO, M., DUFOUR, C., NOTARANGELO, L. D., LOUGARIS, V., PLEBANI, A., CASANOVA, J. L., GANAL, S. C., DIEFENBACH, A., AROSTEGUI, J. I., JUAN, M., YAGUE, J., MAHLAOUI, N., DONADIEU, J., CHEN, K. & CERUTTI, A. 2012. B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. *Nat Immunol,* 13**,** 170-80.

RAKHMANOV, M., KELLER, B., GUTENBERGER, S., FOERSTER, C., HOENIG, M., DRIESSEN, G., VAN DER BURG, M., VAN DONGEN, J. J., WIECH, E., VISENTINI, M., QUINTI, I., PRASSE, A., VOELXEN, N., SALZER, U., GOLDACKER, S., FISCH, P., EIBEL, H., SCHWARZ, K., PETER, H. H. & WARNATZ, K. 2009. Circulating CD21low B cells in common variable immunodeficiency resemble tissue homing, innate-like B cells. *Proc Natl Acad Sci U S A,* 106**,** 13451-6.

RATANATHARATHORN, V., LOGAN, B., WANG, D., HOROWITZ, M., UBERTI, J. P., RINGDEN, O., GALE, R. P., KHOURY, H., ARORA, M., SPELLMAN, S., CUTLER, C., ANTIN, J., BORNHAUSER, M., HALE, G., VERDONCK, L., CAIRO, M., GUPTA, V., PAVLETIC, S., CENTER FOR INTERNATIONAL, B. & MARROW TRANSPLANT RESEARCH, M. W. I. U. S. A. 2009. Prior rituximab correlates with less acute graft-versus-host disease and better survival in B-cell lymphoma patients who received allogeneic peripheral blood stem cell transplantation. *Br J Haematol,* 145**,** 816-24.

RAY, A., BASU, S., WILLIAMS, C. B., SALZMAN, N. H. & DITTEL, B. N. 2012. A novel IL-10-independent regulatory role for B cells in suppressing autoimmunity by maintenance of regulatory T cells via GITR ligand. *J Immunol,* 188**,** 3188-98.

REPARON-SCHUIJT, C. C., VAN ESCH, W. J., VAN KOOTEN, C., EZENDAM, N. P., LEVARHT, E. W., BREEDVELD, F. C. & VERWEIJ, C. L. 2001. Presence of a population of CD20+, CD38- B lymphocytes with defective proliferative responsiveness in the synovial compartment of patients with rheumatoid arthritis. *Arthritis Rheum,* 44**,** 2029-37.

RESNICK, I. B., SHAPIRA, M. Y. & SLAVIN, S. 2005. Nonmyeloablative stem cell transplantation and cell therapy for malignant and non-malignant diseases. *Transpl Immunol,* 14**,** 207-19.

REZVANI, K., MIELKE, S., AHMADZADEH, M., KILICAL, Y., SAVANI, B. N., ZEILAH, J., KEYVANFAR, K., MONTERO, A., HENSEL, N., KURLANDER, R. & BARRETT, A. J. 2006. High donor FOXP3-positive regulatory T-cell (Treg) content is associated with a low risk of GVHD following HLA-matched allogeneic SCT. *Blood,* 108**,** 1291-7.

RICHARDS, S. J., MORGAN, G. J. & HILLMEN, P. 2000. Immunophenotypic analysis of B cells in PNH: insights into the generation of circulating naive and memory B cells. *Blood,* 96**,** 3522-8.

RINGDEN, O., BARRETT, A. J., ZHANG, M. J., LOBERIZA, F. R., BOLWELL, B. J., CAIRO, M. S., GALE, R. P., HALE, G. A., LITZOW, M. R., MARTINO, R., RUSSELL, J. A., TIBERGHIEN, P., URBANO-ISPIZUA, A. & HOROWITZ, M. M. 2003. Decreased treatment failure in recipients of HLA-identical bone marrow or peripheral blood stem cell transplants with high CD34 cell doses. *Br J Haematol,* 121**,** 874-85.

ROCHA, V., FRANCO, R. F., PORCHER, R., BITTENCOURT, H., SILVA, W. A., JR., LATOUCHE, A., DEVERGIE, A., ESPEROU, H., RIBAUD, P., SOCIE, G., ZAGO, M. A. & GLUCKMAN, E. 2002. Host defense and inflammatory gene polymorphisms are associated with outcomes after HLA-identical sibling bone marrow transplantation. *Blood,* 100**,** 3908-18.

RODRIGUEZ, V., ANDERSON, P. M., TROTZ, B. A., ARNDT, C. A., ALLEN, J. A. & KHAN, S. P. 2007. Use of infliximab-daclizumab combination for the treatment of acute and chronic graft-versus-host disease of the liver and gut. *Pediatr Blood Cancer,* 49**,** 212-5.

ROMEU, M. A., MESTRE, M., GONZALEZ, L., VALLS, A., VERDAGUER, J., COROMINAS, M., BAS, J., MASSIP, E. & BUENDIA, E. 1992. Lymphocyte immunophenotyping by flow cytometry in normal adults. Comparison of fresh whole blood lysis technique, Ficoll-Paque separation and cryopreservation. *J Immunol Methods,* 154**,** 7-10.

ROSILLO, M. C., ORTUNO, F., RIVERA, J., MORALEDA, J. M. & VICENTE, V. 1995. Cryopreservation modifies flow-cytometric analysis of hemopoietic cells. *Vox Sang,* 68**,** 210-4.

ROWE, V., BANOVIC, T., MACDONALD, K. P., KUNS, R., DON, A. L., MORRIS, E. S., BURMAN, A. C., BOFINGER, H. M., CLOUSTON, A. D. & HILL, G. R. 2006. Host B cells produce IL-10 following TBI and attenuate acute GVHD after allogeneic bone marrow transplantation. *Blood,* 108**,** 2485-92.

SAGE, D., STANWORTH, S., TURNER, D. & NAVARRETE, C. 2005. Diagnosis of transfusion-associated graft-vs.-host disease: the importance of short tandem repeat analysis. *Transfus Med,* 15**,** 481-5.

SAITO, T., CHIBA, S., ICHIKAWA, M., KUNISATO, A., ASAI, T., SHIMIZU, K., YAMAGUCHI, T., YAMAMOTO, G., SEO, S., KUMANO, K., NAKAGAMI-YAMAGUCHI, E., HAMADA, Y., AIZAWA, S. & HIRAI, H. 2003. Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. *Immunity,* 18**,** 675-85.

SAITO, Y., MIYAGAWA, Y., ONDA, K., NAKAJIMA, H., SATO, B., HORIUCHI, Y., OKITA, H., KATAGIRI, Y. U., SAITO, M., SHIMIZU, T., FUJIMOTO, J. & KIYOKAWA, N. 2008. B-cell-activating factor inhibits CD20-mediated and B-cell receptor-mediated apoptosis in human B cells. *Immunology,* 125**,** 570-90.

SALE, G. E., ALAVAIKKO, M., SCHAEFERS, K. M. & MAHAN, C. T. 1992. Abnormal CD4:CD8 ratios and delayed germinal center reconstitution in lymph nodes of human graft recipients with graft-versus-host disease (GVHD): an immunohistological study. *Exp Hematol,* 20**,** 1017-21.

SANCHEZ-GARCIA, J., SERRANO, J., GOMEZ, P., MARTINEZ, F., MARTIN, C., ROMAN-GOMEZ, J., RODRIGUEZ, A., HERRERA, C., GARCIA, J. M., ALVAREZ, M. A. & TORRES, A. 2006. The impact of acute and chronic graft-versus-host disease on normal and malignant B-lymphoid precursors after allogeneic stem cell transplantation for B-lineage acute lymphoblastic leukemia. *Haematologica,* 91**,** 340-7.

SANZ, I., ANOLIK, J. H. & LOONEY, R. J. 2007. B cell depletion therapy in autoimmune diseases. *Front Biosci,* 12**,** 2546-67.

SANZ, I., WEI, C., LEE, F. E. & ANOLIK, J. 2008. Phenotypic and functional heterogeneity of human memory B cells. *Semin Immunol,* 20**,** 67-82.

SARANTOPOULOS, S., STEVENSON, K. E., KIM, H. T., BHUIYA, N. S., CUTLER, C. S., SOIFFER, R. J., ANTIN, J. H. & RITZ, J. 2007. High levels of B-cell activating factor in patients with active chronic graft-versus-host disease. *Clin Cancer Res,* 13**,** 6107-14.

SARANTOPOULOS, S., STEVENSON, K. E., KIM, H. T., CUTLER, C. S., BHUIYA, N. S., SCHOWALTER, M., HO, V. T., ALYEA, E. P., KORETH, J., BLAZAR, B. R., SOIFFER, R. J., ANTIN, J. H. & RITZ, J. 2009. Altered B-cell homeostasis and excess BAFF in human chronic graft-versus-host disease. *Blood,* 113**,** 3865-74.

SASAKI, H., SCHMITT, D. A., MATSUMOTO, K., POLLARD, R. B. & SUZUKI, F. 1993. Demonstrations of a B-cell population that regulates the immune response in spleens of mice infected with herpes simplex virus type I. *Clin Immunol Immunopathol,* 66**,** 169-75.

SAUSSINE, A., TAZI, A., FEUILLET, S., RYBOJAD, M., JUILLARD, C., BERGERON, A., DESSIRIER, V., BOUHIDEL, F., JANIN, A., BENSUSSAN, A., BAGOT, M. & BOUAZIZ, J. D. 2012. Active chronic sarcoidosis is characterized by increased transitional blood B cells, increased IL-10-producing regulatory B cells and high BAFF levels. *PLoS One,* 7**,** e43588.

SCHEEREN, F. A., NAGASAWA, M., WEIJER, K., CUPEDO, T., KIRBERG, J., LEGRAND, N. & SPITS, H. 2008. T cell-independent development and induction of somatic hypermutation in human IgM+ IgD+ CD27+ B cells. *J Exp Med,* 205**,** 2033-42.

SCHIOPPA, T., MOORE, R., THOMPSON, R. G., ROSSER, E. C., KULBE, H., NEDOSPASOV, S., MAURI, C., COUSSENS, L. M. & BALKWILL, F. R. 2011. B regulatory cells and the tumor-promoting actions of TNF-alpha during squamous carcinogenesis. *Proc Natl Acad Sci U S A,* 108**,** 10662-7.

SCHROEDER, M. A. & DIPERSIO, J. F. 2011. Mouse models of graft-versus-host disease: advances and limitations. *Dis Model Mech,* 4**,** 318-33.

SCHULTZ, K. R., PAQUET, J., BADER, S. & HAYGLASS, K. T. 1995. Requirement for B cells in T cell priming to minor histocompatibility antigens and development of graft-versus-host disease. *Bone Marrow Transplant,* 16**,** 289-95.

SCHWAB, J. & ILLGES, H. 2001a. Regulation of CD21 expression by DNA methylation and histone deacetylation. *Int Immunol,* 13**,** 705-10.

SCHWAB, J. & ILLGES, H. 2001b. Silencing of CD21 expression in synovial lymphocytes is independent of methylation of the CD21 promoter CpG island. *Rheumatol Int,* 20**,** 133-7.

SEGUNDO, C., MEDINA, F., RODRIGUEZ, C., MARTINEZ-PALENCIA, R., LEYVA-COBIAN, F. & BRIEVA, J. A. 1999. Surface molecule loss and bleb formation by human germinal center B cells undergoing apoptosis: role of apoptotic blebs in monocyte chemotaxis. *Blood,* 94**,** 1012-20.

SEIFERT, M. & KUPPERS, R. 2009. Molecular footprints of a germinal center derivation of human IgM+(IgD+)CD27+ B cells and the dynamics of memory B cell generation. *J Exp Med,* 206**,** 2659-69.

SENGSTAKE, S., BONEBERG, E. M. & ILLGES, H. 2006. CD21 and CD62L shedding are both inducible via P2X7Rs. *Int Immunol,* 18**,** 1171-8.

SHANNON-LOWE, C. & ROWE, M. 2011. Epstein-Barr virus infection of polarized epithelial cells via the basolateral surface by memory B cell-mediated transfer infection. *PLoS Pathog,* 7**,** e1001338.

SHE, K., GILMAN, A. L., ASLANIAN, S., SHIMIZU, H., KRAILO, M., CHEN, Z., REID, G. S., WALL, D., GOLDMAN, F. & SCHULTZ, K. R. 2007. Altered Toll-like receptor 9 responses in circulating B cells at the onset of extensive chronic graft-versus-host disease. *Biol Blood Marrow Transplant,* 13**,** 386-97.

SHI, Y., AGEMATSU, K., OCHS, H. D. & SUGANE, K. 2003. Functional analysis of human memory B-cell subpopulations: IgD+CD27+ B cells are crucial in secondary immune response by producing high affinity IgM. *Clin Immunol,* 108**,** 128-37.

SHI, Y., YAMAZAKI, T., OKUBO, Y., UEHARA, Y., SUGANE, K. & AGEMATSU, K. 2005. Regulation of aged humoral immune defense against pneumococcal bacteria by IgM memory B cell. *J Immunol,* 175**,** 3262-7.

SHIMABUKURO-VORNHAGEN, A., HALLEK, M. J., STORB, R. F. & VON BERGWELT-BAILDON, M. S. 2009. The role of B cells in the pathogenesis of graft-versus-host disease. *Blood,* 114**,** 4919-27.

SHIMAMURA, T., HABU, S., HASHIMOTO, K. & SASAKI, S. 1984. Feedback suppression of the immune response in vivo. III. Lyt-1+ B cells are suppressor-inducer cells. *Cell Immunol,* 83**,** 221-4.

SHIMAMURA, T., HASHIMOTO, K. & SASAKI, S. 1982a. Feedback suppression of the immune response in vivo. I. Immune B cells induce antigen-specific suppressor T cells. *Cell Immunol,* 68**,** 104-13.

SHIMAMURA, T., HASHIMOTO, K. & SASAKI, S. 1982b. Feedback suppression of the immune response in vivo. II. Involvement of prostaglandins in the generation of suppressor-inducer B lymphocytes. *Cell Immunol,* 69**,** 192-5.

SHLOMCHIK, W. D. 2007. Graft-versus-host disease. *Nat Rev Immunol,* 7**,** 340-52.

SHONO, Y., UEHA, S., WANG, Y., ABE, J., KURACHI, M., MATSUNO, Y., SUGIYAMA, T., NAGASAWA, T., IMAMURA, M. & MATSUSHIMA, K. 2010. Bone marrow graft-versus-host disease: early destruction of hematopoietic niche after MHC-mismatched hematopoietic stem cell transplantation. *Blood,* 115**,** 5401-11.

SHUBINSKY, G., SCHLESINGER, M., POLLIACK, A. & RABINOWITZ, R. 1997. Pathways controlling the expression of surface CD21 (CR2) and CD23 (Fc(epsilon)IIR) proteins in human malignant B cells. *Leuk Lymphoma,* 25**,** 521-30.

SHULMAN, H. M., SULLIVAN, K. M., WEIDEN, P. L., MCDONALD, G. B., STRIKER, G. E., SALE, G. E., HACKMAN, R., TSOI, M. S., STORB, R. & THOMAS, E. D. 1980. Chronic graft-versus-host syndrome in man. A long-term clinicopathologic study of 20 Seattle patients. *Am J Med,* 69**,** 204-17.

SHUSTOV, A., LUZINA, I., NGUYEN, P., PAPADIMITRIOU, J. C., HANDWERGER, B., ELKON, K. B. & VIA, C. S. 2000. Role of perforin in controlling B-cell hyperactivity and humoral autoimmunity. *J Clin Invest,* 106**,** R39-47.

SIMS, G. P., ETTINGER, R., SHIROTA, Y., YARBORO, C. H., ILLEI, G. G. & LIPSKY, P. E. 2005. Identification and characterization of circulating human transitional B cells. *Blood,* 105**,** 4390-8.

SLAVIN, S. 2003. Graft-versus-host disease, the graft-versus-leukemia effect, and mixed chimerism following nonmyeloablative stem cell transplantation. *Int J Hematol,* 78**,** 195-207.

SOHN, H. W., KRUEGER, P. D., DAVIS, R. S. & PIERCE, S. K. 2011. FcRL4 acts as an adaptive to innate molecular switch dampening BCR signaling and enhancing TLR signaling. *Blood,* 118**,** 6332-41.

SONG, H. & CERNY, J. 2003. Functional heterogeneity of marginal zone B cells revealed by their ability to generate both early antibody-forming cells and germinal centers with hypermutation and memory in response to a T-dependent antigen. *J Exp Med,* 198**,** 1923-35.

SONODA, K. H. & STEIN-STREILEIN, J. 2002. CD1d on antigen-transporting APC and splenic marginal zone B cells promotes NKT cell-dependent tolerance. *Eur J Immunol,* 32**,** 848-57.

SRINIVASAN, M., FLYNN, R., PRICE, A., RANGER, A., BROWNING, J. L., TAYLOR, P. A., RITZ, J., ANTIN, J. H., MURPHY, W. J., LUZNIK, L., SHLOMCHIK, M. J., PANOSKALTSIS-MORTARI, A. & BLAZAR, B. R. 2012. Donor B-cell alloantibody deposition and germinal center formation are required for the development of murine chronic GVHD and bronchiolitis obliterans. *Blood,* 119**,** 1570-80.

SRIVASTAVA, B., QUINN, W. J., 3RD, HAZARD, K., ERIKSON, J. & ALLMAN, D. 2005. Characterization of marginal zone B cell precursors. *J Exp Med,* 202**,** 1225-34.

STELZER, G. T., MARTI, G., HURLEY, A., MCCOY, P., JR., LOVETT, E. J. & SCHWARTZ, A. 1997. U.S.-Canadian Consensus recommendations on the immunophenotypic analysis of hematologic neoplasia by flow cytometry: standardization and validation of laboratory procedures. *Cytometry,* 30**,** 214-30.

STOREK, J., FERRARA, S., KU, N., GIORGI, J. V., CHAMPLIN, R. E. & SAXON, A. 1993. B cell reconstitution after human bone marrow transplantation: recapitulation of ontogeny? *Bone Marrow Transplant,* 12**,** 387-98.

STOREK, J., WELLS, D., DAWSON, M. A., STORER, B. & MALONEY, D. G. 2001. Factors influencing B lymphopoiesis after allogeneic hematopoietic cell transplantation. *Blood,* 98**,** 489-91.

STOREK, J., WITHERSPOON, R. P. & STORB, R. 1997. Reconstitution of membrane IgD(-) (mIgD(-)) B cells after marrow transplantation lags behind the reconstitution of mIgD(+) B cells. *Blood,* 89**,** 350-351.

SUGIMOTO, K., OGAWA, A., SHIMOMURA, Y., NAGAHAMA, K., MIZOGUCHI, A. & BHAN, A. K. 2007. Inducible IL-12-producing B cells regulate Th2-mediated intestinal inflammation. *Gastroenterology,* 133**,** 124-36.

SUN, C. M., DERIAUD, E., LECLERC, C. & LO-MAN, R. 2005. Upon TLR9 signaling, CD5+ B cells control the IL-12-dependent Th1-priming capacity of neonatal DCs. *Immunity,* 22**,** 467-77.

SURYANI, S., FULCHER, D. A., SANTNER-NANAN, B., NANAN, R., WONG, M., SHAW, P. J., GIBSON, J., WILLIAMS, A. & TANGYE, S. G. 2010. Differential expression of CD21 identifies developmentally and functionally distinct subsets of human transitional B cells. *Blood,* 115**,** 519-29.

SVEGLIATI, S., OLIVIERI, A., CAMPELLI, N., LUCHETTI, M., POLONI, A., TRAPPOLINI, S., MORONCINI, G., BACIGALUPO, A., LEONI, P., AVVEDIMENTO, E. V. & GABRIELLI, A. 2007. Stimulatory autoantibodies to PDGF receptor in patients with extensive chronic graft-versus-host disease. *Blood,* 110**,** 237-41.

TAKAHASHI, H., FURUKAWA, T., HASHIMOTO, S., SUZUKI, N., KUROHA, T., YAMAZAKI, F., INANO, K., TAKAHASHI, M., AIZAWA, Y. & KOIKE, T. 2000. Contribution of TNF-alpha and IL-10 gene polymorphisms to graft-versus-host disease following allo-hematopoietic stem cell transplantation. *Bone Marrow Transplant,* 26**,** 1317-23.

TAKAHASHI, T., CHIBA, S., MATSUMURA, T. & HIRAI, H. 2002. A case of severe B cell deficiency after allogeneic stem cell transplantation. *Bone Marrow Transplant,* 30**,** 397-400.

TAKIZAWA, M., SUGANE, K. & AGEMATSU, K. 2006. Role of tonsillar IgD+CD27+ memory B cells in humoral immunity against pneumococcal infection. *Hum Immunol,* 67**,** 966-75.

TAN, S. S., UYL-DE GROOT, C. A., HUIJGENS, P. C. & FIBBE, W. E. 2007. Stem cell transplantation in Europe: trends and prospects. *Eur J Cancer,* 43**,** 2359-65.

TANGYE, S. G. & GOOD, K. L. 2007. Human IgM+CD27+ B cells: memory B cells or "memory" B cells? *J Immunol,* 179**,** 13-9.

TANGYE, S. G., LIU, Y. J., AVERSA, G., PHILLIPS, J. H. & DE VRIES, J. E. 1998. Identification of functional human splenic memory B cells by expression of CD148 and CD27. *J Exp Med,* 188**,** 1691-703.

TANGYE, S. G. & TARLINTON, D. M. 2009. Memory B cells: effectors of long-lived immune responses. *Eur J Immunol,* 39**,** 2065-75.

TAY, J., ALLAN, D., TINMOUTH, A., COYLE, D. & HEBERT, P. 2013. Donor selection for patients undergoing allogeneic hematopoietic SCT: assessment of the priorities of Canadian hematopoietic SCT physicians. *Bone Marrow Transplant,* 48**,** 314-6.

TAYEBI, H., LAPIERRE, V., SAAS, P., LIENARD, A., SUTTON, L., MILPIED, N., ATTAL, M., CAHN, J. Y., KUENTZ, M., BLAISE, D., HERVE, P., TIBERGHIEN, P., ROBINET, E. & SOCIETE FRANCAISE DE GREFFE DE MOELLE ET DE THERAPIE, C. 2001. Enhanced activation of B cells in a granulocyte colony-stimulating factor-mobilized peripheral blood stem cell graft. *Br J Haematol,* 114**,** 698-700.

TEDDER, T. F., POE, J. C., FUJIMOTO, M., HAAS, K. M. & SATO, S. 2005. The CD19-CD21 signal transduction complex of B lymphocytes regulates the balance between health and autoimmune disease: systemic sclerosis as a model system. *Curr Dir Autoimmun,* 8**,** 55-90.

TERRIER, B., JOLY, F., VAZQUEZ, T., BENECH, P., ROSENZWAJG, M., CARPENTIER, W., GARRIDO, M., GHILLANI-DALBIN, P., KLATZMANN, D., CACOUB, P. & SAADOUN, D. 2011. Expansion of functionally anergic CD21-/low marginal zone-like B cell clones in hepatitis C virus infection-related autoimmunity. *J Immunol,* 187**,** 6550-63.

THIEL, J., KIMMIG, L., SALZER, U., GRUDZIEN, M., LEBRECHT, D., HAGENA, T., DRAEGER, R., VOLXEN, N., BERGBREITER, A., JENNINGS, S., GUTENBERGER, S., AICHEM, A., ILLGES, H., HANNAN, J. P., KIENZLER, A. K., RIZZI, M., EIBEL, H., PETER, H. H., WARNATZ, K., GRIMBACHER, B., RUMP, J. A. & SCHLESIER, M. 2012. Genetic CD21 deficiency is associated with hypogammaglobulinemia. *J Allergy Clin Immunol,* 129**,** 801-810 e6.

TIAN, J., ZEKZER, D., HANSSEN, L., LU, Y., OLCOTT, A. & KAUFMAN, D. L. 2001. Lipopolysaccharide-activated B cells down-regulate Th1 immunity and prevent autoimmune diabetes in nonobese diabetic mice. *J Immunol,* 167**,** 1081-9.

TIERENS, A., DELABIE, J., MICHIELS, L., VANDENBERGHE, P. & DE WOLF-PEETERS, C. 1999. Marginal-zone B cells in the human lymph node and spleen show somatic hypermutations and display clonal expansion. *Blood,* 93**,** 226-34.

TILG, H., VAN MONTFRANS, C., VAN DEN ENDE, A., KASER, A., VAN DEVENTER, S. J., SCHREIBER, S., GREGOR, M., LUDWICZEK, O., RUTGEERTS, P., GASCHE, C., KONINGSBERGER, J. C., ABREU, L., KUHN, I., COHARD, M., LEBEAUT, A., GRINT, P. & WEISS, G. 2002. Treatment of Crohn's disease with recombinant human interleukin 10 induces the proinflammatory cytokine interferon gamma. *Gut,* 50**,** 191-5.

TOLLERUD, D. J., BROWN, L. M., CLARK, J. W., NEULAND, C. Y., MANN, D. L., PANKIW-TROST, L. K. & BLATTNER, W. A. 1991. Cryopreservation and long-term liquid nitrogen storage of peripheral blood mononuclear cells for flow cytometry analysis: effects on cell subset proportions and fluorescence intensity. *J Clin Lab Anal,* 5**,** 255-61.

TOYAMA, H., OKADA, S., HATANO, M., TAKAHASHI, Y., TAKEDA, N., ICHII, H., TAKEMORI, T., KURODA, Y. & TOKUHISA, T. 2002. Memory B cells without somatic hypermutation are generated from Bcl6-deficient B cells. *Immunity,* 17**,** 329-39.

TRETTER, T., VENIGALLA, R. K., ECKSTEIN, V., SAFFRICH, R., SERTEL, S., HO, A. D. & LORENZ, H. M. 2008. Induction of CD4+ T-cell anergy and apoptosis by activated human B cells. *Blood,* 112**,** 4555-64.

TSUIJI, M., YURASOV, S., VELINZON, K., THOMAS, S., NUSSENZWEIG, M. C. & WARDEMANN, H. 2006. A checkpoint for autoreactivity in human IgM+ memory B cell development. *J Exp Med,* 203**,** 393-400.

VALE, A. M. & SCHROEDER, H. W., JR. 2010. Clinical consequences of defects in B-cell development. *J Allergy Clin Immunol,* 125**,** 778-87.

VAN DER VLUGT, L. E., LABUDA, L. A., OZIR-FAZALALIKHAN, A., LIEVERS, E., GLOUDEMANS, A. K., LIU, K. Y., BARR, T. A., SPARWASSER, T., BOON, L., NGOA, U. A., FEUGAP, E. N., ADEGNIKA, A. A., KREMSNER, P. G., GRAY, D., YAZDANBAKHSH, M. & SMITS, H. H. 2012. Schistosomes induce regulatory features in human and mouse CD1d(hi) B cells: inhibition of allergic inflammation by IL-10 and regulatory T cells. *PLoS One,* 7**,** e30883.

VASKOVA, M., FRONKOVA, E., STARKOVA, J., KALINA, T., MEJSTRIKOVA, E. & HRUSAK, O. 2008. CD44 and CD27 delineate B-precursor stages with different recombination status and with an uneven distribution in nonmalignant and malignant hematopoiesis. *Tissue Antigens,* 71**,** 57-66.

VELARDI, A., CUCCIAIONI, S., TERENZI, A., QUINTI, I., AVERSA, F., GROSSI, C. E., GRIGNANI, F. & MARTELLI, M. F. 1988. Acquisition of Ig isotype diversity after bone marrow transplantation in adults. A recapitulation of normal B cell ontogeny. *J Immunol,* 141**,** 815-20.

VENKEN, K., THEWISSEN, M., HELLINGS, N., SOMERS, V., HENSEN, K., RUMMENS, J. L. & STINISSEN, P. 2007. A CFSE based assay for measuring CD4+CD25+ regulatory T cell mediated suppression of auto-antigen specific and polyclonal T cell responses. *J Immunol Methods,* 322**,** 1-11.

VISENTINI, M., CAGLIUSO, M., CONTI, V., CARBONARI, M., CIBATI, M., SICILIANO, G., CRISTOFOLETTI, C., RUSSO, G., CASATO, M. & FIORILLI, M. 2012. Clonal B cells of HCV-associated mixed cryoglobulinemia patients contain exhausted marginal zone-like and CD21 low cells overexpressing Stra13. *Eur J Immunol,* 42**,** 1468-76.

VOGELSANG, G. B. 2001. How I treat chronic graft-versus-host disease. *Blood,* 97**,** 1196-201.

VOS, Q., LEES, A., WU, Z. Q., SNAPPER, C. M. & MOND, J. J. 2000. B-cell activation by T-cell-independent type 2 antigens as an integral part of the humoral immune response to pathogenic microorganisms. *Immunol Rev,* 176**,** 154-70.

WAGNER, M., POECK, H., JAHRSDOERFER, B., ROTHENFUSSER, S., PRELL, D., BOHLE, B., TUMA, E., GIESE, T., ELLWART, J. W., ENDRES, S. & HARTMANN, G. 2004. IL-12p70-dependent Th1 induction by human B cells requires combined activation with CD40 ligand and CpG DNA. *J Immunol,* 172**,** 954-63.

WALKER, J. A. & SMITH, K. G. 2008. CD22: an inhibitory enigma. *Immunology,* 123**,** 314-25.

WARDEMANN, H., YURASOV, S., SCHAEFER, A., YOUNG, J. W., MEFFRE, E. & NUSSENZWEIG, M. C. 2003. Predominant autoantibody production by early human B cell precursors. *Science,* 301**,** 1374-7.

WARNATZ, K., WEHR, C., DRAGER, R., SCHMIDT, S., EIBEL, H., SCHLESIER, M. & PETER, H. H. 2002. Expansion of CD19(hi)CD21(lo/neg) B cells in common variable immunodeficiency (CVID) patients with autoimmune cytopenia. *Immunobiology,* 206**,** 502-13.

WASSERSTROM, H., BUSSEL, J., LIM, L. C. & CUNNINGHAM-RUNDLES, C. 2008. Memory B cells and pneumococcal antibody after splenectomy. *J Immunol,* 181**,** 3684-9.

WATANABE, R., ISHIURA, N., NAKASHIMA, H., KUWANO, Y., OKOCHI, H., TAMAKI, K., SATO, S., TEDDER, T. F. & FUJIMOTO, M. 2010. Regulatory B cells (B10 cells) have a suppressive role in murine lupus: CD19 and B10 cell deficiency exacerbates systemic autoimmunity. *J Immunol,* 184**,** 4801-9.

WEHR, C., EIBEL, H., MASILAMANI, M., ILLGES, H., SCHLESIER, M., PETER, H. H. & WARNATZ, K. 2004. A new CD21low B cell population in the peripheral blood of patients with SLE. *Clin Immunol,* 113**,** 161-71.

WEHR, C., KIVIOJA, T., SCHMITT, C., FERRY, B., WITTE, T., EREN, E., VLKOVA, M., HERNANDEZ, M., DETKOVA, D., BOS, P. R., POERKSEN, G., VON BERNUTH, H., BAUMANN, U., GOLDACKER, S., GUTENBERGER, S., SCHLESIER, M., BERGERON-VAN DER CRUYSSEN, F., LE GARFF, M., DEBRE, P., JACOBS, R., JONES, J., BATEMAN, E., LITZMAN, J., VAN HAGEN, P. M., PLEBANI, A., SCHMIDT, R. E., THON, V., QUINTI, I., ESPANOL, T., WEBSTER, A. D., CHAPEL, H., VIHINEN, M., OKSENHENDLER, E., PETER, H. H. & WARNATZ, K. 2008. The EUROclass trial: defining subgroups in common variable immunodeficiency. *Blood,* 111**,** 77-85.

WEI, B., VELAZQUEZ, P., TUROVSKAYA, O., SPRICHER, K., ARANDA, R., KRONENBERG, M., BIRNBAUMER, L. & BRAUN, J. 2005. Mesenteric B cells centrally inhibit CD4+ T cell colitis through interaction with regulatory T cell subsets. *Proc Natl Acad Sci U S A,* 102**,** 2010-5.

WEI, C., ANOLIK, J., CAPPIONE, A., ZHENG, B., PUGH-BERNARD, A., BROOKS, J., LEE, E. H., MILNER, E. C. & SANZ, I. 2007. A new population of cells lacking expression of CD27 represents a notable component of the B cell memory compartment in systemic lupus erythematosus. *J Immunol,* 178**,** 6624-33.

WEILL, J. C., WELLER, S. & REYNAUD, C. A. 2009. Human marginal zone B cells. *Annu Rev Immunol,* 27**,** 267-85.

WEISS, G. E., CROMPTON, P. D., LI, S., WALSH, L. A., MOIR, S., TRAORE, B., KAYENTAO, K., ONGOIBA, A., DOUMBO, O. K. & PIERCE, S. K. 2009. Atypical memory B cells are greatly expanded in individuals living in a malaria-endemic area. *J Immunol,* 183**,** 2176-82.

WELLER, S., BRAUN, M. C., TAN, B. K., ROSENWALD, A., CORDIER, C., CONLEY, M. E., PLEBANI, A., KUMARARATNE, D. S., BONNET, D., TOURNILHAC, O., TCHERNIA, G., STEINIGER, B., STAUDT, L. M., CASANOVA, J. L., REYNAUD, C. A. & WEILL, J. C. 2004. Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. *Blood,* 104**,** 3647-54.

WELLER, S., FAILI, A., AOUFOUCHI, S., GUERANGER, Q., BRAUN, M., REYNAUD, C. A. & WEILL, J. C. 2003. Hypermutation in human B cells in vivo and in vitro. *Ann N Y Acad Sci,* 987**,** 158-65.
WELLER, S., REYNAUD, C. A. & WEILL, J. C. 2005. Splenic marginal zone B cells in humans: where do they mutate their Ig receptor? *Eur J Immunol,* 35**,** 2789-92.

WEN, L., BRILL-DASHOFF, J., SHINTON, S. A., ASANO, M., HARDY, R. R. & HAYAKAWA, K. 2005. Evidence of marginal-zone B cell-positive selection in spleen. *Immunity,* 23**,** 297-308.

WIJESURIYA, R., MARUO, S., ZOU, J. P., OGAWA, M., UMEHARA, K., YAMASHITA, M., ONO, S., FUJIWARA, H. & HAMAOKA, T. 1998. B cell-mediated down-regulation of IFN-gamma and IL-12 production induced during anti-tumor immune responses in the tumor-bearing state. *Int Immunol,* 10**,** 1057-65.

WILLENBROCK, K., JUNGNICKEL, B., HANSMANN, M. L. & KUPPERS, R. 2005. Human splenic marginal zone B cells lack expression of activation-induced cytidine deaminase. *Eur J Immunol,* 35**,** 3002-7.

WIRTHS, S. & LANZAVECCHIA, A. 2005. ABCB1 transporter discriminates human resting naive B cells from cycling transitional and memory B cells. *Eur J Immunol,* 35**,** 3433-41.

WOJCIECHOWSKI, W., HARRIS, D. P., SPRAGUE, F., MOUSSEAU, B., MAKRIS, M., KUSSER, K., HONJO, T., MOHRS, K., MOHRS, M., RANDALL, T. & LUND, F. E. 2009. Cytokine-producing effector B cells regulate type 2 immunity to H. polygyrus. *Immunity,* 30**,** 421-33.

WOLF, S. D., DITTEL, B. N., HARDARDOTTIR, F. & JANEWAY, C. A., JR. 1996. Experimental autoimmune encephalomyelitis induction in genetically B cell-deficient mice. *J Exp Med,* 184**,** 2271-8.

WON, W. J. & KEARNEY, J. F. 2002. CD9 is a unique marker for marginal zone B cells, B1 cells, and plasma cells in mice. *J Immunol,* 168**,** 5605-11.

YAMADA, H., MARUO, R., WATANABE, M., HIDAKA, Y., IWATANI, Y. & TAKANO, T. 2010. Messenger RNA quantification after fluorescence activated cell sorting using intracellular antigens. *Biochemical and Biophysical Research Communications,* 397**,** 425-428.

YANABA, K., BOUAZIZ, J. D., HAAS, K. M., POE, J. C., FUJIMOTO, M. & TEDDER, T. F. 2008. A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. *Immunity,* 28**,** 639-50.

YANABA, K., BOUAZIZ, J. D., MATSUSHITA, T., TSUBATA, T. & TEDDER, T. F. 2009. The development and function of regulatory B cells expressing IL-10 (B10 cells) requires antigen receptor diversity and TLR signals. *J Immunol,* 182**,** 7459-72.

YOUNG, J. S., WU, T., CHEN, Y., ZHAO, D., LIU, H., YI, T., JOHNSTON, H., RACINE, J., LI, X., WANG, A., TODOROV, I. & ZENG, D. 2012. Donor B cells in transplants augment clonal expansion and survival of pathogenic CD4+ T cells that mediate autoimmune-like chronic graft-versus-host disease. *J Immunol,* 189**,** 222-33.

ZAGO, M. A. 1989. The evaluation of spleen function in man. *Braz J Med Biol Res,* 22**,** 159-69.
ZANDVOORT, A., LODEWIJK, M. E., DE BOER, N. K., DAMMERS, P. M., KROESE, F. G. & TIMENS, W. 2001. CD27 expression in the human splenic marginal zone: the infant marginal zone is populated by naive B cells. *Tissue Antigens,* 58**,** 234-42.

ZENZ, T., RITGEN, M., DREGER, P., KROBER, A., BARTH, T. F., SCHLENK, R., BOTTCHER, S., HALLEK, M. J., KNEBA, M., BUNJES, D., DOHNER, H. & STILGENBAUER, S. 2006. Autologous graft-versus-host disease-like syndrome after an alemtuzumab-containing conditioning regimen and autologous stem cell transplantation for chronic lymphocytic leukemia. *Blood,* 108**,** 2127-30.

ZHANG, C., TODOROV, I., ZHANG, Z., LIU, Y., KANDEEL, F., FORMAN, S., STROBER, S. & ZENG, D. 2006. Donor CD4+ T and B cells in transplants induce chronic graft-versus-host disease with autoimmune manifestations. *Blood,* 107**,** 2993-3001.

ZHOU, Z., CHEN, Z., LI, H., CHEN, X., XU, J., GU, D., DU, W., ZHENG, C., ZHANG, L., HUANG, Y., REN, Q. & YANG, R. 2009. BAFF and BAFF-R of peripheral blood and spleen mononuclear cells in idiopathic thrombocytopenic purpura. *Autoimmunity,* 42**,** 112-9.

ZHOU, Z., NIU, H., ZHENG, Y. Y. & MOREL, L. 2011. Autoreactive marginal zone B cells enter the follicles and interact with CD4+ T cells in lupus-prone mice. *BMC Immunol,* 12**,** 7.

ZORN, E., KIM, H. T., LEE, S. J., FLOYD, B. H., LITSA, D., ARUMUGARAJAH, S., BELLUCCI, R., ALYEA, E. P., ANTIN, J. H., SOIFFER, R. J. & RITZ, J. 2005. Reduced frequency of FOXP3+ CD4+CD25+ regulatory T cells in patients with chronic graft-versus-host disease. *Blood,* 106**,** 2903-11.

List of publications

Publications:

Khoder A,* de Lavallade H*, Hart H et al. Tyrosine kinase inhibitors impair B cell immune responses in CML through off-target inhibition of kinases important for cell signalling. ***Blood in press***

Khoder A, Alsuliman A, Sekine T et al. Evidence for expansion of CD21 negative B cells with an exhausted phenotype in patients with active chronic GVHD. **Submitted** to Leukemia

In preparation

Khoder A, Alsuliman A et al, $CD19^{+}CD27^{+}IgM^{hi}$ B cells exhibit IL10 dependent regulatory capacity in healthy individuals but are functionally impaired in cGVHD patients.

Abstracts and presentations:

Khoder A, Alsuliman A, Sekine T, Stringaris K, de Lavallade H, Sarvaria A, MacDonald, Jane Apperley, Rezvani K. Evidence for Expansion of $CD21^{-}$ B Cells with an Exhausted Phenotype in Patients with Active Chronic GVHD. **Oral abstract ASBMT 2013.**

Khoder A, Alsuliman A, Sekine T, Sarvaria A, de Lavallade H, Cooper N, Marin D, Stringaris K, Rezvani K. Evidence for Expansion of CD21-negative B Cells with an Exhausted Phenotype in Patients with Active Chronic GVHD. **Poster EBMT 2013.**