Elucidating the role of regulatory B cells in early pregnancy

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A thesis submitted to the University of South Australia in fulfillment of the requirements for admission to the degree of Doctor of Philosophy

June 2020

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Glossary of Terms and Abbreviations

ACK	Ammonium-Chloride-Potassium
ANOVA	Analysis of variance
B10	IL-10-producing B cell
B-CM	B cell conditioned medium
Bregs	Regulatory B cell
BSA	Bovine serum albumin
CD	Cluster of differentiation
CpG ODN	5'—C—phosphate—G—3'oligodeoxynucleotides
CTLA	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cells
DMEM	Dulbecco's Modified Eagle Media
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FBS	Fetal bovine serum
FO	Follicular
Foxp3	Forkhead box P3
FSC	Forward scatter
GFP	Green fluorescent protein
GITRL	Glucocorticoid-induced tumour-necrosis-factor-receptor ligand
HBV	Hepatitis B virus
hCG	Human chorionic gonadotropin
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IFNγ	Interferon-gamma
Ig	Immunoglobulin

IL	Interleukin
IP	Intraperitoneal
IV	Intravenous
LPS	Lipopolysaccharide
M	Medium
mAb	Monoclonal antibody
MDSC	Myeloid-derived suppressor cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MZ	Marginal zone
NK	Natural killer
PALN	Para-aortic lymph nodes
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PC	Post-coitus
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
pDC	Plasmacytoid dendritic cell
PD-L1	Programmed death-ligand 1
PI	Proliferation index
PMA	Phorbol 12-myristate 13-acetate
RA	Rheumatoid arthritis
rhIL-2	Recombinant human interleukin-2
RIF	Repeated implantation failure
RNA	Ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute 1640 Medium
SLE	Systemic lupus erythematosus
SSC	Side scatter
Sw.71	Swan 71 cell line
T1D	Type 1 diabetes
T2-MZP	Transitional 2-marginal zone precursor
TB	Trophoblast

TB-CM	Trophoblast conditioned medium
TCR	T cell receptor
TE-B	Trophoblast-educated B cell
TGF-β	Transforming growth factor-beta
Th	Helper T cell
TIM-1	T cell immunoglobulin and mucin domain 1
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNFα	Tumor necrosis factor-alpha
Tr1	Type 1 regulatory T cell
Tregs	Regulatory T cell
Tstim	Stimulated T cell
WT	Wild-type

List of Published Manuscripts

Uterine B cells exhibit regulatory properties during the peri-implantation stage of murine pregnancy

RM Guzman-Genuino, P Eldi, P Garcia-Valtanen, JD Hayball, KR Diener

Frontiers Immunology 10:2899; 2019

Trophoblasts promote induction of a regulatory phenotype in B cells that can protect against detrimental T cell-mediated inflammation

RM Guzman-Genuino, T Dimova, Y You, P Aldo, JD Hayball, G Mor, KR Diener

American Journal of Reproductive Immunology 82(6):e13187; 2019

Regulatory B Cells

RM Guzman-Genuino and KR Diener. 2018. In: eLS. John Wiley & Sons Ltd: Chichester. DOI: 10.1002/9780470015902.a0026235

Regulatory B cells in pregnancy: lessons from autoimmunity, graft tolerance, and cancer

RM Guzman-Genuino, KR Diener

Frontiers Immunology 8:172; 2017

List of Published Abstracts

Maternal B cells contribute to the regulation of inflammatory processes required for successful implantation

RM Guzman-Genuino, P Eldi, P Garcia-Valtanen, T Dimova, Y You, P Aldo, J Hayball, G Mor, KR Diener

American Journal of Reproductive Immunology 81(1 Supplement) OR13:32-33; 2019

Lymphatic and uterine B cells contribute to maternal immunological tolerance during murine pregnancy

RM Guzman-Genuino, P Eldi, P Garcia-Valtanen, JD Hayball, KR Diener

Journal of Immunology 200(1 Supplement) 118.9; 2018

Summary of Thesis

The establishment and maintenance of pregnancy primarily requires a balancing act between maintaining tolerance to the fetal allograft while retaining the immune mechanisms for protection against pathogens. This unique predicament necessitates a conditional maternal immune tolerance to prevent any undesirable reactions against paternal antigens. Research has shown that various maternal leukocyte subsets are altered during early pregnancy in order to accommodate this immune shift. Natural killer cells, macrophages, monocytes, and regulatory T cells are among the most studied subsets, however in recent years, a newly re-discovered subgroup of B cells – the regulatory B cells or Bregs – have emerged as a potential key player in the pregnancy immune network. Bregs, termed as such due to their capacity to negatively regulate other immune cell types, have been extensively studied in the fields of autoimmunity, transplantation tolerance, and cancer, with the consensus that despite their low numbers, Bregs can potently modulate immune responses.

To date, the nature and mechanism of action of Bregs in the context of pregnancy remains ill-defined. Studies in pregnancy have been few and far between over the past decade, but the seminal findings suggest that Bregs may be involved in restoring maternal immune tolerance in abortion-prone conditions in murine pregnancy and that there is a positive clinical correlation between Breg impairment and poor pregnancy outcome. Taken together with the lessons gleaned from other fields, we hypothesized that Bregs are pertinent immune cells within the pregnancy microenvironment that contribute to the overall immune conditions required in a healthy gestation.

The main objective of this thesis was to bring regulatory B cells to the fore, in terms of their involvement within the immune network during pregnancy. We investigated the basic characteristics of Bregs within the uterine microenvironment during pregnancy (Chapter 3). Herein we demonstrated that B cells acquire a regulatory phenotype by virtue of their IL-10 production and high expression of co-stimulatory molecules at the peri-implantation period. We explored the possible fetal-maternal conditions that enable a proportion of B cells to become regulatory at this timepoint (Chapter 4). We have shown here that fetal trophoblast cells can educate B cells to alter their phenotype and function to better fit the requirements for a successful embryo implantation. Lastly, we evaluated the possibility of harnessing these Bregs as a therapeutic tool to address immune-based pregnancy pathology (Chapter 5). Interestingly, our results provide preliminary evidence that Bregs are capable of compensating for the lack of regulatory T cells during pregnancy.

In *toto*, the findings obtained from the three studies support the emerging insight of a tailored B cell response during pregnancy, geared towards promoting fetal tolerance. The identification of uterine Bregs' phenotypic and functional characteristics, as well as the interaction between Bregs and other leukocytes and fetal cells are critical fundamental data that can serve as a springboard for future research aimed at elucidating the prevailing cellular network during pregnancy. Likewise, the protective effect of adoptively transferred Bregs during compromised murine pregnancy propositions Bregs as a potential therapeutic agent for an immune-based intervention. Combined with the latest innovations of allogeneic therapy and genomic editing, the future of a Breg-based therapy shows great promise and could provide a unique approach to treating immune-related pregnancy pathologies.

Declaration

I, Ruth Marian Guzman Genuino, declare that:

This thesis presents work carried out by myself and does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; to the best of my knowledge it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, is clearly acknowledged.



Acknowledgments

Undertaking this PhD has been a wonderful experience for me, and it would not have been possible to do without the support and guidance that I received from many people.

First, I would like to give my deepest gratitude and appreciation to my PhD supervisor Dr Kerrilyn Diener. You have graciously imparted your time, wisdom, kindness, and patience in moulding me to become a better scientist, and also a better person; for that I am eternally grateful. I can only aspire to be like you – a strong and successful scientist but also a nurturing mother to your biological (Oscar) and adoptive (graduate students) offspring.

I am equally grateful for Prof John Hayball, for taking a chance on a random applicant for an RA position back in 2015. I will never forget reciting my Master's thesis summary over the phone interview, shouting over the noise of the thunderstorm. Thank you for taking me under your wing. It has been a joy to be part of your lab family.

I would also like to thank my co-supervisors, Dr Preethi Eldi and Dr Pablo Garcia-Valtanen for your invaluable contributions, especially for teaching and training me in labwork and writing. Together with the ETL postdocs, Dr Tamara Cooper, Dr Liang Liu, and Dr Natalie Prow, thank you for always fostering a conducive environment for us to grow as budding researchers and for always being there to help, encourage, and cheer us on during the tough times.

Many thanks to our collaborators at the Mor Lab of Yale School of Medicine, most especially to Dr Gil Mor, Ms Paulomi Aldo, Mr Yuan You, and Dr Tanya Dimova. I had the most delightful experience working with the most brilliant, yet ever so humble, bunch of people I have come across with.

I am eternally grateful for my PhD colleagues and friends, Dr Jessica Day, Ms Abi Trinidad, Ms Nerissa Lakhan, Ms Fan Jia, Dr Noralyn Tan, Ms Jamie Zhang, Ms Robyn Kievet and Mr Gary Heinemann for accompanying me in my PhD journey and for keeping me sane throughout it all. I shall now pass on the A-OK Lucky China Man tin can over to you guys.

I gratefully acknowledge the funding sources that made my PhD work possible – the UniSA scholarship, the Australian Government RTPd scholarship, the Robinson Research Institute Seeding Fund Program, the ETL lab, and the Mor Lab. I also greatly appreciate the financial support received from UniSA, ASI, and ASRI towards travel as a visiting researcher and conference attendee.

Special thanks to my family: to my parents Ruth and Roger, siblings Cheng, Chee, Rina, and Rocky, and my nephews Arden and Andric. To my parents who raised me with a love for science and supported me in all my pursuits. Thank you for all your prayers, love and encouragement.

Finally, I would like to extend my deepest appreciation to my husband, Ryan Genuino, for your unwavering support, patience, and love throughout this journey. For being my rock and my person – I cannot even begin to express how grateful I am. And last but certainly not least, to little Lukas, my darling baby boy, for always inspiring mum to keep on keeping on. Thank you.

Funding

I have received scholarship and research grant funding support for my doctoral candidature from the following bodies:

Australian Government Research Training Program domestic (RTPd) Scholarship

University of South Australia President's Scholarship (UPS)

UniSA Research Degree Students International Travel Grant

Australian and New Zealand Society of Immunology (ASI) Travel Grants

American Society for Reproductive Immunology Travel Grant

The research project has been funded by the following bodies:

Robinson Research Institute Innovation Seed Funding Program

Experimental Therapeutics Laboratory, University of South Australia

Mor Laboratory, Yale School of Medicine, Yale University

Chapter One

GENERAL INTRODUCTION

This is an updated, combined, and modified version of the following publications:

Guzman-Genuino RM, Diener KR. Regulatory B cells in pregnancy: Lessons from autoimmunity, graft tolerance, and cancer. *Frontiers Immunol*. *8:172; 2017.*

Guzman-Genuino RM, Diener KR. Regulatory B Cells. 2018. In: eLS. John Wiley & Sons, Ltd: Chichester. DOI: 10.1002/9780470015902.a0026235

Overview

Pregnancy is a remarkable biological process wherein the mother carries an offspring that is semi-allogenic, that is, half of its genome is derived from the foreign paternal parent. It imposes significant physiological stress, requiring the mother's body to undergo immense functional changes. Pregnancy in mammals represents a unique compromise in the context of immunity – the maternal immune system must accommodate the semi-allogenic fetus by dampening its immune responses to maintain a state of immunological tolerance throughout gravidity, whilst retaining the capacity to identify pathogens and destroy them with appropriate control of resultant inflammation. This dichotomy necessarily requires a fine and highly regulated balance between immune tolerance and immune activation.

In pregnancy, the immune profiles of dendritic cells, natural killer cells, macrophages, and T cells are by necessity modified (Parham, 2004;Segerer et al., 2012;Faas et al., 2014;Ruocco et al., 2014). Recently, it has been shown that the B cell profile is also modified in response to the immunological needs of the mother during pregnancy (Muzzio et al., 2014). The role of B cells in pregnancy holds much interest, especially in light of the recent identification of a B cell subset capable of negative regulation, that is, can suppress immune inflammation. Due to their unique role amongst the B cell types, this subset is aptly designated as 'regulatory B cells' or 'Bregs'. The role of Bregs in pregnancy has been subject to very limited investigation to date. However, research pursuits in other fields involving immune dysregulation such as autoimmunity, transplantation tolerance, cancer, allergy, and infectious diseases have kindled a growing interest in the field of pregnancy. Current literature demonstrates that Breg mechanisms of function and effect on other immune components are similar across conditions of immune dysfunction and thus, may be also applicable in the context of pregnancy. Moreover, the current state of knowledge on the functions of Bregs in these different disease conditions provide pointers as to the possible role of Bregs may play in aiding the maternal immune system's capacity for selective tolerance during pregnancy. For example, autoimmune diseases reflect an inadequate ability to control an overactive immune response to self while cancer develops from an enthusiastic suppression of the immune system that allows a mutated self to survive and proliferate. Bregs, as mediators of immune suppression, have been shown to inhibit immune flares or chronic inflammation in the former while acting as pro-tumorigenic agents that inhibit the action of effector immune cells and foster a favourable environment for tumour growth in the latter. Pregnancy may be viewed as a reconciliation of these two extreme conditions

wherein Bregs can induce maternal immune tolerance while also allowing the growth and development of a semi-allogeneic fetus in the mother's womb.

1.1 Regulatory B cells: characteristics and general properties

B cells are best known to organize immune responses by production of antibodies. Representing the humoral arm of adaptive immunity, they are the primary facilitators of antigen-specific immune responses via antibody production and differentiation into memory cells that provide long-lasting immunity. However, reports over the past 40 years indicate that not all B cells function for that purpose. The earliest studies (1974) found that B cells could suppress delayed-type hypersensitivity reactions in guinea pigs, implying an inhibitory effect of B cells on T cell function (Katz et al., 1974;Neta and Salvin, 1974). Further evidence of this B cell regulatory phenotype eventuated more than two decades later, with the observation in a murine autoimmune model that inflammation was exacerbated in the absence of B cells (Wolf et al., 1996). Whilst this suggested that B cells may play a down-modulating role in the inflammatory response, it was only in 2000 that Mizoguchi et al. formally described and reported a subset of B cells that inhibited, rather than promoted, the inflammatory response in a mouse model of inflammatory bowel disease (Mizoguchi et al., 2000). This peculiarly suppressive B cell subset was classified as 'regulatory B cells' or 'Bregs'. Since then, defective Breg function or deficiency in Breg levels have been implicated in conditions involving uncontrolled pro-inflammatory immune responses; most extensively in autoimmune diseases and renal transplantation cases (Fillatreau et al., 2002;Matsushita et al., 2008;Newell et al., 2010;Silva et al., 2012).

1.1.1 Breg phenotypic identification

Defining a specific Breg phenotype has proven to be a difficult as multiple B cell 'subsets' have been reported to function as negative regulators of the immune response. Whilst there is no unifying characteristics with respect to cell surface activation and lineage markers as of yet, initial reports indicated that the regulative properties of these unique B cells were attributed exclusively to the production of the anti-inflammatory cytokine interleukin-10 (IL-10) (Fillatreau et al., 2002;Mizoguchi et al., 2002;Mauri et al., 2003). However, more recent studies have revealed B cell subsets with IL-10-independent regulatory functions, indicating that some Bregs employ a multi-mechanistic, and possibly cooperative, approach for regulating immune responses. Given the lack of a unified approach and as IL-10

production is the most reported mechanism of suppressive action; IL-10 production remains the defining feature of Bregs.

Different B-cell subsets that have been attributed with regulatory function in mice include the transitional 2 marginal-zone precursor (T2-MZP) cells, $CD5^{+}CD1d^{hi}IL10^{+}$ B (B10) cells, follicular (FO) B cells, marginal-zone (MZ) B cells, $CD5^{+}$B1a cells, $CD5^{+}CD178^{+}$ killer B cells, GIFT-15 B cells, plasma cells, plasmablasts, TIM-1^{+} B cells, and PD-$L1^{hi}$ B cells (Mauri and Menon, 2015;Rosser and Mauri, 2015) (Table 1.1). In humans, immature B cells, IL-10^{+} B cells (B10), GrB^{+} B cells, Br1 cells, and plasmablasts are reported to play immunosuppressive roles (Mauri and Menon, 2015). Despite the diversity in phenotype, most B cell subsets that carry out negative regulation produce anti-inflammatory cytokines, with the majority of the cell surface marker-defined subsets enriched with IL-10 producing cells. In mice, the suppressive IL-10 producing Bregs, also known as B10 cells are characterized by the $CD1d^{hi}CD5^{+}$ phenotype (Yanaba et al., 2008). Among the splenic B10 cells, both marginal-zone (MZ) B cells and transitional 2-MZ precursor (T2-MZP) B cells have been shown to have a protective effect in mouse models of lupus and autoimmune arthritis due to their IL-10 competency (Lenert et al., 2005;Evans et al., 2007). The peritoneal cavity contains B-1a cells which are also a major source of IL-10 (O'Garra et al., 1992). In humans, $CD19^{+}CD24^{hi}CD38^{hi}$ B cells isolated from human peripheral blood are classified as Bregs due to their ability to suppress inflammation by a combination of IL-10 production and CD80 and CD86 co-stimulation (Blair et al., 2010), while the IL-10-competent $CD24^{hi}CD27^{+}$ B cells are proposed as the Breg subset analogous to the mouse regulatory B10 cells (Iwata et al., 2011). Bregs in humans include immature or transitional B cells, B10 cells, memory B cells, plasmablasts, GrB^{+} B cells, Br1 cells and TIM-1^{+} B cells (Table 1.2). The heterogeneity of these subsets suggests that Bregs are not derived from one specific lineage; rather they may acquire their regulatory ability through exposure to environmental stimuli.

Since surface markers identifying these subsets are varied, there are currently ongoing attempts to identify a unique global indicator for Bregs, analogous to the transcription factor Foxp3, a defining feature of regulatory T cells. Evidence suggests that the T cell immunoglobulin and mucin domain 1 (TIM-1) may be an inclusive marker for Bregs as it identifies about 70% of all IL-10^{+} B cells in all subsets mentioned (Ding et al., 2011). However, CD9 has also been reported as a marker for murine IL-10-competent regulatory B cells by virtue of transcriptomic analysis which strongly associated its expression with Breg

biogenesis and function (Sun et al., 2015). A recent study distinguished between B cells that are immediately prone and developmentally committed to IL-10 production and B cells that would require a longer stimulation to produce IL-10 via their DNA methylation profile within the IL10 gene locus, with the former group bearing an *early IL10 regulatory region* (*eIL10rr*) and the latter characterized by a *delayed IL10 regulatory region* (*dIL10rr*) (Tonon et al., 2019) The differences in the methylation signatures signify that Bregs may also be an epigenetically controlled functional state of mature B cell subsets.

In principle, identification of a unique marker signifying a direct regulatory function in B cells would reduce the reliance on functional assays and increase our understanding of the role that Bregs may play in different diseases, and allow exploration of therapeutic options. To date, elucidating the Breg phenotype and underlying ontology continues to be an active research endeavour.

Table 1.1: Murine regulatory B cell subsets

Subsets	**Phenotype**	**Mechanism**
B10	$CD5^{+}CD1d^{hi}$	Produce IL-10; suppress effector $CD4^{+}$ T cell and monocytes
MZ	$CD19^{+}CD21^{hi}CD23^{-}$ $CD24^{hi}IgM^{hi}IgD^{hi}IgD^{lo}CD$ $1d^{hi}$	Produce IL-10; induce Treg cells and suppress effector $CD8^{+}$ and $CD4^{+}$ T cells
T2-MZP	$CD19^{+}CD21^{hi}CD23^{hi}$	Produce IL-10: induce Treg cells and suppress effector $CD8^{+}$ and $CD4^{+}$ T cells
B-1a cells	$CD19^{+}CD5^{+}$	Produce IL-10; suppress effector $CD4^{+}$ T cells
TIM-1^{+} B cells	TIM-$1^{+}CD19^{+}$	Produce IL-10; Suppress effector $CD4^{+}$ T cells
Plasma cells	$CD138^{+}IgM^{+}CD1d^{hi}CXC$ $R4^{+}MHC\text{-}11^{lo}$	Produce IL-10 and IL-35; suppress NK cells, neutrophils and effector $CD4^{+}$ T cells
Plasmablasts	$CD138^{+}CD44^{hi}$	Produce IL-10; suppress DCs and effector $CD4^{+}$ T cells
GIFT-15 B cells	$B220^{+}CD21^{+}CD22^{+}CD23^{+}$ $CD24^{+}CD1d^{+}CD138^{+}IgD^{+}$ IgM^{+}	Produce IL-10; suppress IFN-γ response
PD-L1hi B cells	$PD\text{-}L1^{hi}CD19^{+}B220^{+}$	Regulate humoral responses through cognate interactions with PD-L1
Killer B cells	$CD19^{+}CD5^{+}CD178^{+}$	Induce cell death through FasL

Table 1.2: Human regulatory B cell subsets

Subsets	Phenotype	Mechanism
B10	$CD24^{hi}CD27^{+}$	Produce IL-10; suppress effector $CD4^{+}$ T cells and monocytes
Transitional B cells	$CD19^{+}CD24^{hi}CD38^{hi}$	Produce IL-10; induce Tregs, suppress Th1 and Th17 cells
Memory B cells	$CD19^{+}CD27^{+}IgM^{+}$	Produce IL-10; suppress $CD4^{+}$ T cell proliferation and effector function
Plasmablast	$CD19^{+}CD38^{hi}CD24^{hi}CD27^{int}$	Produce IL-10; suppress DCs and effector $CD4^{+}$ T cells
GrB+ B cells	$CD19^{+}CD38^{+}CD1d^{hi}IgM^{+}CD147^{+}GrB^{+}$	Express Granzyme B; suppress T cell proliferation
Br1 cells	$CD19^{+}CD25^{hi}CD71^{hi}CD73^{lo}$	Produce IL-10 and IgG4
TIM-1+ B cells	$CD19^{+}TIM\text{-}1^{+}$	Produce IL-10; suppress T cell function

1.1.2 *Breg suppression mechanisms*

The best studied mechanism for negative regulation of Bregs is their capacity to produce anti-inflammatory cytokines, especially IL-10. The principal function of IL-10 is to repress excessive inflammation and maintain homeostasis through numerous mechanisms, which includes hampering antigen presentation and expression of co-stimulatory molecules CD80, CD86, and MHC Class II by macrophages and dendritic cells, as well as limiting the activation of and cytokine release by T cells in immune responses and promoting the differentiation of regulatory T cells (Pestka et al., 2004).

While IL-10 production remains the hallmark of Breg's negative regulatory function, other independent suppressive mechanisms have come to light in recent years and include transforming growth factor beta (TGF-β), IL-35, and IL-17. Production of TGF-β by B cells leads to promotion of Treg development in diabetes, transplant tolerance, allergic diseases, and colitis (Ray et al., 2015). Moreover, about 60% of IL-10 producing B cells in mice also express IL-35, a cytokine identified as a key player in the recovery phase of experimental autoimmune encephalomyelitis (EAE), and necessary for improved resistance to *Salmonella*

infection (Shen et al., 2014). Secretion of IL-17 by activated B cells has been reported to occur upon contact with parasite-derived trans-sialidase, a feature shown to be critical in the control and resolution of infection (Bermejo et al., 2013). This result emphasizes the importance of Breg function in a disease that has a high potential to develop into chronic inflammation.

Various surface molecules on B cells have also been identified as the main instigators of negative regulation upon cognate interaction with pro-inflammatory cells. Among the most studied are programmed death receptor ligand 1 (PD-L1), Granzyme B, and Fas ligand (FasL). Upregulated PD-L1 expression on B cells is critical in down-modulating T cell activity. In EAE, adoptive transfer studies demonstrate that PD-L1$^+$ B cells confer protection and reduce disease severity via restriction of helper T cells' inflammatory role and induction of Treg activity (Bodhankar et al., 2011;Bodhankar et al., 2012;Bodhankar et al., 2013). Furthermore, PD-L1$^+$ B cells have been shown to regulate cytotoxic T cells in both *Salmonella* infection and prostate cancer (Lopez-Medina et al., 2015;Shalapour et al., 2015), with high expression shown to directly suppress the follicular helper T cell response and differentiation via attenuation of downstream signalling pathways concomitant to PD-1 ligation (Khan et al., 2015). Similarly, B cells expressing FasL were found to be critical in preventing graft rejection in mice and modulating the induction of autoimmune disease through a myriad of mechanisms including promotion of apoptosis of autoreactive T cells and the generation of Tregs (Minagawa et al., 2004;Lundy and Fox, 2009). Lastly, IL-21 induced granzyme B expression in B cells was shown to impart a regulatory function against effector T cells in the tumour microenvironment (Lindner et al., 2013). As to the exact mechanism by which Bregs facilitate modifications in T cell phenotype and function, there is evidence that Bregs-T cell contact is enhanced by antigen-specific priming and that IL-10-producing Bregs establish frequent and longer cell-cell contacts with T cells compared to non-Bregs (Mohib et al., 2019).

Thus far, the mechanisms of suppression associated with Bregs include the following: inhibit cytotoxic CD8$^+$ T cells, hinder activation of naïve CD4$^+$ T cells, promote the generation of Foxp3$^+$ Tregs and Tr1 cells; suppress helper T (Th) cell differentiation; inhibit the activation of monocytes and plasmacytoid dendritic cells; maintain the immature state of dendritic cells and stimulate natural killer T-cell activity (Figure 1.1).

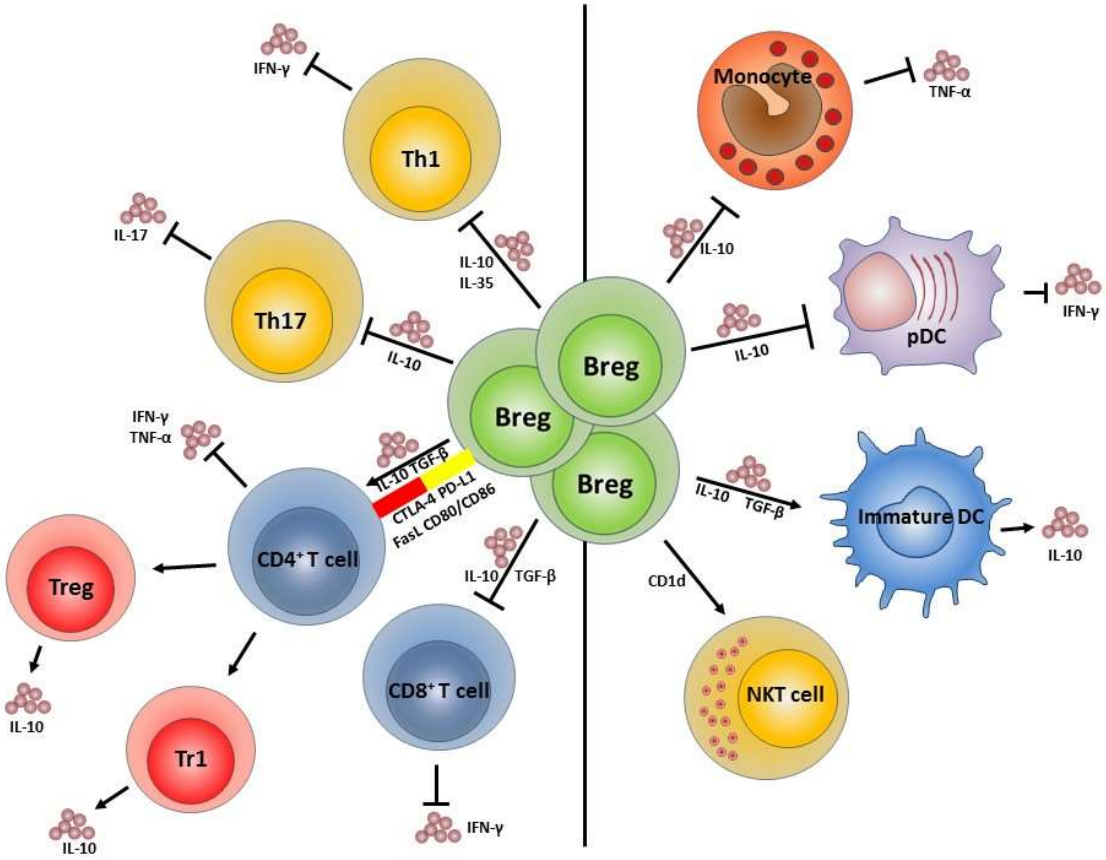


Figure 1.1. Mechanisms of Breg suppression. Bregs inhibit Th1 and Th17 responses, inducing a Th2-like bias to the T-cell response; Bregs also inhibit cytotoxic $CD8^+$ T cells, curtail effector $CD4^+$ T-cell proliferation, and abrogate the proinflammatory cytokine response, pushing instead the differentiation of $CD4^+$ T cells into Tregs and Tr1s (left panel). Bregs likewise affect other immune cells such as monocytes, dendritic cells and natural killer cells (right panel). (Guzman-Genuino and Diener, 2018)

1.1.3 *Breg detection and expansion*

Due to the inconsistencies in specific surface markers used to classify Breg subsets, the most common approach for Breg identification remains a combination of surface marker staining and intracellular IL-10 staining. In mice, isolated Bregs do not readily produce IL-10 *ex vivo*, therefore to identify this specific subset of B cells, cells are subjected to acute B cell stimulation – 5 hours of culture with a cocktail of lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA), and ionomycin to enhance the translation of cytokine genes, and thus detectable IL-10 production (Matsushita and Tedder, 2011). In the spleen and other lymphoid tissues, Breg progenitor cells have also been identified (Yanaba et al., 2009).

These are defined as B cells that do not express IL-10 following the short-term stimulation but may be induced to Breg maturation and IL-10 competency by culture with agonistic CD40 monoclonal antibody for 48 hours. This technique has proven beneficial for expanding this rare subset for subsequent *in vivo* applications such as adoptive transfer experiments. Together, active and progenitor Bregs account for 3-8% of basal B cell levels in the murine spleen, of which 1-3% are IL-10 competent Bregs. Lower percentages are found in the blood, lymph nodes, intestinal tissues, Peyer's patches, and the central nervous system (Yanaba et al., 2008;Matsushita et al., 2010;Maseda et al., 2013). In humans, IL-10-producing B cells isolated from the peripheral blood are found to express increased levels of CD19, IgD, CD27, CD48, and/or CD148 at varying proportions and phenotypic combinations. IL-10 competent Bregs have also been identified in peripheral tissues such as spleen, tonsils, and umbilical cord blood at less than 1% of the total B cell population (Iwata et al., 2011).

1.2 Regulatory B cells: current state of knowledge

1.2.1 Bregs in autoimmunity

In the last decade, numerous studies have been conducted to determine the operating mechanisms that impart Breg-mediated suppressive function. The majority of what is currently known stems from the multitude of studies focusing on autoimmune diseases where it has been demonstrated that Bregs interact and regulate the function of T cells, dendritic cells, monocytes and macrophages, and natural killer cells.

The regulatory role of B cells in EAE, a mouse model for multiple sclerosis, was first apparent when exacerbation of disease was observed in B cell-deficient B10.PLμMT mice (Wolf et al., 1996). The current model posits that Bregs regulate EAE severity through IL-10 production and expression of costimulatory molecules CD40, CD80, and CD86, thus limiting the type 1 cytokine response via cross-talk between B cells and T cells, and recruitment of $FoxP3^+$ Tregs into the central nervous system (Mann et al., 2007;Matsushita et al., 2008). B cells expressing toll-like receptors (TLR)-2 and TLR-4 also suppressed inflammatory T cell responses (Th1 and Th17) and stimulated the recovery phase of EAE (Lampropoulou et al., 2008). Other mechanisms such as the presence of glucocorticoid-induced TNF ligand (GITRL) and elevated levels of B and T lymphocyte attenuator (BTLA) on B cells also play a role in inducing and maintaining the Treg pool in the central nervous system during EAE, a process which ultimately facilitates the protection against or

amelioration of the disease (Ray et al., 2012;Huarte et al., 2016). Interestingly, recent evidence indicates that Bregs can also directly access the CNS. Bone marrow cells transiently stimulated with the TLR9 agonist CpG generate proB (CpG-proB) cells, which upon transfer to recipient mice at the onset of EAE symptoms differentiate into mature B cells with regulatory function. Some of these mature B cells home to the inflamed CNS for local production of IL-10 thus instigating a switch of the host cytokine profile from inflammatory to immunoregulatory, whilst others enter reactive lymph nodes and restrain encephalitogenic T cells via CCL19 expression. Together these functions co-operatively enhanced amelioration of active EAE (Korniotis et al., 2016). MicroRNAs (miRNAs) have also been shown to influence the development of IL-10^{+} Breg cells, particularly that of miR-21 expression in B cells identified as a regulator of Breg cell differentiation. Silencing of miR-21 in the B cells led to an increased number of IL-10^{+} Bregs which in turn dramatically alleviated the clinical signs of EAE, thus providing evidence of a B cell-intrinsic miRNA pathway that may be explored as a potential therapeutic target for the control of autoimmune inflammation (Wang et al., 2017). Evidence of the regulatory function of B cells in human disease is also accumulating, with B cells from multiple sclerosis (MS) patients observed to have diminished capacity for IL-10 production on activation as opposed to healthy controls. This suggests that dysregulation of this pathway contributes to MS pathogenesis (Duddy et al., 2007;Hirotani et al., 2010;Knippenberg et al., 2011).

Type 1 diabetes (T1D) is characterized by the autoimmune-mediated destruction of pancreatic β cells. Circulating $CD19^{+}CD27^{-}CD24^{hi}CD38^{hi}$ Bregs have been found to decrease with age and as such are hypothesized to be a major contributing factor to the prevalence of T1D in older age groups (Thompson et al., 2014). Patients with T1D also exhibit the lowest frequency of B10 cells compared with patients with Type 2 diabetes, latent autoimmune diabetes in adults, and healthy controls (Deng et al., 2016). These results highlight the importance of B cells in the immunopathogenesis of autoimmune diabetes and suggest that antigen-activated B10 cells may have a role to play in inhibiting autoreactive T cell responses to islet-specific antigen in healthy individuals, as the absence of this population leads to hyperglycaemia in T1D patients. In non-obese diabetic (NOD) mice, adoptive transfer of $CD1d^{hi}CD5^{+}$IL-10^{+} Bregs prevented T1D development. Further, the addition of tolerogenic dendritic cells (DC) to the transferred therapeutic mix reversed the onset of T1D by augmenting the frequency of IL-10^{+} Bregs (Di Caro et al., 2014). In the same manner, adoptive transfer of CpG-proB cells, either directly or indirectly after maturation into mature Bregs, protected against the pathogenesis of T1D in NOD mice via

suppression of IL-21 and induction of apoptosis in effector T cell populations, including specific diabetogenic T cells (Montandon et al., 2013). The DC- and CpG-mediated suppression of T1D through Breg activity reiterates the notion that B cells have an intrinsic suppressive potential, which is only activated when the environmental cues are appropriate.

In systemic lupus erythematosus (SLE), regulatory B cells are emerging as vital players during disease initiation. Adoptive transfer of splenic $CD5^{+}CD1d^{hi}$ Bregs from wild type mice extended the survival period of $CD19^{-/-}$ mice in this spontaneous lupus model (Watanabe et al., 2010). In humans, SLE patients exhibited similar frequencies of circulating $CD24^{hi}CD38^{hi}$ B cells but had significantly lower $IL10^{+}$ percentages compared to normal healthy controls (Heinemann et al., 2016). Further, there is evidence of functional impairment in Bregs from SLE patients. Isolated Bregs were unresponsive to CD40 ligation and exhibited lower IL-10 production compared to Bregs obtained from normal patients, which resulted in inadequate suppression of T cell activation and proliferation (Gao et al., 2014). Compromised cross-talk between IL-10-producing $CD24^{+}CD38^{hi}$ Bregs and interferon alpha (IFNα)-producing plasmacytoid dendritic cells (pDCs) has also been documented as a key contributor to the pathogenesis of SLE (Menon et al., 2016).

Studies of rheumatoid arthritis (RA) suggest that Bregs are responsible for the primary induction of Tregs. Specifically, IL-10 from B cells aids in the establishment of the Treg to inflammatory Th1 and Th17 cell ratio as seen in chimeric ($IL10^{-/-}$ B cell) mice which exhibit a significant decrease in Foxp3-expressing $CD4^{+}$ T cells; the low number of Tregs occurred in parallel with an increase in Th1 and Th17 cell populations in the draining lymph nodes of the inflamed joints (Carter et al., 2011). Furthermore, the adoptive transfer of Bregs into these chimeric mice restored the Treg population to normal levels and decreased inflammation by inhibiting Th1 and Th17 differentiation, thus decreasing IFNγ and IL-17 cytokine levels, and reinstating a balanced Th1/Th2 response (Carter et al., 2012). This notion of Bregs influencing T cell plasticity was similarly demonstrated by injection of exogenous B10 cells which effectively suppressed the development of arthritis by suppressing Th17 cell generation (Yang et al., 2012). Clinical studies report decreased frequency of $CD24^{hi}CD38^{hi}$ Bregs in RA patients compared to unaffected individuals; it was shown that normal levels of Bregs effectively inhibited $CD4^{+}CD25^{-}$ T cell differentiation into Th1 and Th17 cells and promoted the differentiation of T cells into Tregs via IL-10 production (Flores-Borja et al., 2013;Mielle et al., 2018). Similarly, $PD\text{-}L1^{+}$ Bregs capable of suppressing $CD8^{+}$ T cell proliferation were significantly lower in untreated RA patients

compared to healthy controls, but demonstrated significant increase in numbers after treatment in good responder patients (Zacca et al., 2018). Moreover, it has been demonstrated that many RA patients achieve remission during pregnancy, presumably due to a shift in T cell function from a Th1 to a Th2 phenotype, but relapse postpartum, a phenomenon possibly correlated to the increase of Breg levels during pregnancy and consequent decrease upon parturition (Ostensen et al., 2005;de Man et al., 2008;Rolle et al., 2012).

The regulatory function of human B cells in autoimmune diseases is realized in case studies where complete B cell depletion therapy with rituximab (anti-CD20 antibody) has resulted in aggravation of the disease or onset of new immune-mediated pathologies. For instance, a patient with intractable ulcerative colitis experienced severe clinical aggravation of disease upon treatment with rituximab, a result ascribed to the depletion of both the intestinal and systemic suppressive B cell population (Goetz et al., 2007). Similarly, rituximab was an effective treatment in a patient with Graves' disease; however development of ulcerative colitis and arthritis was observed shortly thereafter. Again, the depletion of colonic B cells was implicated, with symptoms alleviated upon repopulation (El Fassi et al., 2008b).

Therefore, in autoimmune disease, the suppressive role of Bregs in the framework of T cell function occurs at two levels: (1) control of T cell plasticity by invoking the production of Tregs, inhibiting activation of naïve T cells, and suppressing helper T cell (Th) differentiation; and (2) manipulation of the production of pro- and anti-inflammatory cytokines and their balance thereof.

1.2.2 Bregs in transplantation tolerance

Graft tolerance has been best studied in liver and kidney transplantations. Post-operative care involves continuous treatment with immunosuppressive drugs to prevent the immune response to the alloantigen, which may lead to organ rejection. Some recipients require immunosuppression medication for their entire lifetime; however there are reports of operationally tolerant recipients maintaining graft function despite immunosuppressive drug withdrawal (Roussey-Kesler et al., 2006). The containment of the inflammatory response that typically ensues without immunosuppressive drugs points to the valuable function of regulatory immune cells – the Tregs which have an established role in graft tolerance, and the Bregs, whose role in transplantation has recently emerged.

Initial reports implicating a role for B cells as facilitators of graft tolerance resulted from studies investigating the effect of CD40 blockade on resting B cells. Permanent survival of mouse pancreatic islet allografts was demonstrated upon pre-treatment with allogeneic non-T cell small lymphocytes (elutriated from spleen cell suspensions) plus blocking antibody to CD40L (Parker et al., 1995). Moreover, pre-treatment of resting B cells with anti-CD40 monoclonal antibody prolonged fully allogeneic mouse cardiac allografts and augmented the hyporesponsiveness to MHC molecules *in vivo* (Niimi et al., 1998), suggesting a dominant role of the CD40/CD40L pathway in tolerance induction. The mechanism by which anti-CD40 antibodies can elicit a tolerogenic response was elucidated a decade later when it was demonstrated that CD40 ligation resulted in the differentiation of IL-10^+ T2 Bregs, the rescue of B cells and transitional B cell subsets from apoptosis, as well as prevention of differentiation into mature follicular B cells. Specifically, anti-CD40 Ab administration induced the differentiation of T2 and MZ B cells and expanded the population of IL-10^+ Bregs *in vitro* and *in vivo*, resulting in significantly improved renal disease and controlled progression of lupus in mice (Blair et al., 2009). This was also reflected in tolerant kidney and cord blood transplant patients where increased CD40 ligation potentiated IL-10 production by naïve and transitional B cells (Nova-Lamperti et al., 2016;Sarvaria et al., 2016).

In a rat model of kidney allografts, intravenous injection of bulk donor B cells to the recipient at the time of transplantation was by itself proven to be effective in inducing long-term acceptance (more than 300 days) of the graft. This was a significantly longer time period than when donor T cells were administered 17 days after transplantation (Yan et al., 2002). Studies of islet allografts in mice identified TIM-1 as an inclusive marker for IL-10 and IL-4 expressing regulatory B cells in all major B cell subpopulations including the transitional, marginal zone, follicular, and $CD5^+CD1d^{hi}$ cells. More importantly, ligation by anti-TIM-1 induced the regulatory function of these B cells constitutively expressing TIM-1 via IL-10 production. Adoptive transfer of TIM-1^+ B cells from untreated BALB/c allograft recipients into chemically diabetic B cell-deficient allograft recipients prolonged graft survival by activation of transferred IL-10 producing TIM-1^+ B cells and augmented the frequency of $FoxP3^+$ Tregs (Ding et al., 2011). Moreover, the mucin domain of TIM-1 has been identified as being primarily responsible for Breg induction and maintenance in prolonged allograft survival (Yeung et al., 2015). A follow-up study demonstrated that $CD5^+CD1d^+$ B10 cells played an important role in the early stages of islet transplantation tolerance induction but

were not necessarily involved with long-term graft survival, however TIM-1^+ B cells were found to increase at a later time post-transplantation and correlates with the maintenance stage of tolerance induction (Li et al., 2018). Aside from TIM-1^+ Bregs, tolerance in a mouse model of MHC class I mismatched skin transplantation was associated with a transient expansion in transitional-2 (T2) B cells in mice tolerized by donor splenocyte transfusion (DST) and ligation with CD40L. These tolerized B cells were characterized by expression of downregulated levels of CD86 and upregulated levels of TIM-1, and prolonged skin allograft survival *in vivo*, as well as suppressed T cell activation *in vitro*. Moreover, it was seen in this study that tolerized B cells and graft-specific Tregs worked synergistically in prolonging graft survival, albeit in an IL-10-independent fashion (Moreau et al., 2015). Similarly, the adoptive transfer of TGF-β producing Bregs induced the expression of FoxP3 in $CD4^+CD25^-$ T cells, thus activating and expanding the Treg population which contributed to protracted graft survival (Lee et al., 2014). These reports reinforce the multi-mechanistic mode of suppression by Bregs in that through IL-10, TGF-β, CD86 costimulation, and other surface molecules, antigen-specific tolerized B cells no longer stimulate allospecific T cell activation, but instead support the induction of Treg function to enact a tolerogenic response.

In humans, research has also indicated a critical role for B cells in regulating alloimmunity. For operationally tolerant renal transplant recipients, renal allograft tolerance was strongly associated with a B cell signature consisting of an increased expression of multiple B cell differentiation and activation genes over that observed in non-tolerant patients receiving immunosuppression and non-transplanted controls. The 3 genes identified as reliable predictors of operational tolerance – *IGKV4-1, IGLL1,* and *IGKV1D-13* – are genes that are upregulated during transition from pre-B to mature B cells and during class switching post-antigen stimulation, suggesting that transitioning B cells may be highly involved in tolerance induction and/or maintenance (Newell et al., 2010). Moreover, operationally tolerant patients displayed higher transitional ($CD24^{hi}CD38^{hi}$) and naïve B cell frequencies, lower numbers of memory B cells, and an enriched IL-10^+ B cell population – all phenotypes that were strongly associated with reduced allograft rejection rates (Leibler et al., 2014;Newell et al., 2015;Shabir et al., 2015;Bigot et al., 2016). Together these recent findings suggest assigning a prospective biomarker status on transitional B cells. Additionally, operationally tolerant patients retain the capacity to activate the CD40 pathway essential to Breg activation (Silva et al., 2012), and although IL-10 producing Bregs are higher in these patients, other mechanisms such as granzyme B^+ and TGF-β production may also account for the inhibitory

effect on T cells essential to maintaining transplantation tolerance (Lee et al., 2014;Chesneau et al., 2015).

1.2.3 Bregs in cancer

Oncogenesis is primarily a battle between the immune system and the cancer stem cells. Tumor development and progression is dependent on the lenience of the microenvironment, to which leukocytes play a critical role. Both anti-tumorigenic and pro-tumorigenic immune mechanisms transpire in the early stages of cancer development, with the net effect dictating whether the tumour develops or not (Grivennikov et al., 2010). In particular, the induction of immune tolerance within the tumour environment has been shown to curtail immune-enhancing anti-tumorigenic efforts and encourage tumour progression and subsequent metastases. Immunosuppressive cells such as Bregs and Tregs have been implicated in facilitating immune tolerance, and ultimately, cancer escape.

For instance, tumour growth is contingent on the immune profile within the local microenvironment. Strong infiltration of anti-tumour $CD8^+$ T cells and natural killer cells and decreased levels of regulatory B and T cells and myeloid-derived suppressor cells all result in tumour destruction, whereas reversed proportions support tumour growth. In the tumour-draining lymph nodes, increased accumulation of B and T cells resulted in lymphangiogenesis and increased lymph flow (Harrell et al., 2007), which contributed to and accelerated the rate of metastasis (Ruddell et al., 2011). Moreover, B cell accumulation in lymph nodes of preneoplastic Eμ-*c-myc* mice was positively correlated with expansion of the lymphatic sinuses and enhanced tumour growth, as opposed to preneoplastic B cell-deficient μMT mice which did not undergo lymphangiogenesis and exhibited minimal tumour growth (Ganti et al., 2015). However, tumour growth was accelerated in the B cell-deficient μMT mice upon adoptive transfer of naïve T2-MZP cells, highlighting the role of transitional and suppressive B cells in cancer escape. Furthermore, B cells bearing downregulated MHC class II and CD86 expression and upregulated Ly6A/E, PD-L1, and CD39 selectively accumulated in the draining lymph nodes of mice with HPV-related cancer. The presence of PD-L1 and CD39 on the B cell surface confers regulative properties via direct inhibition of T cell function upon cognate interaction. The depletion of B cells in this model resulted in a robust Th1 cytokine response and decline in recruitment of Tregs, which was accompanied by a strong infiltration into the tumour of $CD8^+$ T cells and ultimately tumour rejection (Tang et al., 2016). Interestingly, although primary colorectal

tumours contain a significant memory and plasma B cell infiltrate suggestive of an active anti-tumour response, $CD24^{hi}CD38^{hi}$ Bregs were significantly increased in metastatic tissue indicating a shift towards active immunosuppression in the local microenvironment (Shimabukuro-Vornhagen et al., 2014). In parallel, tumour-infiltrating lymphocyte aggregates found in the marginal regions of invasive ductal carcinoma of the breast were observed to be platforms for the tumour-induced production of $IL\text{-}10^{+}$ Bregs and their subsequent induction of Tregs (Ishigami et al., 2019). Thus the current literature suggests that B cell infiltration and accumulation in the tumour site and draining lymph nodes establishes an immunosuppressive microenvironment that leads to inhibition of anti-tumour immune responses and expansion of the critical lymphatic network, thereby facilitating tumour growth and metastasis.

Investigations into the specific role of Bregs in cancer have recently increased following the discovery of a unique B cell subset termed tumour-evoked Bregs (tBregs) in Balb/c mice harbouring 4T1 carcinoma cells (Olkhanud et al., 2011). In this model it was shown that cancer-eradicating B cells were 'hijacked' by resident cancer cells within the tumour microenvironment and converted into immunosuppressive Bregs. These tBregs do not conform to any of the established Breg phenotypes; instead they resemble activated mature B2 cells ($CD19^{+}CD25^{hi}CD69^{hi}$ and $B7\text{-}H1^{hi}CD81^{hi}CD86^{hi}CD62L^{lo}IgM^{int}$). Incubation of non-regulatory $CD4^{+}$ T cells with tBregs bearing high expression levels of CD40, CD80, CD86, MHC class I and II molecules, and TGF-β production capacity led to the significant generation of $CD4^{+}CD25^{+}FoxP3^{+}$ Tregs which in turn inhibited $CD8^{+}$ T cell proliferation – thereby facilitating breast cancer escape and metastasis (Olkhanud et al., 2011). More recently, it has been demonstrated that B cells infiltrating the mammary tumour bed are 'educated' through cognate interactions with resident tumour cells and myeloid-derived suppressor (MDSCs) within the tumour. These tumour-infiltrating B cells acquired PD-L1 expression and TGF-β competency which contributed to an immunosuppressive phenotype characterized by an enhanced inhibitory capacity against $CD4^{+}CD25^{-}$ T cells, $CD8^{+}$ T cells, and $CD49b^{+}$NK cells, as well as promotion of Treg expansion (Zhang et al., 2016;Shen et al., 2018). Similarly, IL-18 derived from pancreatic cancer cells were shown to promote cell proliferation, induce IL-10 production, as well as upregulate PD-L1 expression in B cells, which enhanced the immune tolerance towards pancreatic cancer cells (Zhou et al., 2017). Therefore, infiltrating B cells subverted into Bregs by the tumour microenvironment facilitates the necessary critical changes in the local immune profile to support a pro-tumorigenic outcome.

In humans, studies reveal that a higher frequency of Bregs is indicative of enhanced tumour aggressiveness and poorer prognosis. For instance, hepatocellular carcinoma patients harbor significantly more peripheral Bregs compared to healthy controls (Shao et al., 2014); malignancy in non-small cell lung cancer is associated with an increased frequency of IL-10 producing Bregs, $CD4^+CD25^{+/high}CD127^{low/-}$ Tregs, and MDSCs (Zhou et al., 2014); patients with tongue squamous cell carcinoma, gastric cancer, and colorectal cancer also exhibit a higher frequency of Bregs within the tumour itself compared to unaffected neighbouring tissues, with numbers positively correlated with an increased frequency of Tregs (Shimabukuro-Vornhagen et al., 2014;Wang et al., 2015;Zhou et al., 2016). The coexistence of Bregs and Tregs within the tumour-infiltrating lymphocyte aggregates were also evaluated as an independent prognostic factor in breast cancer patients, with their dual presence associated with increased rates of metastasis and therefore, worse overall survival (Ishigami et al., 2019). Moreover, *in vitro* studies of lung cancer cells demonstrated their direct capacity to upregulate Treg and Breg phenotypes in lymphocyte co-cultures (Zhou et al., 2014). As for the mechanism, IL-10 and TGF-β competency appear to be the primary facilitators of suppression. For example, peripheral blood $CD19^+CD24^{hi}CD38^{hi}$ Bregs isolated from gastric cancer patients converted $CD4^+CD25^-$ effector T cells into $CD4^+FoxP3^+$ Tregs via expression of TGF-β (Wang et al., 2015). Moreover, IL-10 facilitates cross talk between $CD19^+IL\text{-}10^+$ Bregs and $CD4^+CD25^-$ T cells in tongue squamous cell carcinoma patients, resulting in the conversion of these resting T cells into Tregs (Zhou et al., 2016). Likewise, IL-10 has been shown to induce the expansion of Tregs in peripheral lymphoid organs in gastric cancer patients (Wang et al., 2015).

1.2.4 Bregs in allergy

Bregs are strongly implicated in the development of allergen tolerance, as patients with allergic asthma and allergic rhinitis exhibit reduced frequencies of IL-10-producing $CD24^{hi}CD27^+$ Bregs, as well as Bregs with impaired capacity for IL-10 production (van der Vlugt et al., 2014a;Kamekura et al., 2015). Moreover, functional B10 cells produce about 15-fold more of allergen-specific IgG4 (antibodies that suppress allergy-induced symptoms) than non-IL-10 producing B cells. Patients allergic to phospholipase A2 (PLA), the major bee venom allergen, who successfully completed allergen-specific desensitisation therapy displayed up to a five-fold increase in PLA-specific B cells characterized by high production of IL-10 and mature IgG4 mRNA. These resultant levels were comparable to that of healthy

beekeepers (van de Veen et al., 2013). Further, in murine asthma models where initial allergic airway disease can be resolved by ongoing chronic antigen exposure, local inhalation tolerance can be transferred to allergic recipients by adoptive transfer of TGF-β producing $CD5^{+}CD1d^{hi}$ B cells. Tolerance could only be transferred by Breg cells obtained from lung lymph nodes, suggesting that tolerance induction was locally induced and maintained after chronic antigen exposure. These Bregs were able to induce Foxp3 expression in $CD4^{+}$ T cells in vitro, and were found co-localized with Tregs in vivo in local lung lymph nodes (Singh et al., 2008;Natarajan et al., 2012). Adoptive transfer of $CD5^{+}$ B cells also provided protection from allergic airway disease development, with the degree of protection commensurate to the capacity of transferred B cells to produce IL-10 (Taitano et al., 2018). In a similar manner, TGF-β produced by $CD5^{+}CX3CR1^{+}$ Bregs are also pertinent in promoting the differentiation of Tregs and suppressing T cell proliferation in food allergy-related intestinal inflammation (Liu et al., 2013b).

1.2.5 Bregs in infectious diseases

Bregs have been implicated in the pathogenesis of bacterial, viral, and parasitic infections. In general, Bregs numbers are positively correlated with bacterial or viral load, as their suppressive mechanisms can alter the immune response in favour of pathogen survival thus resulting in a worse prognosis.

In viral infections, studies on human immunodeficiency virus (HIV) and hepatitis B virus (HBV) have shown that Bregs can regulate disease-specific $CD8^{+}$ T cells, induce and expand Treg populations, and impair helper T cell function through IL-10 production and PD-L1 cognate interactions (Das et al., 2012;Siewe et al., 2013;Wang et al., 2018). This deleterious effect of Bregs was recently highlighted in studies of HIV-infected individuals, whose increased circulating Breg and Treg frequencies were associated with poor immune responses to influenza vaccination (Weinberg et al., 2013). A more recent study demonstrated that HIV-1 particles induce B cells to develop the $CD19^{+}CD24^{hi}CD38^{hi}$ Breg phenotype and the expression of immunosuppressive cytokines IL-10, TGF-β1, and IL-35. Moreover, these induced Bregs efficiently reduced T cell proliferation and effector T cell TNFα production through cognate interactions, providing evidence that Breg induction is one of the mechanisms of immune dysregulation in HIV infection (Lopez-Abente et al., 2018).

In bacterial infections, lipopolysaccharide and lipoteichoic acid found in the outer membranes of Gram-negative and Gram-positive bacteria respectively, have the capacity to engage B cells via TLR (TLR 2, 4, 5, 7/8 and 9) expression. Following TLR activation, populations of B cells such as $CD5^+$, $CD138^+$, and MZ B cells can produce IL-10 and interact with T cells and macrophages in a negative regulatory manner. Bregs can thus facilitate pathogen survival and increase disease severity. For instance, *Salmonella typhimurium* infection induces IL-10 production in $CD138^+$ B cells which suppresses effector cells such as natural killer cells, neutrophils, and effector T cells (Neves et al., 2010). Susceptibility to *Listeria* is also supported by the high production of IL-10 by marginal zone B cells encountered by the bacteria in the spleen (Lee and Kung, 2012).

Lastly, the persistence of helminth and protozoan infections is largely due to the reduced or lack of inflammatory responses necessary for eradication. This is due in part to the capacity of regulatory cells to polarize the Th1/Th2 cytokine balance towards a more Th2-like phenotype. Exposure of B cells to protozoan and helminth infections leads to induction of regulatory properties and consequent suppression of Th1 function, as seen in cases of *Schistosoma* and *Leishmania* infections. *Schistosoma*-induced Bregs secrete IL-10 and increase Th2 responses while inhibiting the production of pro-inflammatory cytokines IFN-γ, IL-4, and IL-17 (van der Vlugt et al., 2014b). Protozoan parasite *Plasmodium berghei*, the causative agent of malaria, has also been shown to expand B10 cells that in turn reduce populations of natural killer cells and cytotoxic $CD8^+$ T cells to aid survival (Liu et al., 2013a).

1.3 Regulatory B cells: Emerging role in pregnancy

1.3.1 Bregs and female sex hormones

The two major sex hormones involved in pregnancy are estrogen and progesterone, with each hormone playing a central role at different time points during gravidity. Progesterone promotes endometrial decidualization for the implantation of the embryo and maintains uterine relaxation throughout pregnancy (Tan et al., 2012). Estrogen on the other hand, plays a central role in angiogenesis which is needed for placentation and sustenance of the fetus (Enninga et al., 2014). High estrogen levels and the withdrawal of progesterone promote the onset of labour, and ultimately, human parturition. Female sex hormones were found to have a significant influence on inflammatory responses when women with autoimmune

conditions were reported to experience a change in their disease activity upon pregnancy. For instance, women that acquired SLE during pregnancy generally experienced a more severe affliction compared to non-pregnant patients (Wei et al., 2011;Zhao et al., 2013). Existing rheumatoid arthritis is often ameliorated by the surge of immunoinhibitory estrogen during pregnancy, which relapses post-partum upon an increase in the immunostimulatory hormone prolactin (Jara et al., 1991;Jorgensen and Sany, 1994). There is also evidence that female sex hormones are capable of modifying decidual immune cells, such as dendritic cells and uterine natural killer cells, from being actively pro-inflammatory to being tolerogenic (Kyurkchiev et al., 2010). Considering that pregnancy outcomes essentially rely on the effective control of the inflammatory response, essential pregnancy hormones should be investigated in any study of immune cell changes during pregnancy.

Estrogen regulation of immune cells, whether innate or adaptive, has been established in recent years. Its presence can increase splenic neutrophil numbers, alter the phagocytic capacity of macrophages, and enhance the maturation of dendritic cells (Segerer et al., 2012). It has also been shown to regulate the activities of $CD4^+$ and $CD8^+$ T cells, and promote the expansion and activity of Tregs by increasing Foxp3, programmed death receptor (PD-1), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) expression (Polanczyk et al., 2005;Tai et al., 2008;Bodhankar et al., 2012). Rheumatoid arthritis and multiple sclerosis can both be ameliorated by estrogen-induced Treg expansion and activation which leads to immune suppression (Polanczyk et al., 2003;Polanczyk et al., 2005). B cells are likewise affected by the presence of estrogen. During lymphopoiesis, a rise in estrogen levels leads to a reduction of B cell precursors and an expansion of mature B cells. Estrogen activation therefore affects B cell maturation, and ensures that the immune system is equipped to defend the body against pathogens if required (Muzzio et al., 2014).

Two receptors are responsible for estrogen-mediated signalling – estrogen receptor α (ERα) and estrogen receptor β (ERβ) and upon activation act as transcription factors for a range of estrogen-sensitive genes, with ERα more predominant on B cells (Phiel et al., 2005). Studies of EAE demonstrate that the protective effect of 17β-estradiol (E2) comes from its interaction with ERα on immune cells, including B cells (Polanczyk et al., 2003). Whilst many studies have explored the protective effect of estrogen in autoimmune diseases, primarily by focusing on effects on Tregs (Polanczyk et al., 2004;Bodhankar et al., 2012), others have highlighted the role of B cells by showing that E2 conferred protection against the induction of EAE in mice even after ablation of Treg; a result unsuccessful in B cell-

deficient mice (Mann et al., 2007;Subramanian et al., 2011). Specifically, E2 augmented the frequency of splenic IL-10^{+} Bregs via the PD-1/PD-L1 mechanism (Bodhankar et al., 2015;Zhang et al., 2015a) which led to a decline in the frequency of CD11b^{+}CD45^{hi} activated macrophages, dendritic cells, and infiltrating CD4^{+} cells in the spinal cord (Zhang et al., 2015b). Together, these results suggest that Bregs may have carried out this protective effect via their responsiveness to estrogen. This was confirmed by studies which demonstrated that estrogen-receptor positive B cells upregulate Treg function in EAE (Bodhankar et al., 2012).

The effect of progesterone on innate immune cells, mainly monocytes, dendritic cells, and natural killer cells, has also been elucidated (Kyurkchiev et al., 2010), and in a manner similar to estrogen, depends on the presence of progesterone receptors. These receptors also act as nuclear transcription factors upon activation, and may either be progesterone receptor A (PR-A) or progesterone receptor B (PR-B) (Szekeres-Bartho et al., 2009). The specific actions of progesterone relevant to myometrial function are mediated by the combined and distinct genomic actions of PR-A and PR-B. Myometrial cells express PR-B for most of the pregnancy, facilitating progesterone-mediated Th2-like maternal immune responses, resulting in reduced production of pro-inflammatory cytokines and increased secretion of IL-10 (Lin et al., 1993;Tan et al., 2012). At the latter part of pregnancy nearing the onset of parturition, PR-A gene expression becomes more predominant, blocking the transcriptional anti-inflammatory activity of PR-B and consequently increasing pro-inflammatory gene expression and promoting labour (Tan et al., 2012). Progesterone function in cancer and autoimmune diseases occurs comparably to that in pregnancy, in that progesterone inhibits T cell proliferation and pro-inflammatory cytokines IL-2, IL-17, and IFN-γ expression while enhancing IL-4, IL-5, IL-6, and IL-10 cytokine production (Garay et al., 2009;Enninga et al., 2014). Moreover, there is a concurrent expansion of B cells associated with the increase in IL-10, and a lack of effect of progesterone on regulatory T cells (Yates et al., 2010). Collectively, this suggests a possible signalling pathway wherein progesterone elicits IL-10 augmentation by activating Bregs.

The molecular mechanisms by which estrogen and progesterone operate appear to be distinct from one another. Whereas estrogen elicits a strong effect on Tregs, progesterone does not. However, both have been documented to affect the B cell immune profile. The current literature gives insights as to how the female sex hormones shape the immune response in various clinical conditions, and overall indicate that estrogen and progesterone foster a tolerant environment within the pregnancy milieu by expanding all regulatory cells. Changes

in the circulating amount and distribution of these cells during pregnancy indicates that adaptations are in place to ensure that the mother's body is well equipped to conceive and grow a semi-allogeneic fetus.

1.3.2 Bregs in pregnancy

Previous studies of Bregs in the context of autoimmune disease, graft transplantation, cancer, allergy, and infectious diseases all indicate one thing – Bregs dampen the inflammatory response and foster a stable tolerant immune profile within the local microenvironment. Based on the current literature, it is therefore reasonable to speculate that Bregs may be a key player in pregnancy. Their anti-inflammatory role in autoimmunity, graft tolerance, and allergy suggest relevance to the induction of maternal immunological tolerance, whilst their pro-tumorigenic and pro-pathogenic role in cancer and infectious diseases may be applicable in pregnancy; i.e. maternal-fetal cognate interactions may hijack naïve B cells and educate them to become Bregs thus ensuring the fetus' unperturbed growth. The pregnancy hormones estrogen and progesterone are likewise critically involved in the establishment, maintenance, and termination of pregnancy.

During pregnancy, as the mother's womb houses and protects the semi-allogeneic fetus, the response of the maternal immune system is the key determinant to pregnancy success, with failure to induce selective immune tolerance toward the fetus resultant in pregnancy loss. Modification of the maternal immune response is orchestrated by multiple cytokines that influence the nature and abundance of leukocyte subsets in the uterus and placenta and includes the reduction of antigen-presenting function of monocytes, macrophages, and dendritic cells; inhibition of natural killer cells, T cells, and B cells; proliferation of uterine killer cells; maintenance of tolerogenic dendritic cells; and the induction of Tregs (Schumacher et al., 2014). These immunological adaptations during early pregnancy pave the way for two main objectives – to protect the fetus from immune rejection and to facilitate the tissue remodelling processes needed for placental development (Robertson et al., 2007).

Primarily, Bregs are considered highly relevant in the pregnancy milieu as they are a major cellular source of the potent anti-inflammatory cytokine IL-10. This cytokine is vital for optimal pregnancy outcomes, with IL-10 deficiency associated with fetal resorption, growth restriction, and even death (Chaouat et al., 1995;Murphy et al., 2005;Robertson et al., 2007). IL-10 is found in abundant amounts in the uterus and placenta during pregnancy and has

been identified as a critical player for counteracting the pro-inflammatory cytokine response. Even at the initial conception stage, inflammation resulting from of the recognition of paternal antigens is thwarted by the presence of IL-10 (White et al., 2004). Furthermore, administration of a sub-clinical dose of LPS to pregnant IL-$10^{-/-}$ mice resulted in a ten-fold reduction in viability of semi-allogeneic fetuses compared to unaffected controls. Analysis of maternal serum and uterine tissue in these LPS challenged $IL10^{-/-}$ mice demonstrated high levels of the pro-inflammatory cytokines TNFα, IL6, IL1A, and IL12p40, a result which could be abrogated by administration of exogenous IL-10 (Terrone et al., 2001;Robertson et al., 2006;Robertson et al., 2007). Therefore IL-10 confers pregnancy protection by multiple mechanisms including inhibition of pro-inflammatory cells such as macrophages and monocytes, and suppression of pro-inflammatory cytokine and chemokine production such as TNF-α, IL-6, IL-1a, IL-8, and IL-12 (de Waal Malefyt et al., 1991;Fiorentino et al., 1991;Robertson et al., 2007). These findings demonstrate the capacity of IL-10 to regulate the inflammatory response in the uterus and placenta during gestation.

Evidence of a direct role for Bregs was indicated in comparative mouse studies of normal and abortion-prone pregnancies which showed a diminished frequency of B10 cells in the maternal spleen of abortion-prone mice, whereas normal pregnancies harboured an increased number of B10 cells. Additionally, human chorionic gonadotropin (hCG) hormone was shown to mediate the generation of B10 cells during pregnancy, a mechanism which may be severely hampered in the abortion-prone mouse model (Liu et al., 2019). Adoptive transfer of B10 cells rescued pregnancies in abortion-prone mice, by inhibition of dendritic cell maturation and expansion of the Treg population (Jensen et al., 2013). Furthermore, adoptive transfer of B10 cells, but not IL-10 negative B effector cells nor B cells from IL-10 deficient mice, rescued fetal loss against lethal LPS-driven inflammation by reducing IL-17A and IL-6 production by T cells and expanding the Treg population (Busse et al., 2019). Another Breg phenotype reported as critical to pregnancy success are the marginal zone B (MZ B) cells that are found expanded in the spleen of normal pregnant mice, but lacking in mice undergoing pregnancy complications. Successful pregnancy was correlated with the capacity of MZ B cells to produce enhanced levels of immuglobulin (IgM and IgA) which likely shifts the immune response from a Th1-like to a Th2-like profile (Muzzio et al., 2016). Although also IL-10 competent (Lenert et al., 2005), there is no evidence yet linking MZ B cell cytokine production to their role in pregnancy wellbeing. In humans, IL-10 producing $CD19^{+}CD24^{hi}CD27^{+}$ B cells in the peripheral blood have been shown to be significantly higher in women undergoing normal pregnancies as opposed to non-pregnant women, or

women who had suffered spontaneous abortions (Rolle et al., 2013;Liu et al., 2019). Bregs isolated from peripheral blood of pregnant women taken during the first trimester successfully inhibited TNFα secretion by activated T effector cells *ex vivo* (Rolle et al., 2013). Interestingly, $CD24^{hi}CD38^{hi}$ Bregs were found to be significantly lower during the third trimester of pregnancy and on delivery day compared to non-pregnant and post-partum controls, possibly as a result of a drop in female sex hormones that influence B cell activation (Lima et al., 2016;Martins et al., 2017). A study on repeated implantation failure (RIF) in humans suggests impairment in the IL-10 production capacity of B cells as one of the key pathological mechanisms as peripheral B cells from women who have experienced RIF possessed lower IL-10 mRNA levels and produced significantly lower IL-10 upon CpG stimulation relative to healthy controls (Koushaeian et al., 2019). Although limited in number, these studies on Breg function in pregnancy, and the underpinning Breg-mediated immunosuppression, parallels that observed in autoimmune disease, graft tolerance, and cancer. In all conditions, Bregs impose a suppressive environment via induction and maintenance of Tregs, modification of the helper T cell response, and inhibition of effector cell responses including cytolytic T cells, NK cells, and dendritic cells.

As Breg function in pregnancy appears to be interlinked with that of Treg and dendritic cells, defining the working mechanisms is requisite. Both Tregs and dendritic cells are critical players in determining pregnancy outcomes. In normal pregnancy, the majority of decidual dendritic cells remain immature which contributes to the maintenance of a fetal-tolerant local environment. These immature dendritic cells are thus referred to as tolerogenic dendritic cells (Blois et al., 2004). Tregs are likewise crucial contributors in achieving and maintaining maternal fetal immune tolerance. In mice, a natural periodic accumulation of Tregs during the estrus stage is seen, purportedly in preparation for the implantation of a semi-allogeneic fetus (Kallikourdis and Betz, 2007). At the onset of embryo implantation, Tregs are recruited to the para-aortic lymph nodes draining the uterus (Chen et al., 2013). Expansion of the Treg population is critical in improving the rates of successful pregnancy as deficiency or harbouring insufficient numbers of Tregs in the vicinity of the uterus at any time during pregnancy leads to abortion or miscarriage (Zenclussen, 2006;Ruocco et al., 2014). In the context of pregnancy, tolerogenic dendritic cells and Tregs foster a fetal-tolerant environment via production of anti-inflammatory IL-10 and TGF-β (Alijotas-Reig et al., 2014).

During pregnancy, if an anti-inflammatory signal such as IL-10 or TGF-β is lacking, the dendritic cell phenotype remains such that it prevents the activation of T cells that protect the semi-allogeneic fetus (Rutella et al., 2006). It is thus pertinent that dendritic cells retain their immature state to acquire the tolerogenic phenotype. IL-10 and or TGF-β must then be initially present in the pregnancy milieu to either set off the induction of tolerogenic dendritic cells followed by the induction of Tregs, or directly drive the induction of Tregs. However, given that Bregs are (1) established sources of both IL-10 and TGF-β, (2) significantly increased in numbers during normal pregnancy, and (3) are responsive to endocrine modulation, it is plausible that Bregs also play a critical role in pregnancy, especially in the initial induction of maternal immunological tolerance. In support, the findings of Jensen et al. (2013) suggest that IL-10 production from B10 cells likely influences the non-maturation of IL-10-receptor rich dendritic cells which then expand the Treg population in pregnant mice. Based on the current literature indicating similar immunosuppressive roles and direct interface among these three types of immune cells, a Breg-Treg-tolerogenic dendritic cell feedback loop is proposed for the maintenance of a tolerant pregnancy milieu (Figure 1.2).

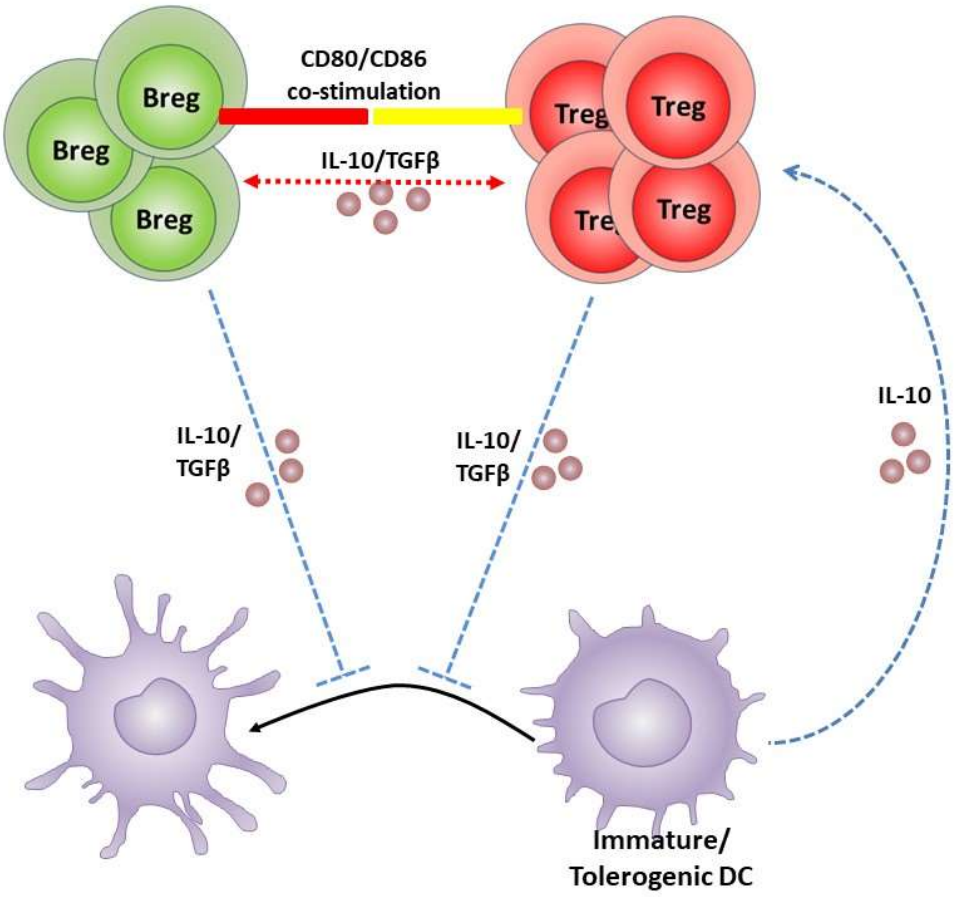


Figure 1.2. Schematic illustration of the hypothetical feedback loop between regulatory B cell (Breg), regulatory T cell (Treg) and dendritic cells (DCs) during pregnancy. Bregs create a tolerant pregnancy milieu either by direct induction and expansion of the Treg

population via CD80/CD86 costimulation and/or IL-10 and TGFβ production (depicted in red); or by inhibition of DC maturation causing retention of a tolerogenic phenotype and in turn expansion of the Treg population via IL-10 (depicted in blue). Tregs and tolerogenic DCs produce IL-10 and may maintain the feedback loop responsible for negative regulation within the pregnancy milieu. The exact mechanism by which Bregs recruit Tregs and inhibit DC maturation in the pregnancy context is, however, yet to be established. (Guzman-Genuino and Diener, 2017).

1.4 Use of Bregs in immune therapy

As negative regulators of immune responses and promoters of immunological tolerance, the use of Bregs to prevent or treat various immune conditions clinically is theoretically a viable and conceivable initiative. In circumstances where inflammation is excessive such as in autoimmune diseases, transplantation, and allergy, induction and expansion of these immunosuppressive cells may restore homeostasis. In cancer and infections however, Breg depletion, rather than expansion may be the required approach. Therefore, the use of Bregs for immune therapy requires careful calibration in order to achieve the desired outcome.

To date, many immune therapies employed in treating diseases are non-selective. In autoimmunity, rituximab-mediated B cell depletion is used as a successful method of treating disease, however as it targets all B cells, beneficial Bregs are also depleted which can result in the generation of new inflammatory conditions (Goetz et al., 2007;El Fassi et al., 2008a). Natalizumab, a humanized monoclonal antibody against the integrin α_4 (Itga4) currently used as an effective MS-modifying therapy, has been shown to neutralize B cells, including Bregs, in peripheral lymphoid organs leading to development of secondary progressive multifocal leukoencephalopathy (Glatigny et al., 2016). In cancer, Bregs have been found to accumulate specifically in the tumour-draining lymph nodes but in some cases they neither produce IL-10 nor recruit Tregs, instead they inhibit T cell function via PD-L1 and CD39 cognate interactions which facilitate tumour progression (Ganti et al., 2015). Non-selective depletion will obliterate the Bregs, however it will also deplete effector B cells that eradicate tumours. Targeted cell therapy where specific B cell subsets are selectively depleted will address these issues; however the lack of specific cell markers for efficient identification and isolation of all subsets presents a challenge to therapy development. In addition, the diversity of Breg subsets across different disease conditions suggest that Breg differentiation and

function are context-dependent, thus any immune therapy needs to be tailored to the specific disease of interest.

Currently, amassed knowledge of Breg induction and function has led to many prospective observational studies exploring the relevance of Bregs in diseases such as SLE and thymoma. Results are eagerly awaited as these endeavours and future research insights will herald further understanding of context-dependent Breg physiology, information requisite in designing novel disease-specific therapeutic approaches.

1.5 Aims and Rationale

There is compelling evidence that Bregs play a role in pregnancy well-being. The field of Bregs in pregnancy is still at its infancy thus very little is known about its specific functions; yet the current state of knowledge points towards a key role in the induction of maternal immunological tolerance and maintenance of a healthy gestation. Previous studies have been limited to examining Bregs in peripheral sites such as the spleen, bone marrow, and peripheral blood. However, the form and function of B cells are known to be immensely influenced by local environmental cues. Therefore, a major knowledge gap in this field is the lack of information on the involvement of Bregs in events taking place within the local microenvironment during pregnancy. The general aim of this thesis was to address this gap by elucidating the nature and function of Bregs lying in closest proximity to the fetus. In Chapters 3 and 4, we investigated the characteristics and functions of uterine B cells, as well as the possibility of fetal factors influencing B cell phenotype and function while in Chapter 5, we present an examination of the potential therapeutic use of Bregs as cell-based treatment for immune-linked pregnancy pathology.

We hypothesized that:

1. B cells acquire regulatory properties during pregnancy that potentially contributes to the induction of maternal immunological tolerance towards the fetus and maintenance of pregnancy well-being.
2. B cells acquire regulatory properties through their interactions with fetal factors such as trophoblast cells and secreted factors.
3. Bregs can interact with and induce the expansion of regulatory T cells within the uterine microenvironment during pregnancy.

4. Bregs may be harnessed as cell therapeutic tools to address and ameliorate immune-based pregnancy disorders.

The specific objectives were:

1. To examine the kinetics of B cells in the local microenvironment during murine pregnancy.
2. To characterize the phenotype and function of uterine B cells at critical points during pregnancy.
3. To investigate the effect of fetal trophoblast cells on B cell phenotype and function.
4. To evaluate whether trophoblast-educated B cells are involved in implantation events.
5. To elucidate the functional interplay of Bregs and Tregs in the uterus during pregnancy.
6. To explore the possibility of Bregs for use as cell therapy in the context of a pregnancy disorder.

Chapter Two

MATERIALS AND METHODS

2.1 Materials

2.1.1 Buffers and solutions

All reagents were purchased from Sigma unless otherwise stated. Common recipes for buffers and solutions are listed in the table below.

Table 2.1. List of buffers and solutions used in this thesis

Buffer / Solution	Recipe
Ammonium-Chloride-Potassium (ACK) lysis buffer	150 mM NH_4Cl_2, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2
DMEM culture medium (Gibco Invitrogen)	Dulbecco's Modified Eagle Medium supplemented with 10% FBS, 2mM L-glutamine, 1 mM sodium pyruvate, and 100 units/mL Penicillin Streptomycin solution
DMEM/F12 culture medium (Gibco Invitrogen)	Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 supplemented with 10% FBS, 2mM L-glutamine, 1 mM sodium pyruvate, and 100 units/mL Penicillin Streptomycin solution
ELISA coating buffer	PBS, pH 7.4
ELISA wash buffer	PBS with 0.05% Tween-20
ELISA assay diluent	PBS with 1% Bovine Serum Albumin (BSA)
FACS buffer	PBS with 2% Fetal Bovine Serum (FBS)
Phosphate buffered saline (PBS)	13 mM NaCl 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 1mM $CaCl_2$ and 0.5 mM $MgCl_2$
RPMI culture medium	Roswell Park Memorial Institute 1640 (RPMI-1640) Medium supplemented with 10% FBS, 2mM L-glutamine, 1 mM HEPES, and 100 units/mL Penicillin/streptomycin/gentamycin

2.1.2 Antibodies and reagents used in flow cytometry and enzyme-linked immunosorbent assay (ELISA)

The following tables contain the details of the anti-mouse and anti-human antibodies used in flow cytometry and other reagents used in this thesis. For antibodies used for immuno-staining, optimal working concentration for best staining with minimum background was determined experimentally using the antibody-immuno-staining optimisation protocol as described in Section 2.2.2.

Table 2.2. List of anti-mouse antibodies used in flow cytometry

Antibody	Conjugate	Clone	Source
B220	APC	RA3-6B2	BioLegend
CD19	APC-Cy7	1D3	BD Biosciences
CD1d	FITC	1B1	BD Biosciences
CD4	FITC	RM4-5	eBioscience
CD5	BV510	53-7.3	BD Biosciences
CD21	BV421	7E9	BioLegend
CD24	BV421	M1/69	BioLegend
CD25	APC	PC61.5	eBioscience
CD27	PE-Cy7	LG.3A10	BioLegend
CD38	BV421	90/CD38	BD Biosciences
CD80	PE-Cy7	16-10A1	BioLegend
CD86	AF488	GL-1	BioLegend
CD138	BV510	281-2	BD Biosciences
Foxp3	PE	FJK-16s	eBioscience
I-A/I-E	AF488	M5/114.15.2	BioLegend
IgD	PE-Cy7	11-26c.2a	BioLegend
IgM	AF488	RMM-1	BioLegend
IL-10	PE	JES5-16E3	BioLegend
IgG2α κ Isotype	PE	eBR2a	eBioscience
IgG2β κ Isotype	PE	RTK4530	BioLegend
PD-L1	PE-Cy7	MIH5	eBioscience
TIM-1	BV421	RMT 1-4	BD Biosciences

Table 2.3. List of anti-human antibodies used in flow cytometry

Antibody	Conjugate	Clone	Source
CD4	APC	OKT4	SungeneBiotech
CD19	FITC	HIB19	BD Biosciences
CD24	PerCP	ML5	BD Biosciences
CD25	PE	HI25A	Sungene Biotech
CD27	APC	O323	BioLegend
CD38	PE	HIT2	BD Biosciences
Foxp3	FITC	PCH101	eBioscience
IgM	PE-Cy7	G20-127	BD Biosciences
IL-10	FITC	JES3-9D7	BioLegend

Table 2.4. List of notable reagents used in this thesis

Reagent	Clone/Conjugate	Source
Purified Mouse Anti-Human CD3	SP34-2	BD Biosciences
Purified Mouse Anti-Human CD28	CD28.2	BD Biosciences
Purified NA/LE Hamster Anti-Mouse CD40	HM40-3	BD Biosciences
Brefeldin A	n/a	Sigma
Cell Proliferation Dye	e450	eBioscience
Cell Stimulation Cocktail	n/a	eBioscience
Cytofix/Cytoperm Kit	n/a	BD Biosciences
Diphtheria toxin (DT)	n/a	Sigma
Dynabeads™ mouse T-activator CD3/CD28	n/a	eBioscience
FcBlock CD16/32	n/a	eBioscience
Fixable Viability Dye	eFluor™ 660	eBioscience
Foxp3 Staining Buffer Set Kit	n/a	eBioscience
Ionomycin	n/a	Sigma
Lipopolysaccharide	LPS 0111:B4	Sigma
Phorbol-myristate-acetate (PMA)	n/a	Sigma
Recombinant human IL-2	n/a	Peprotech
3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA	n/a	Sigma

2.1.3 Cell lines and culture conditions

Table 2.5 lists all cell lines used in this thesis.

Table 2.5. List of cell lines

Cell line	Species	Culture medium	Source/Reference
SWAN 71 (Sw.71)	Human	DMEM/F12	Mor Lab/Straszewski-Chavez et al. 2009
SWAN 71-GFP (Sw.71-GFP)	Human	DMEM/F12	Mor Lab/ Straszewski-Chavez et al. 2009
Human Endometrial Stromal Cells (HESC)	Human	DMEM	Mor Lab/Krikun et al. 2006

The human first-trimester trophoblast cell line, SWAN-71 (Sw.71), was derived from a 7-week placenta and immortalized by ectopic expression of the catalytic subunit of the human telomerase (hTERT) and used for all experiments performed. Cells were maintained in DMEM/F12 (Gibco Invitrogen, Carlsbad, CA, USA) and passaged when approximately 85% confluent. For some experiments, hTERT-immortalized human endometrial stromal cells (HESC) were used and maintained in DMEM (Gibco Invitrogen, Carlsbad, CA, USA) and passaged every 3 days. All culture media were supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 units/mL penicillin-streptomycin and kept at 37°C and 5% CO_2.

2.1.4 Mouse strains

Table 2.6 lists all mouse strains used in this thesis.

Table 2.6 Inbred wildtype and transgenic mouse strains used in thesis experiments

Mouse strain	Source
C57Bl/6J	Animal Resources Centre (WA)
BALB/c	Animal Resources Centre (WA)
Foxp3-DTR	Gift from Prof Jonathan Sprent, Garvan Institute of Medical Research (NSW)

All mice were kept in a specific pathogen free (SPF) animal facility under optimal lighting conditions and supplied with food and water ad libitum.

For experiments in Chapter 3, six- to 9-week old female mice (C57Bl/6J) were checked for estrus cycle stage by vaginal flushing with 20 μL sterile PBS and mounting the volume in a glass slide. Slides were assessed under the microscope to determine the types and ratios of cells present. Females in proestrus or early estrus stages were identified and placed with a BALB/c stud male for mating overnight. The following day, female mice were visually inspected for the presence of a vaginal copulation plug indicative of a successful mating event. Plugged females were designated day 0.5 post-coitus (d0.5 pc). For kinetic analysis, pregnant mice were humanely killed for collection of organs at days 0.5, 2.5, 5.5, 8.5, 12.5, 14.5, 16.5, and 18.5 pc. Subsequent experiments focused on the peri-implantation time point at d5.5 pc. Virgin age-matched females at estrus were used as experimental controls.

For experiments in Chapter 5, spleens were collected from six- to 9-week old female C57Bl/6J mice for the isolation of B cells and generation of CD40-induced regulatory B cells. Spleens from eight-week old BALB/c female mice were used for the isolation of T cells used in the mixed lymphocyte reaction experiments. For the pregnancy studies, C57Bl/6J or Foxp3-DTR female mice were placed with a BALB/c stud male for mating overnight and checked for a vaginal copulation plug the morning thereafter. Plugged females were humanely killed for collection of the spleen, para-aortic lymph nodes, and the uterus at either d5.5 pc or d7.5 pc.

All animal work was conducted in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition (2013) as defined by the National Health and Medical Research Council of Australia. All experiments were conducted with approval from The University of Adelaide and University of South Australia Animal Ethics Committees.

2.2 Methods used on murine tissue and cells

2.2.1 Tissue dissociation and cell purification

The following organs were collected at necropsy: spleen, the uterus draining para-aortic lymph nodes (PALNs), and uterine tissue.

Single cell preparations from the spleen and the lymph nodes were isolated using standard methods. Briefly, using the plunger of a small syringe, spleens or lymph nodes were crushed through a 70 μm cell strainer placed atop a 50 mL tube. The single cell suspension collected in the tube was spun down and then resuspended in 1 mL of ammonium-chloride-potassium (ACK) lysis buffer for 5 minutes to eliminate red blood cells. Approximately 25 mL RPMI culture medium supplemented with 2% fetal bovine serum (FBS) was then added to neutralise the lysis buffer. Splenocytes were washed with phosphate buffered saline (PBS) twice and resuspended at a concentration of 10^7cells/mL.

Cells were gently extracted from uterine tissue using the gentleMACS^{TM} Octo Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Uterine tissue was dissected into small pieces then transferred into gentleMACS^{TM} C tubes suspended in RPMI culture medium supplemented with Collagenase A (10 mg/mL, Roche, Basel, Switzerland) and DNAse I (280 U per sample, Sigma-Aldrich, MO, USA) for efficient connective tissue digestion. Heating adaptors were used for optimal temperature control of tissue dissociation as required by the pre-set dissociation program (37 Multi A). After completion of the program, the homogenized tissue suspension was passed through a 70 μm cell strainer to collect cells in a 50 mL tube. Cells were then resuspended in 25 mL of culture medium and washed twice with PBS. Collected cells were resuspended at 10^7 cells/mL in PBS.

In experiments requiring B or T cells, the harvested mixed cells were further purified by magnetic isolation. B or T cells were negatively selected for using the MojoSort^{TM} Mouse Pan B Cell Isolation Kit or CD4 T cell isolation Kit for Mouse (BioLegend, San Diego, CA, USA) following the manufacturer's instructions. Isolated B cells or $CD4^+$ T cells were routinely achieved >90% purity as assessed by flow cytometry (Figure 2.1).

For uterine tissue, the resultant cell suspension was briefly incubated with anti-mouse CD16/32 (FC block; eBioscience, San Diego, CA, USA) for 15 minutes at 4°C to block Fc

receptors, washed, then stained with conjugated monoclonal antibody anti-mouse CD19-APC-Cy7 (BD Biosciences, NJ, USA) for 40 min in the dark on ice. $CD19^+$ B cells were purified by sorting on a FACS Aria II (BD Biosciences) using a 100 µm nozzle at a flow rate of 2000 events/second and a sorting efficacy between 95 and 100%. Sorted uterine $CD19^+$ B cells were used in the suppression assays described in Chapter 3.

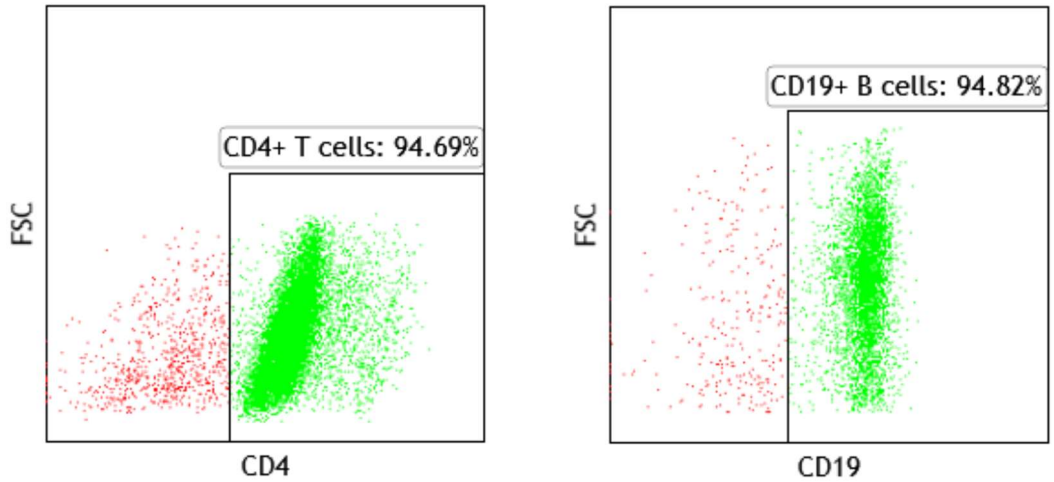


Figure 2.1. Flow cytometric assessment of $CD4^+$ T cell and B cell purification methods. $CD4^+$ T cells were purified from female C57Bl/6J splenocyte single cell suspensions by negative selection via magnetic isolation. Similarly, B cells were purified from single cell preparations of spleen and the para-aortic (uterine draining) lymph nodes by negative selection while B cells from uterine tissue were purified by FACS sorting. Assessment of resultant $CD4^+$ T cell and B cell purity was performed by staining samples of purified cell preparations with anti-CD4 or anti-CD19 antibodies respectively, followed by flow cytometric analysis. Shown are representative dot plots which indicate that each purification technique resulted in >90% purity.

2.2.2 Antibody immune-staining optimisation

The working concentration of fluorophore-conjugated antibodies were determined via antibody titration. Splenocytes were harvested and prepared as described above, then incubated with anti-mouse CD16/32 for 15 minutes on ice and resuspended at 5 x 10^6 cells/mL RPMI culture media. In a 96-well V-bottom microplate, 200 µL of the cell suspension was dispensed per well. Two-fold serial dilution of antibodies were prepared separately, with the highest concentration at 1 µL Ab:50 µL buffer down to 1 µL Ab:6400 µL buffer. A blank control was included to correct for the level of autofluoresence. After spinning down the cell suspensions and removing the supernatant, 100 µL of prepared

antibody dilution was added onto the cells for staining for 40 minutes at 4°C. After incubation, cells were washed twice with FACS buffer and analysed by flow cytometry. The lowest antibody dilution wherein positive and negative populations remain clearly defined was identified as the working concentration to be used in subsequent experiments.

2.2.3 Phenotypic analysis by flow cytometry

Multi-parameter flow cytometry was used to identify the expression of B cell phenotype markers in cells isolated from the organs of interest. Harvested cells were collected, washed, and then incubated with anti-mouse CD16/32 for 15 minutes on ice. After washing once, cells were stained with the cocktail of monoclonal antibodies diluted in buffer for 40 minutes. Cell suspensions were stained with rat/Armenian hamster anti-mouse monoclonal antibodies (as listed in 2.1.2). Stained cells were washed twice with PBS then further stained with a fixable viability dye (eFluor^{TM} 660; eBioscience) for 20 minutes. Washed cells were then transferred into FACS tubes for flow cytometric analysis (Cytoflex; Beckman Coulter, Brea, CA, USA). Post-acquisition analysis was carried out using Kaluza analysis software (Beckman Coulter). Unstained, single colour, FMO, and isotype controls were used to set gates and identify the positive population.

Phenotypes of B cell subsets were identified as follows: $CD19^+B220^+CD80^+CD86^+$ activated B cells, $CD19^+B220^+CD21^+CD27^+$ memory B cells, $CD19^+B220^+CD27^{hi}CD38^+CD138^+$ plasmablasts, and $CD19^+B220^+CD24^+IgM^{hi}IgD^{lo}$ transitional B cells. The following regulatory B cell phenotypes were also identified: $CD19^+$TIM-1^+ B cells, $CD19^+$PD-$L1^+$ B cells, $CD19^+CD5^+CD1d^+$ B cells, and $CD19^+$IL-10^+ B cells. For the phenotyping experiments in Chapter 3, unstained and single colour controls were used to set gates and identify the positive population (Figure 2.2).

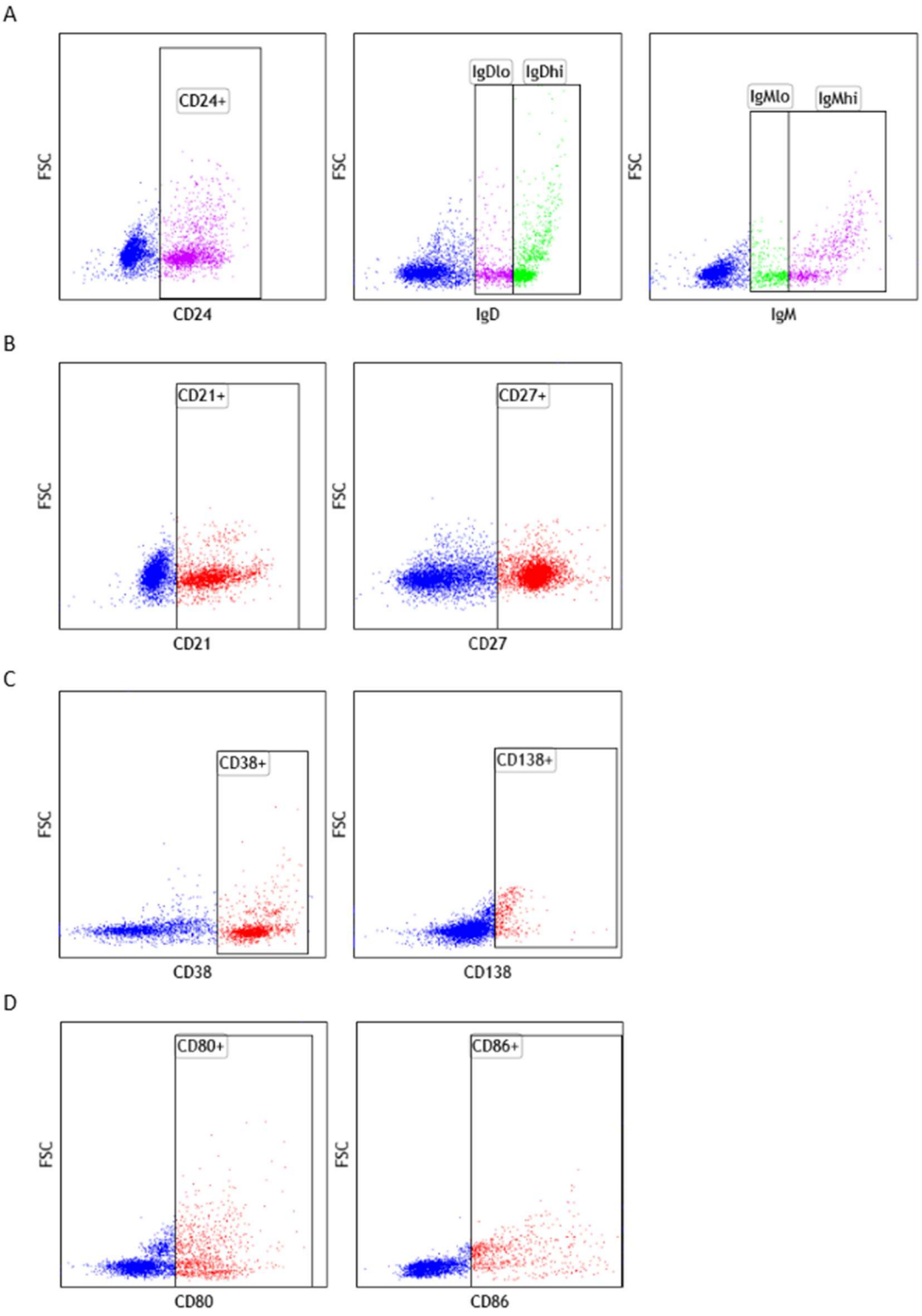


Figure 2.2. Single colour controls for establishing the gating strategies of various B cell populations. Single staining was used to determine the fluorescence parameters of populations positive or negative for surface marker expression. Parameters were applied to $CD19^{+}B220^{+}$ B cells for the identification of the following groups: (A) $CD24^{+}IgM^{hi}IgD^{lo}$ transitional B cells; (B) $CD21^{+}CD27^{+}$ memory B cells; $CD27^{+}CD38^{+}CD138^{+}$ plasma cells; (D) $CD80^{+}CD86^{+}$ activated B cells.

Cell suspensions were assessed for T cell activation by staining for CD4 expression using CD4-FITC Ab (RM4-5; eBioscience) and CD25 expression using CD25-APC Ab (PC61.5; eBioscience) for 40 mins in the dark on ice. CD25 expression on $CD4^+$ T cells was detected via flow cytometry. Further assessment for regulatory T cells involved staining for the transcription factor Foxp3. CD4 and CD25 stained T cells were fixed and permeabilized using the Foxp3 Staining Buffer Set Kit (eBioscience). Permeabilized cells were stained for Foxp3 (FJK-16s; eBioscience) or the IgG2α κ isotype control (eBR2a; eBioscience) for 40 mins in the dark at room temperature, then washed twice with the permeabilization buffer and resuspended in FACS buffer. Unstained, single-color controls, and FMOs were used as gating controls.

2.2.4 Generation of CD40-induced Bregs

Induced Bregs were used as a positive control for IL-10 production. Briefly, B cells magnetically isolated from splenocytes were resuspended in RPMI culture medium and dispensed at 1 x 10^6 cells per well in a 24-well flat bottom plate and stimulated with purified anti-mouse CD40 (1 µg/mL; HM40-3; BD Biosciences) for 48 hrs, as anti-CD40 has been previously described to induce IL-10 production in B cells *in vitro* (Matsushita and Tedder, 2011). The cell culture supernatant was collected and used for the measurement of secreted IL-10 levels by ELISA. For verification of IL-10 competency, an aliquot of the cell culture was non-specifically stimulated with PMA (500 ng/mL) and ionomycin (500 ng/mL), with Brefeldin A (1 µg/mL) in the last 5 hours of the 48-hr incubation. Cells were then intracellularly stained for IL-10 as described below.

2.2.5 Intracellular staining of IL-10^+ B cells

Intracellular production of IL-10 was used to identify the IL-10^+ regulatory B cell population. Single cell suspensions were incubated for 5 hours in a stimulation cocktail (50 ng/mL phorbol-myristate-acetate (PMA), 500 ng/mL ionomycin, and 5 µg/mL lipopolysaccharide (LPS 0111:B4, Sigma)) and 1 µg/mL Brefeldin A, to induce cytokine production and inhibit Golgi transport enabling accumulation of cytokines within the cell. After stimulation, cells were washed twice then incubated with anti-mouse CD16/32 (eBioscience). Cells were washed once and stained with anti-mouse B220/CD45R-BV650

(RA3-6B2; BD Biosciences) for 40 mins in the dark on ice. Post-staining, cells were fixed and permeabilized using the BD Fix/Perm Kit (BD Biosciences) as per manufacturer's instructions. Permeabilized cells were incubated with PE anti-mouse IL-10 Ab (JES5-16E3; BioLegend) or isotype control PE Rat IgG2b κ (RTK4530; BioLegend) for 40 mins at room temperature. Lastly, cells were washed twice with Perm Buffer then resuspended in FACS buffer. Prepared samples were analysed for $B220^{+}IL\text{-}10^{+}$ cells via flow cytometry analysis. Matched isotype controls were used to ensure correct gating for $IL\text{-}10^{+}$ cells.

2.2.6 IL-10 detection by ELISA

IL-10 quantities in supernatants were measured using the ELISA Max™ Standard Set Mouse IL-10 Kit (Biolegend) following the manufacturer's instructions. Briefly, high-binding 96-well plates were coated with 100 µL of the capture antibody and kept at 4°C overnight. After washing, the wells were blocked with 200 µL of 1% BSA-PBS for an hour. Samples and standards were incubated in the wells for 3 hours then detected using 100 µL of biotin-conjugated anti-IL-10 antibodies. After washing, avidin-horseradish peroxidase (HRP) was added for the detection of biotinylated antibodies for 30 minutes. HRP activity was then detected and visualised by incubating with 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution for 30 minutes then stopped by adding 100 µL of 1M HCl. The plates were then read at 450 nm with a differential filter set at 570 nm using the MultiSkan™ FC (Thermo Fisher Scientific, USA) plate reader.

2.2.7 Mixed lymphocyte reaction

B cells and T cells were harvested and isolated from the spleens of C57Bl/6J female mice and BALB/c female mice, respectively. $CD4^{+}$ T cells were used as responder cells and were labelled with Cell Proliferation Dye e450 (10 µM; eBioscience) for 10 mins at 37°C in the dark, then washed with 10% cold RPMI culture medium twice. B cells were added directly to the wells of the responder T cells as stimulator cells, or cultured first with anti-CD40 for 48 hrs to generate an increased proportion of Bregs before adding to the responder T cells. The MLR was performed by seeding responder T cells at 5×10^5 cells/well plus allogeneic B cells or Bregs in varying ratios (1:1, 1:2, 1:4). The mixed culture was kept at 37°C and 5% CO_2 for 5 days, with the cell culture medium replenished at 3 days. On Day 5, the resultant cell culture was collected and spun in a conical tube at 300 x g for 10 minutes to pellet cells. Cells were then washed and stained for viability and surface marker expression and analysed

by flow cytometry. In some experiments, $CD4^+$ T cells were magnetically isolated from the unstained cell culture and prepared for use in subsequent experiments as described in Section 2.2.8.

2.2.8 In vitro suppression assay for Breg/Treg functionality

To examine the suppressive activity of (1) B cells collected from pregnant or virgin mice, (2) CD40-induced Bregs, and (3) Tregs generated from mixed lymphocyte reactions, B or T cells were purified by negative selection and co-cultured with positively selected $CD4^+$ responder T cells magnetically isolated from a syngeneic spleen. $CD4^+$ splenic responder T cells were labelled with Cell Proliferation Dye e450 (10 μM; eBioscience) for 10 mins at 37°C in the dark, then washed with 10% cold RPMI culture medium twice before resuspending in pre-warmed 10% RPMI culture medium supplemented with IL-2 (10 ng/ml, Peprotech, NJ, USA) and Dynabeads™ mouse T-activator CD3/CD28 (eBioscience) for T cell expansion and activation. Labelled $CD4^+$ responder T cells were dispensed into a 96-well round-bottom plate at $1x10^5$ cells/well and purified B cells were added at a ratio of 0.5:1, 1:1, and 2:1 relative to T cell numbers, with unstimulated T cells and T cells with Dynabeads alone as controls. After 3 days in culture, cells were analysed using flow cytometry to determine T cell proliferation as indicated by proliferation dye dilution.

T cell proliferation, as measured by cell proliferation dye dilution, was expressed as the proliferative index (PI) (Moldenhauer et al., 2009). The PI denotes the total number of divisions divided by the number of cells that went into division, and is calculated using the following formula:

$$PI = \frac{(n_{pp}+n_{G1}+n_{G2}+n_{G3}+...n_{Gn})}{[(n_{G1}/2^1)+(n_{G2}/2^2)+(n_{G3}/2^3)+...(n_{Gn}/2^x)]}$$

where, n = number of cells in each fluorescent peak, with the peaks identified as follows:

‘pp’ refers to the parental undivided peak of T cells,

‘G1’ is the first T-cell division peak,

‘G2’ the second,

‘G3’ the third etc.

until peak differentiation is not discernible from the background fluorescence. The PI for each sample was calculated using the fitting and modelling processes of the software FCS Express on cell division profiles (DeNovo Software, California, USA).

2.2.9 In vivo assessment of Treg induction by B cells/Bregs adoptive transfer

The ability of purified B cells and CD40-induced Bregs to induce Treg *in vivo* during the estrus stage and during the peri-implantation period of pregnancy was assessed by evaluating $CD4^{+}Foxp3^{+}$ T cell levels after adoptive transfer of purified cell populations. Estrus and plugged (d0.5 pc pregnant) female C57Bl/6J mice were injected intravenously with $1x10^{6}$ B cells or Breg cells suspended in 300 µl of Dulbecco's PBS. Mice were then monitored for five days and humanely killed on day 5 for collection of their spleen, para-aortic lymph nodes, and the uterus. Cell suspensions from these organs were stained with CD4, CD25, and Foxp3 for the measurement of Treg percentages following the protocol stated above.

2.2.10 Treg cell depletion in Foxp3-DTR female mice

To induce Treg cell depletion *in vivo*, Foxp3-DTR female mice were injected intraperitoneally with 45 ng/g diphtheria toxin (DT) from *Corynebacterium diphtheria* (Sigma) on d3.5 pc and d5.5 pc (Care et al., 2018).

2.2.11 Evaluation of the effect of Breg adoptive transfer on the pregnancy outcome of DT-injected Foxp3-DTR mice

Foxp3-DTR female mice were mated with BALB/c (allogeneic) studs overnight and checked for the presence of a vaginal copulation plug the morning thereafter. Plugged female mice were assigned as d0.5 pc and were injected intravenously with either 1×10^{6} CD40-induced Bregs suspended in 300 µl of Dulbecco's PBS, or PBS diluent alone as control. Tregs in pregnant mice were then depleted by DT injection as described above. At d7.5 pc, pregnant mice were humanely killed and examined for the number of implantations, number of resorptions, and the size of the implants at the uterine horns. The spleen, para-aortic lymph nodes, and the uterus were then harvested and assessed for Treg populations as described previously.

2.2.12 Cytokine/chemokine analysis by MAGPIX

The cytokine/chemokine profiles of sera collected from mice in the *in vivo* assessments were determined using the Th1/Th2/Th9/Th17/Th22/Treg Cytokine 17-Plex Mouse ProcartaPlex™ Panel (eBioscience) following the manufacturer's instructions. Briefly, wells of a 96-well plate were loaded with the magnetic beads and prepared antigen standard solution or serum samples then incubated overnight in the dark at 4°C. Wells were then washed three times and incubated with biotinylated detection antibody mixture for 30 min at room temperature in the dark. After three washes, streptavidin-phycoerythrin was added to each well and incubated for 30 min in the dark. After a final wash, the beads were resuspended in reading buffer for measurement with the Luminex® MAGPIX® System (LUMINEX, Austin, TX, USA).

2.3 Methods used on human/human-derived cells

2.3.1 Peripheral blood mononuclear cells (PBMC) and B cell isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood obtained from consenting healthy non-pregnant female donors as approved by the Human Investigation Committee of the Yale University Institutional Review Board (IRB). Briefly, whole blood collected in EDTA-coated vacutainers was diluted 1:2 with PBS and carefully overlaid into a tube containing equal volume of Lymphoprep™ (StemCell Tech, Vancouver, CA). Cells were spun at 2000 rpm without brake and acceleration for 25 mins at room temperature with resultant mononuclear cells harvested from the interface of plasma and Lymphoprep™ and washed twice with large volumes of PBS. B cells were then purified by negative selection from the PBMCs using the MojoSort™ Human Pan B Cell Isolation Kit (BioLegend, San Diego, CA, USA) following the manufacturer's instructions, resulting in highly purified population of $CD19^+$ Cells (> 90% purity).

2.3.2 Collection of conditioned media

To obtain conditioned media from trophoblast cells, Sw.71 cells were plated at 25% confluency into T-175 flasks. Cells were incubated for 48 hours after which the cell supernatant was collected and centrifuged to remove all cellular debris. Cell-free supernatant was then aliquoted and stored at -80°C until further use.

Highly purified $CD19^+$ B cells were resuspended in RPMI-1640 media (Gibco Invitrogen, Carlsbad, CA, USA) and seeded at 1 x 10^6 cells/well in a 24-well flat-bottom plate (Sigma-Aldrich, MO, USA). Cells were incubated either unstimulated for the non- treated control or stimulated with Sw.71 conditioned media diluted 50% in RPMI medium for 48 hours at 37°C and 5% CO_2. Cell supernatant was collected and spun down to exclude cellular debris in the collection of cell-free supernatant. Aliquots were prepared and stored at -80°C until further use.

2.3.3 Preparation of differentiated and activated T cells

T cells were purified from isolated PBMCs by negative selection using the EasySep™ Human T cell Isolation Kit (StemCell Tech) following the manufacturer's instructions resulting in purity of >95% $CD3^+$ cells. For non-specific T cell receptor (TCR) stimulation, 1 x 10^5 cells T cells were seeded in 96-well flat-bottom plate pre-coated with anti-CD3 (10 ug/ml, BD Biosciences, NJ, USA) and anti-CD28 (1 ug/ml, BD Biosciences) antibodies in RPMI medium supplemented with 10% FBS and IL-2 (2ng/ml, Peprotech, NJ, USA). The cells were left to incubate at 37°C and 5% CO_2 for 3 days after which the stimulated cells were transferred to a new plate in normal 10% RPMI medium and rested for 2 more days. In some experiments, these pre-primed T cells were activated with Cell Stimulation Cocktail (eBioscience, San Diego, CA, USA) containing phorbol 12-myristate 13-acetate (PMA) and ionomycin for 5 hours, to induce differentiation into activated T cells.

2.3.4 Phenotypic analysis by flow cytometry

Multi-parameter flow cytometry was used to assess B cell phenotypes using a panel of conjugated mouse antihuman monoclonal antibodies (mAb) specific for the following markers: CD19, CD24, CD27, CD38, IgM, and IL-10. B cells were collected, washed twice and then incubated with heat-inactivated human serum for 20 min on ice. Cells were washed and stained with the cocktail of mAb in FACS buffer for 30 min in the dark on ice. For IL-10 intracellular staining, cells were stimulated using a Cell Stimulation Cocktail (plus protein transport inhibitors) (eBioscience) for 5 hours prior to fixing and permeabilizing using the BD Cytofix/Cytoperm Kit (BD Biosciences). The cells were then stained with IL-10-FITC for 30 min at room temperature. Cells were washed and transferred to FACS tubes for flow cytometry analysis. For T cells, the following markers were used: CD4, CD25, and Foxp3.

Cell phenotype was determined by four-color flow cytometry analysis (FACSCalibur; BD Biosciences) and the data were analysed using FlowJo software (Treestar, San Carlos, CA, USA). Unstained, single colour, FMO, and isotype controls were used to set gates and identify the positive populations.

The following B cell subsets were identified by previous studies as regulatory in function via their production of IL-10 and thus were used to classify the B cells acquired: regulatory B cells ($CD19^{+}CD24^{+}CD27^{+}$), transitional B cells ($CD19^{+}CD24^{hi}CD38^{hi}$), IgM-memory B cells ($CD19^{+}IgM^{+}CD27^{+}$), and plasmablasts ($CD19^{+}CD27^{hi}CD38^{hi}$) (Blair et al., 2010; Iwata et al., 2011; Khoder et al., 2014; Matsumoto et al., 2014).

2.3.5 Cytokine/chemokine analysis by LUMINEX

The effect of trophoblast supernatant on immune cell chemokine and cytokine production was determined using Bio-Plex Pro™ Human Cytokine 17-plex Assay (Bio-Rad, CA, USA) following the manufacturer's instructions. Briefly, wells of a 96-well filter plate were loaded with the prepared standard solution or cell-free supernatant and incubated with the 17-plex ProBead Mix at ± 800 rpm for 30 min in the dark at room temperature. Wells were then vacuum-washed three times then incubated with 25µl of biotinylated detection antibody at ± 800 rpm for 30 min at room temperature in the dark. After three washes, streptavidin-phycoerythrin was added to each well and incubated for 10 min at ± 800 rpm in the dark. After a final wash, the beads were resuspended in sheath buffer for measurement with the LUMINEX 200 (LUMINEX, Austin, TX, USA).

2.3.6 RNA isolation, RT-PCR, and quantitative PCR analysis

RNA was extracted from purified B cells cultured in TB-CM, or normal medium as controls, using the RNeasy kit (Qiagen, Valencia, CA). RNA concentration and purity were quantified via spectrophotometric analyses of 260/280 ratios with a cut-off ratio of 1.8 or above for samples to be used for analysis. Pellets were resuspended in 20µL of DNAse/RNAse-free water and quantitation using NanoDrop 2000 (Thermo Fisher, MA, USA). For quantitative analysis of messenger RNA (mRNA), 1 µg of RNA from each sample was reverse-transcribed using iSCript reverse transcription Supermix (Bio-Rad, CA, USA). The complementary DNA (cDNA) was diluted to 1:5 in nuclease-free water, and 5 µL was used in the polymerase chain reaction (PCR). iTaq Universal SYBR green Supermix (Bio-Rad,

CA. USA) and gene-specific primers were added to the reverse transcriptase reactions and run on the CFX96, C1000 system quantitative (qPCR) machine (Bio-Rad, CA, USA). The primer pair sequences are as follows: IL-10 forward 5'-GTGATGCCCCAAGCTGAGA-3' and reverse 5'-CACGGCTTGCTCTTGTTTT-3'; internal normalizer gene GADPH forward 5'-TTAAAAGCAGCCCTGGTGAC-3' and reverse 5'-CTCTGCTCCTCCTGTTCGAC-3'. Data were collected in the SYBR channel and the final cycle was followed by a thermal melt curve. The relative expression to reference GAPDH levels were calculated using the ΔΔCt method (ΔCt = ΔCt treated – ΔCt control), and the results expressed as the fold change over control (Fold change=$2^{-\Delta\Delta Ct}$).

2.3.7 Formation of trophoblast spheroids

For formation of trophoblast spheroids, 4 x 10^5 Sw.71-GFP cells were seeded into each well of a 96-well ultra-low attachment plate (Sigma-Aldrich, MO, USA) and centrifuged at 1500 rpm for 5 minutes to cluster the cells together. The plate was incubated for 48 hours to allow the clumped trophoblast to form into a spheroid. The spheroids were individually collected and transferred onto other plates for migration or invasion assays. This *in vitro* model of trophoblast implantation is described in full by You et al. 2019.

2.3.8 Trophoblast spheroid invasion assay

The matrigel invasion assay directly measures the invasive capability of the trophoblasts as conditioned by the environment. Briefly, a trophoblast spheroid was carefully plated into the centre of a well layered with a matrigel (Corning Inc, NY, USA) solution comprised of matrigel and the treatment medium. Serum-free media supplemented with treatment medium was used to drive trophoblast invasion as measured by the presence of cellular protrusions within the matrigel layer. Decidualized stromal cell conditioned medium and serum-free culture medium were used as positive and negative controls for invasion, respectively. The invasion of trophoblast cells into the matrigel matrix was monitored from 2 days to 4 days and visualized under the Revolve Pro microscope (Echo Laboratories, San Diego, CA, USA). To indicate the level of activity of individual spheroids across biologically independent experiments, a scoring system was devised based on calculated area and visual characteristics of 2D images. Differences in spheroid activity assessed at 48 and 96 hours of culture were scored as follows: 0=cell death; 1=viable spheroid without change in area; 2=

>10% increase in area; 3= >10% increase in area with peripheral protrusions; 4= >10% increase in area and prominent peripheral protrusions. Area ($A = \pi r^2$) was calculated from 2D images where radius (r) was set as half the average of two perpendicular diameter lengths of the horizontal and vertical axes.

2.3.9 Trophoblast spheroid migration assay

For the migration assay, trophoblast spheroids were individually seeded in a 96-well plate and grown in serum-free culture media supplemented with experimental treatment medium. The spheroids were monitored for 48 hrs with time-lapse documentation at 2-hr intervals using the IncuCyte ZOOM System (Essen BioScience, Ann Arbor, MI, USA). Conditioned medium from decidualized stromal cells and normal culture medium were used as positive and negative controls, respectively. To indicate the level of activity of individual spheroids across biologically independent experiments, a scoring system was devised based on visual characteristics of 2D images. Differences in spheroid activity assessed at 0 and 48 hours of culture were scored as follows: 0=Cell death; 1=viable spheroid; 2=viable spheroid with migration of trophoblast cells; 3=viable spheroid with extensive migration of trophoblast cells.

2.4 Statistical analysis

All experiments were performed at least in triplicate. The results were analysed for significant differences using the Student's t test or the Mann-Whitney test for parametric and nonparametric samples, respectively. For multiple comparisons, one-way ANOVA with Dunnett's post-hoc test was used. Data are presented as the mean ± standard error of the mean. Differences between groups were considered statistically significant at p-value <0.05 as analysed using GraphPad Prism 7.03 software (GraphPad, San Diego, CA, USA).

Chapter Three

UTERINE B CELLS EXHIBIT REGULATORY PROPERTIES DURING THE PERI-IMPLANTATION STAGE OF MURINE PREGNANCY

This is a modified version of the following publication:

Guzman-Genuino RM, Eldi P, Garcia-Valtanen P, Hayball JD, Diener KR. Uterine B cells exhibit regulatory properties during the peri-implantation stage of murine pregnancy. *Frontiers Immunol*. *10: 2899; 2019*

Chapter 3: Uterine B cells exhibit regulatory properties during the peri-implantation stage of murine pregnancy

3.1 Abstract

A successful outcome to pregnancy is dependent on the ability of the maternal uterine microenvironment to regulate the inflammation processes and establish maternal tolerance. Recently, B cells have been shown to influence pregnancy outcomes as aberrations in their numbers and functions are associated with obstetric complications. In this study, we aimed to comprehensively examine the population frequency and phenotypic profile of B cells over the course of murine pregnancy. Our results demonstrated a significant expansion in B cells within the uterus during the peri-implantation period (day 2.5-8.5 post-coitus), accompanied by alterations in B cell phenotype. Functional evaluation of purified uterine B cells from pregnant mice at implantation (day 5.5 post-coitus) established their regulatory capacity as evidenced by effective suppression of proliferation and activation of syngeneic $CD4^+$ T cells. Flow cytometry profiles revealed that the uterine B cell population has an expanded pool of IL-10-producing B cells bearing upregulated expression of co-stimulatory molecules CD80 and CD86 and activation marker CD27. Our investigations herein demonstrate that during the critical stages surrounding implantation, uterine B cells are amplified and phenotypically modified to act in a regulatory manner that potentially contributes towards the establishment of maternal immunological tolerance in early pregnancy.

3.2 Introduction

Pregnancy is a model of immune tolerance wherein the mother carries a semi-allogeneic fetus that in principle triggers a maternal immune response due to the presence of paternally derived fetal alloantigens; however the inflammation is kept at a tolerable level and the pregnancy progresses. In early pregnancy, it is well accepted that for implantation to occur, a regulated pro-inflammatory microenvironment that permits tissue remodelling and angiogenesis at the maternal-fetal interface is required. This is then followed by a shift to an immune tolerogenic profile that allows for fetal growth and development (Mor and Cardenas, 2010; Ghaebi et al., 2017). The necessity of a controlled inflammatory environment during this time implies a dynamic feto-maternal crosstalk of immune, endometrial, and fetal cells. A multitude of preclinical and clinical pregnancy studies show immune cells such as uterine dendritic cells, decidual macrophages, decidual natural killer cells, and regulatory T cells undergoing changes during pregnancy for the purposes of calibrating inflammation and mediating maternal immune tolerance (Schumacher et al., 2018b). For B cells, their role in the induction of maternal immune tolerance in pregnancy to date remains poorly understood.

Although studies on B cells in pregnancy are limited, there is an emerging view of their involvement in the physiology of pregnancy. Studies on murine models have demonstrated that percentages of B cell subsets are altered throughout gravidity. In the bone marrow and the spleen, murine B cells undergo lymphopenia mid-pregnancy as numbers of bone marrow pre/pro and immature B cells as well as splenic follicular B cells drop substantially at day 14 post-coitus (Muzzio et al., 2014). However, splenic marginal zone B cells and mature B cells in the para-aortic lymph nodes that drain the uterus were reported to expand (Muzzio et al., 2014; Muzzio et al., 2016). A normal pregnancy was also associated with an enhanced production of immunoglobulins by marginal zone B cells, speculated to reduce the occurrence of autoreactive B cells that may cause pregnancy complications (Muzzio et al., 2016). Similarly, changes in the proportions of B cell subsets during pregnancy have been documented in the clinical setting. Analysis of peripheral blood showed a significant decrease in the percentages of $CD19^{+}CD24^{bright}CD38^{bright}$ transitional B cells during normal pregnancy and relatively higher percentages of non-switched memory B cells were associated with recurrent miscarriages (Noviski et al., 2018; Ziegler et al., 2018).

Recently, there has been interest in B cells taking on a regulatory role during pregnancy as a specific subset of B cells called 'regulatory B cells' or Bregs have been implicated in the network of immune cells crucial for maintaining pregnancy. Interestingly, Bregs do not have a specific phenotypic marker, rather they are defined by their functional capacity for suppressing other immune cells (Guzman-Genuino and Diener, 2017). Among the known Breg subtypes, IL-10-producing B cells or B10 cells are the most commonly studied. Adoptive transfer of B10 cells induced from splenic B cells was previously shown to restore tolerance and rescue pregnancies in abortion-prone mice (Jensen et al., 2013). Peritoneal $CD19^{+}IL\text{-}10^{+}$ cells were also shown to increase throughout normal pregnancy whereas a significant reduction was seen in abortion-prone pregnant females (Schumacher et al., 2018a). Additionally, clinical studies correlate inadequate numbers of circulating IL-10-producing $CD24^{hi}CD^{27}$ B cells with higher risk of spontaneous abortion (Rolle et al., 2013). These studies however have been restricted to B cells in the spleen, peritoneum, and in the blood while studies of B cell function within the pregnancy microenvironment is noticeably lacking. The main hindrance to studying the B cells in the uterine mucosa and the decidua stem from the paucity of B cells within these tissues. Uterine cell profiling of the murine estrous cycle showed that B cells constituted 1.5% of viable cells at estrus (Diener et al., 2016). In humans, B cells are considered rare lymphocyte subpopulations in the uterus with a median of 2% in pre-implantation uterine tissue and 5% in the decidua (Rieger et al., 2009; Feyaerts et al. 2017). Moreover, the functional promiscuity of B cells also poses an added layer of complexity in describing the specific role of B cells in pregnancy.

In the present work, we used a C57Bl/6J x BALB/c semi-allogeneic murine pregnancy model to describe the phenotypes and functions of B cells within the uterine microenvironment throughout gestation. Our results indicate that these uterine B cells undergo changes in frequency and phenotype, particularly during the peri-implantation phase of pregnancy between days 2.5 to 8.5 post-coitus (pc). B cells were shown to be significantly expanded as well as express markers and cytokines indicative of regulatory qualities. Furthermore, we show in *ex vivo* experiments that uterine B cells collected from pregnant females at day 5.5 pc significantly suppressed proliferation and activation of syngeneic $CD4^{+}$ T cells via cell-cell interactions. We thus posit that uterine B cells at peri-implantation exhibit immunosuppressive characteristics and likely take part in fostering the generation of maternal immune tolerance during early pregnancy.

3.3 Results

3.3.1 B cell proportions are increased in the uterine tissue at peri-implantation

To investigate the impact of pregnancy on B cell populations throughout gestation, we used a murine model of allogeneic mating where C57Bl/6J females were mated with BALB/c male studs. The percentages of $B220^+$ B cells in the spleen, para-aortic (uterine draining) lymph nodes, and the uterus itself were determined at succeeding time points and compared to basal B cell numbers found in comparable organs of virgin female controls at estrus.

Representative flow cytometric panels indicating the gating strategy and cell surface staining of viable $B220^+$ B cells found within the spleen, para-aortic lymph nodes, and uterus organs at day 5.5pc are shown in Figure 3.1A. Kinetic assessment of organ B cells clearly showed the induction of lymphopenia in the spleen commenced mid-pregnancy, around day 12.5pc (Figure 3.1B, left panel), with a contrasting significant increase in B cells in the uterine draining lymph nodes occurring around day 14.5pc (Figure 3.1B, middle panel), results that concurred with data shown in previous studies (Muzzio et al. 2014). In the uterus however, a statistically significant increase in B cell proportions commenced pre-implantation, at day 2.5pc, and continued post-implantation to day 8.5pc (Figure 3.1B, right panel). This increase in uterine $B220^+$ B cells was limited to this peri-implantation phase as assessment of later time points revealed similar B cell frequencies to that of virgin mice in estrus. Based on these results, we focused on the day 5.5pc time point, i.e. just after implantation, for all subsequent analyses.

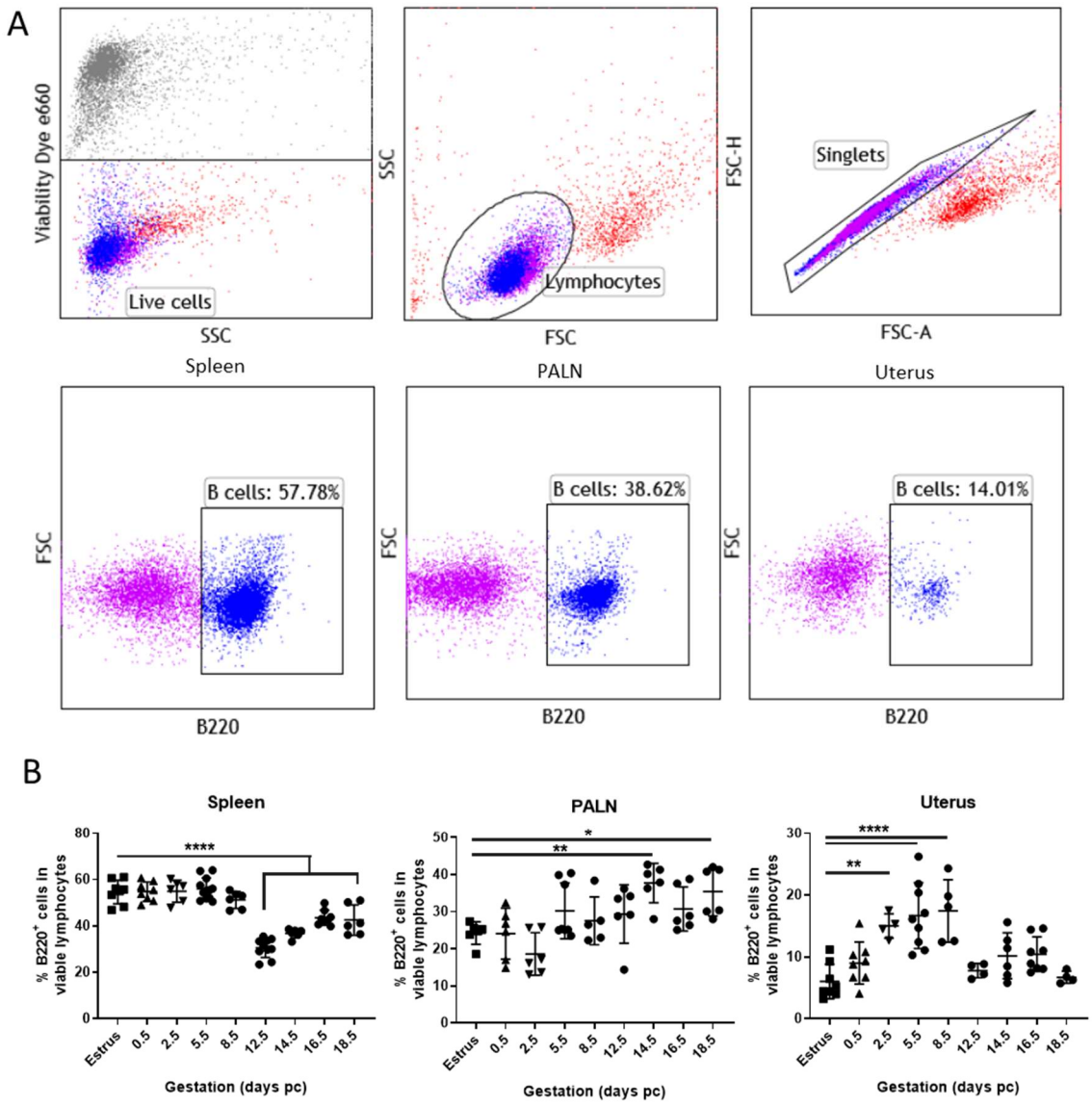


Figure 3.1. Proportions of uterine B cells expand significantly at peri-implantation. (A) Flow cytometric gating strategy used to identify B cells in the isolated cell suspensions from spleen, para-aortic lymph nodes, and the uterus taken at day 5.5 pc. Single viable lymphocyte cells were gated as per exclusion of the viability dye, FSCvSSC plots showing lymphocyte population, doublet discrimination, and single colour controls. (B) Graphical summary of the percentages $B220^{+}$ B cells in the viable lymphocyte cell populations obtained from estrus and mated mice at various days pc (n=4-9 per time point). Data is shown as mean +/- SEM and compared by one-way ANOVA, followed by Dunnett's *post-hoc* multiple comparisons test. *p≤0.05, **p≤0.01, ****p≤0.0001

3.3.2 Alterations in B cell subsets during the peri-implantation stage of pregnancy

Assessment of B cell subsets from spleen, uterus draining lymph nodes, and the uterus itself at day 5.5pc indicated that proportions of $CD24^{+}IgM^{hi}IgD^{lo}$ transitional B cells and $CD21^{+}CD27^{+}$ memory B cells remained the same as those obtained from virgin female controls (Figure 3.2). In contrast, levels of $CD27^{+}CD38^{+}CD138^{+}$ plasma B cells decreased only in the uterine tissue, with no observed changes in the spleen and the para-aortic lymph nodes. However, $CD80^{+}CD86^{+}$ activated B cells were found to be significantly higher in both the uterus and the para-aortic lymph nodes at day 5.5pc. Together this suggests that expanded numbers of B cells at the site of implantation, and associated draining lymph nodes, are activated and upregulate co-stimulatory markers indicative of an increased capacity to interact and engage with other immune cells.

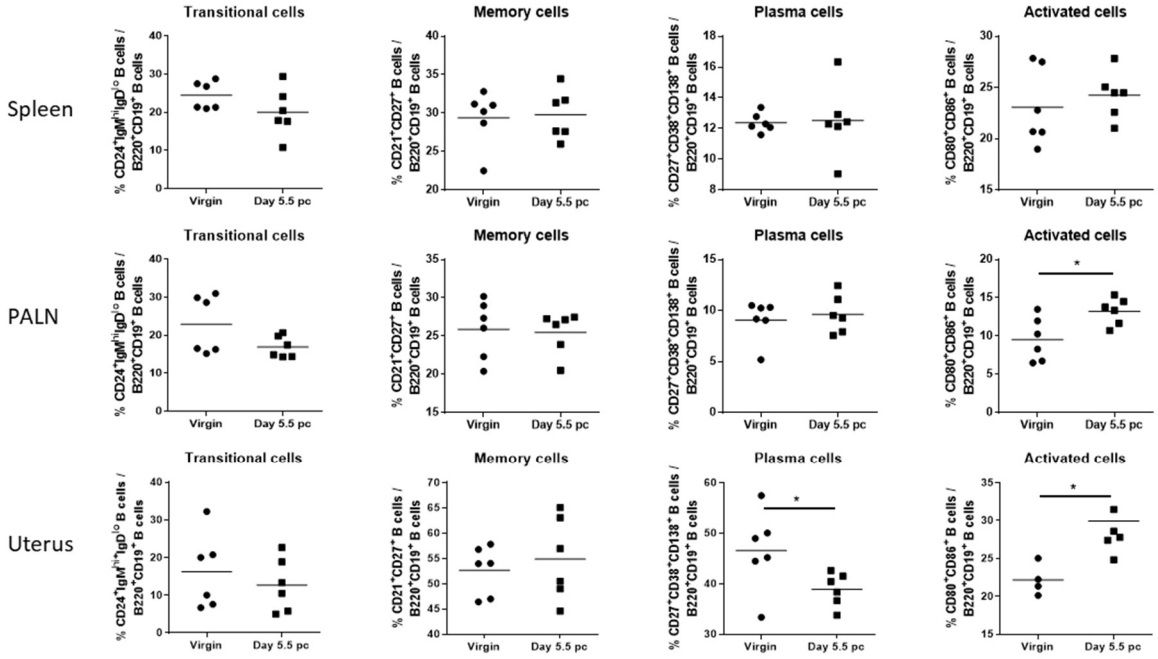


Figure 3.2. B cell subsets in the para-aortic lymph nodes and uterus are altered post-implantation. Percentages of transitional or mature naive B cells ($CD24^{+}IgM^{hi}IgD^{lo}$), memory B cells ($CD21^{+}CD27^{+}$), plasma B cells ($CD27^{+}CD38^{+}CD138^{+}$), and activated B cells expressing co-stimulatory molecules ($CD80^{+}CD86^{+}$) were investigated in the spleen, para-aortic (uterus draining) lymph nodes, and uterus of virgin and pregnant mice at day 5.5 pc (n=6, p values calculated by unpaired t-test). *$p \leq 0.05$

3.3.3 Uterine B cells from pregnant mice suppress CD4$^+$ T cell proliferation and activation

Given the observed changes in B cell percentages and subset composition, we hypothesized that the upregulated expression of co-stimulatory molecules CD80 and CD86 in uterine B cells prompted a directed engagement with T cells. Potentially, co-stimulation of T cells by B cells is a pathway for T cell activation that directly affects their rate of proliferation. Thus, we sought to examine whether uterine B cells influenced CD4$^+$ T cell activation and proliferation.

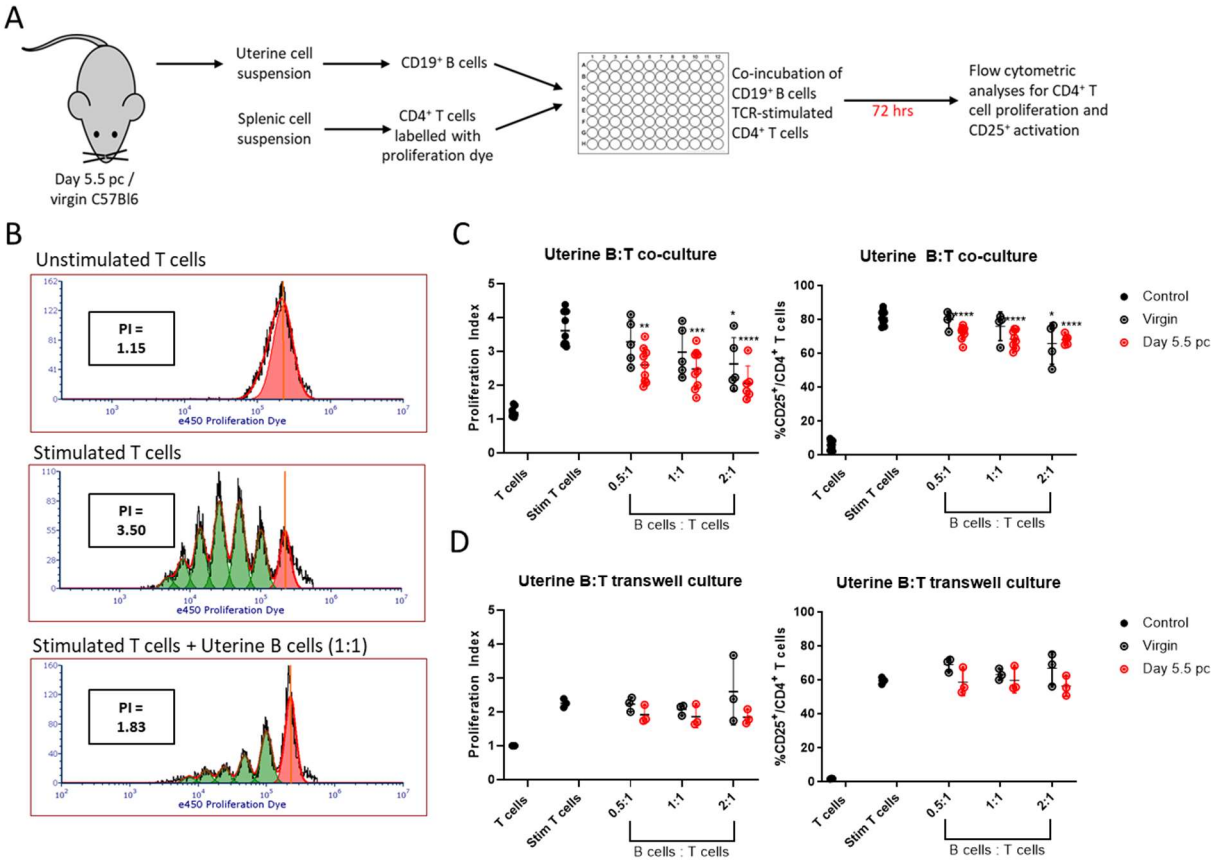


Figure 3.3. Uterine B cells from pregnant mice post-implantation suppress CD4$^+$ T cell proliferation and activation. (A) Schematic diagram of the suppression assay used to assess the effect of B cells on CD4$^+$ T cell proliferation and activation. (B) Representative flow cytometric panels illustrating the inhibitory effect of B cells on T cell proliferation quantified via calculation of individual proliferation index (denoted as PI). (C) Graphical summaries of biologically independent experiments assessing amount of CD4$^+$ T cell proliferation and CD25 expression with and without co-culture of increasing ratio of uterine B cells. (D) The same experiments as in (C) except T and B cells were kept physically apart by a 0.4µm transwell membrane (n=3-8). Data is presented as mean +/- SEM and compared by one-way ANOVA followed by Dunnett's *post-hoc* test. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001. Data were combined from five independent experiments.

Uteri from day 5.5pc pregnant and virgin non-pregnant control mice were collected and processed for single cell suspensions. Viable $CD19^+$ B cells sorted from the uterine cell suspensions were co-cultured with syngeneic TCR-stimulated $CD4^+$ splenic T cells for 72 hours, after which the T cells were assessed for cell proliferation, and activation by upregulation of CD25 expression (Figure 3.3A). Representative histograms of T cell proliferation from unstimulated and stimulated controls, and T and B cell co-cultures are shown with corresponding proliferation index (Figure 3.3B). Combined results clearly indicated the ability of uterine B cells from pregnant mice to significantly inhibit the proliferation of syngeneic $CD4^+$ T cells in a dose-dependent manner, with the proliferation indices significantly decreased in response to an increase in ratio of uterine B cells to T cells (Figure 3.3C, left panel). Similarly, inhibition of proliferation was also associated with decreased numbers of $CD4^+$ T cells expressing the activation marker CD25 (Figure 3.3C, right panel). No such inhibition of T cell proliferation or activation was seen when T cells were co-cultured with B cells obtained from virgin control mice. Furthermore, this inhibitory ability of B cells from pregnant mice was tissue-specific, as no inhibitory capacity was observed by B cells obtained from the spleen or uterus-draining lymph nodes in the same pregnant mice (Figure 3.4A).

To determine whether the inhibitory effect was evoked by B-T cell cognate interactions or via soluble molecules produced by B cells, we conducted the same experiment but with the B cells and T cells physically separated by a 0.4 μm transwell membrane. No significant increase in inhibition of proliferation or CD25 activation was observed (Figure 3.3D and Figure 3.4B), which suggested that the suppressive effect of uterine B cells on T cells is mediated by cognate cell-to-cell interactions rather than through soluble mediators.

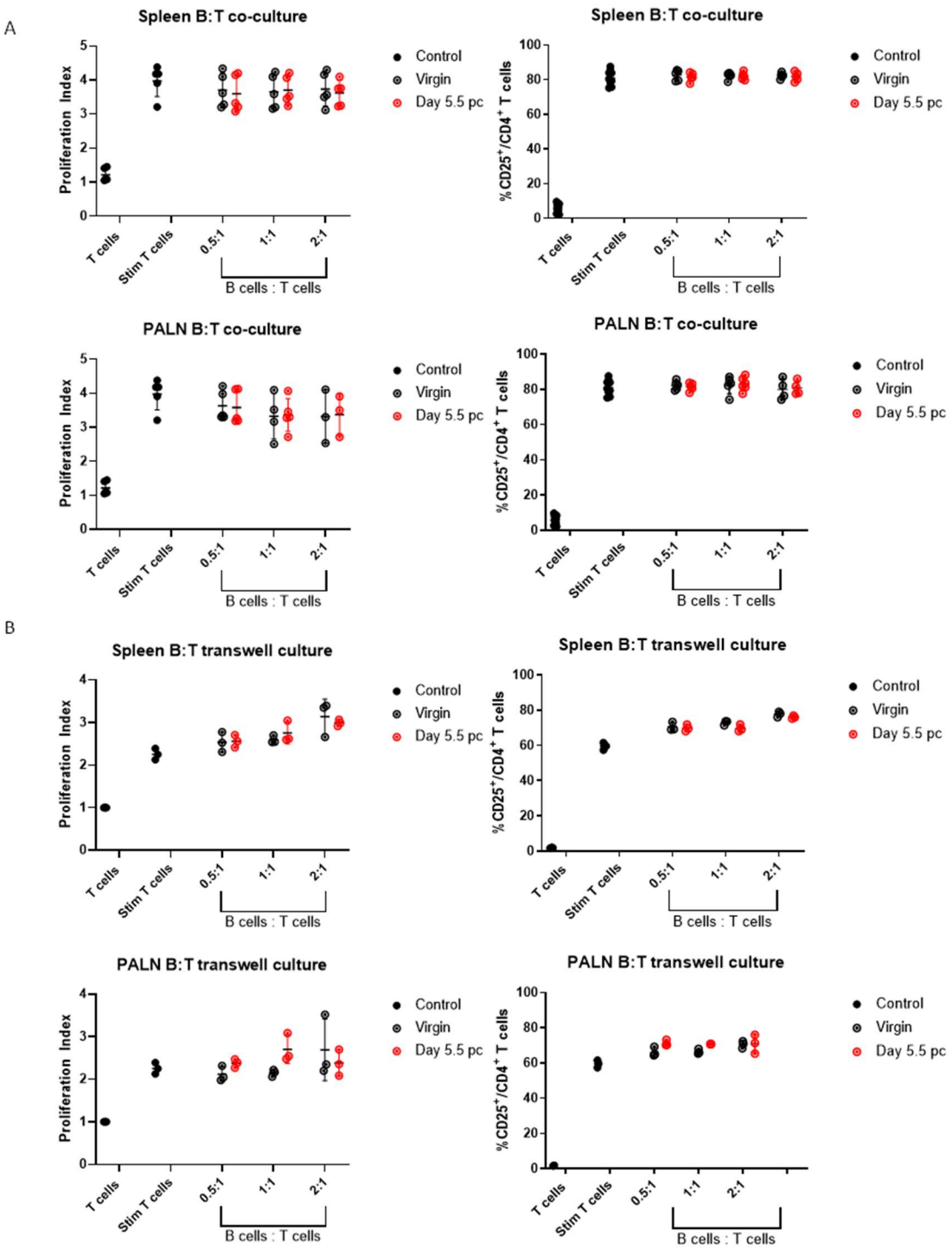


Figure 3.4. B cells from the spleen and lymph nodes do not affect CD4$^+$ T cell proliferation and activation. (A) No statistical differences in proliferation indices of CD4$^+$ T cells incubated with increasing ratios of B cells isolated from the spleen and para-aortic lymph nodes (PALN) in a co-culture (n=5). (B) No observed differences in CD25$^+$ activation of CD4$^+$ T cells incubated with increasing ratios of B cells isolated from the spleen and para-aortic lymph nodes (PALN), separated physically via transwell membranes (n=3). Data presented from three to five independent experiments and compared using one-way ANOVA.

3.3.4 Uterine B cells at peri-implantation stage exhibit an enhanced regulatory phenotype

The previous results indicated that uterine B cells exhibited a suppressive phenotype at day 5.5pc, post-implantation. We next sought to investigate whether these uterine B cells possessed surface and functional markers typically seen in Bregs such as the expression of TIM-1, PD-L1, CD1d, CD5, as well as the anti-inflammatory cytokine IL-10 (Guzman-Genuino and Diener, 2017). We focused particularly on investigating if there were differences in the frequencies of these known Breg phenotypes between uterine B cells from virgin mice and uterine B cells from pregnant mice that could account for their differing effects on $CD4^+$ T cells.

Assessment of surface markers indicated statistically similar proportions of TIM-1^+ B cells, PD-$L1^+$ B cells, and $CD1d^{hi}CD5^+$ B cells within the uterus of virgin and pregnant mice. In contrast, IL-10 cytokine expression in uterine B cells was found to be significantly increased after implantation (Figure 3.5A). Furthermore, *ex vivo* stimulated uterine B cells at day 5.5pc produced levels of IL-10 that were significantly higher than unstimulated controls, stimulated splenic B cells, and stimulated uterine B cells from virgin mice, although levels are 4-5-fold less than the amount of IL-10 produced by the positive control, *in vitro* generated splenic IL-10^+ B cells (Figure 3.5B). These results indicated that the capacity of uterine B cells from pregnant mice to regulate T cell proliferation and activation may be due to a significant increase in the proportion of IL-10-producing B cells within the uterine B cell population.

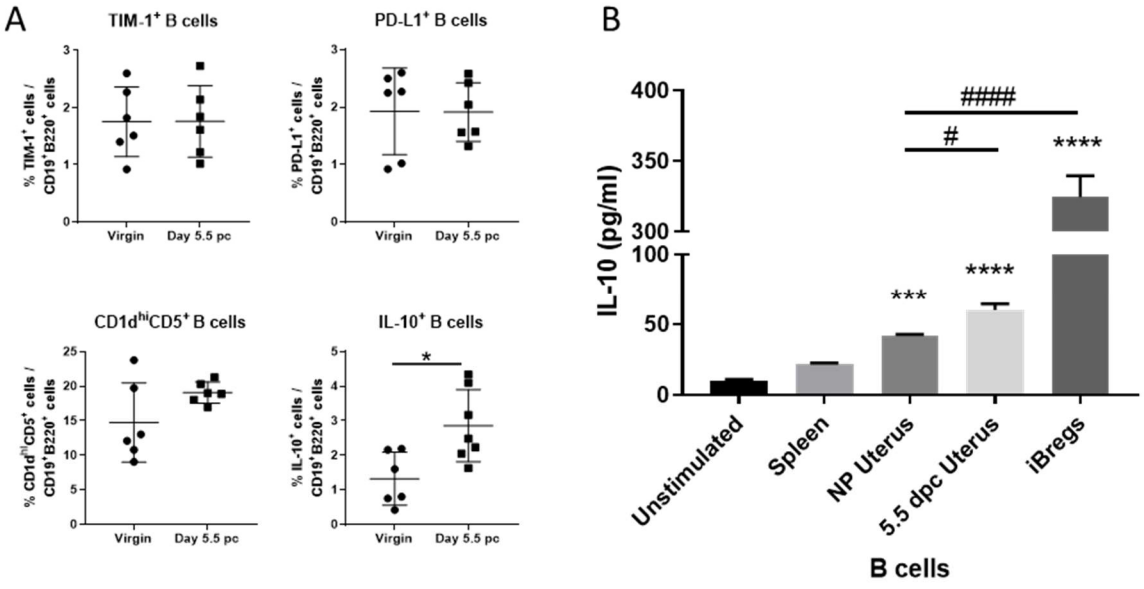


Figure 3.5. An expanded pool of IL-10$^+$ B cells are present in the uterus after implantation. (A) Known regulatory B cell subsets were analysed from B cells collected from non-pregnant and pregnant uteri (n=6-7, unpaired t-test). (B) IL-10 production by stimulated populations of purified B cells obtained from the spleen, virgin uterus, day 5.5 pc uterus, or *in vitro* induced Bregs was assessed by ELISA. Data represent pooled supernatants of duplicate wells from three independent experiments. Data is presented as mean +/- SEM and compared by one-way ANOVA, followed by Dunnett's *post-hoc* multiple comparisons test. *p≤0.05, ***p≤0.001, ****p≤0.0001. Additional Dunnett's analysis assessed against non-pregnant virgin uterus reveal a significant increase in IL-10 produced by day 5.5 pc uterine B cells and iBregs ($^{\#}$p≤0.05, $^{\#\#\#\#}$ p≤0.0001).

3.3.5 Phenotype profile of uterine IL-10$^+$ B cells at peri-implantation show increased activation and co-stimulatory markers

Phenotypic analysis of uterine B cells, as identified by their expression of CD19 and B220, showed that at day 5.5pc they co-expressed two B cell receptor isotypes IgM and IgD, as well as major histocompatibility complex class II (MHC class II), signifying their antigen-driven development and maturity, as well as their capacity to act as professional antigen-presenting cells. These cells also expressed CD24, CD21, and CD38, surface markers associated with B cell differentiation and activation (Figure 3.6). However, multi-parameter flow cytometric analysis of uterine IL-10$^+$ B cells (Figure 3.7A), indicated that these cells expressed higher levels of co-stimulatory molecules CD80 and CD86 compared to non-IL-10 expressing B cells. Notably, expression of activation marker CD27 was also significantly increased in uterine IL-10$^+$ B cell cells (Figure 3.7B). The differential expression of these markers suggests the involvement of uterine IL-10$^+$ B cells in regulation of the maternal immune response at implantation, particularly through their increased capacity to engage and interact with T cells. Together with the previous findings, we posit that the enhanced expression of these co-stimulatory and activation markers accompanied with the increased production of anti-inflammatory IL-10, enable uterine B cells to regulate the proliferation and activation of CD4$^+$ T cells.

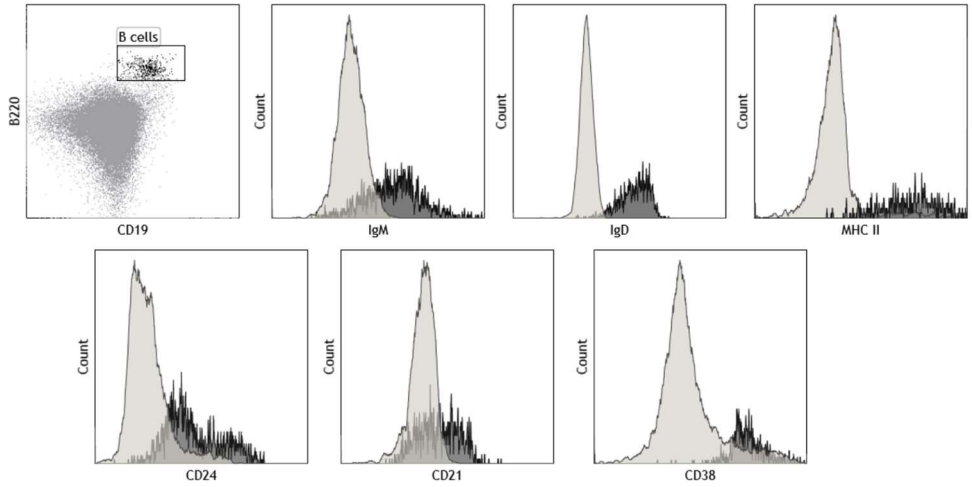


Figure 3.6. Uterine B cells positively express IgM, IgD, CD24, CD21, and CD38. Representative flow cytometric overlays of day 5.5 pc uterine B cells (dark grey) compared to non-B cells stained with B cell receptor markers B220, CD19, IgM, IgD, and differentiation markers CD24 and CD21, and activation marker CD38. Uterine B cells exhibit high expression of these phenotypic markers compared to non-B cells. Surface marker expression was verified in three independent experiments.

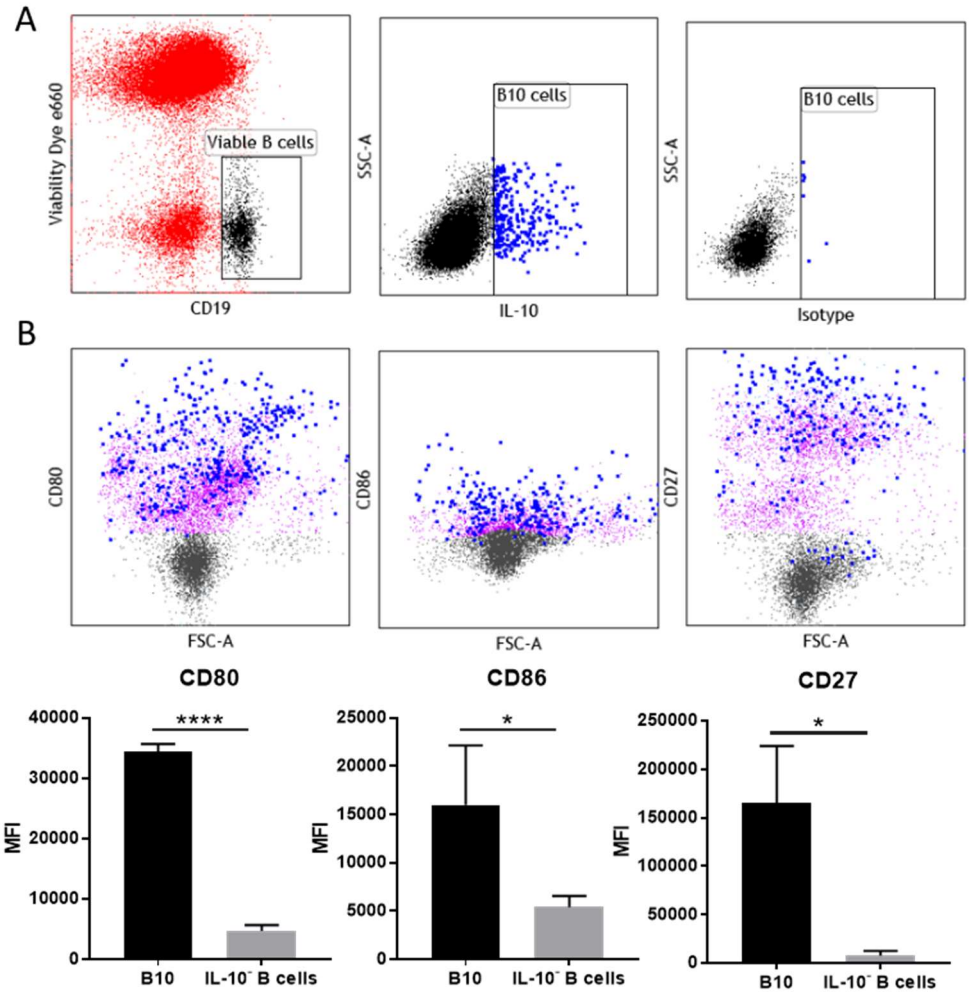


Figure 3.7. IL-10$^+$ B cells at day 5.5 pc have upregulated expression of CD27, CD80, and CD86. (A) Gating strategy for IL-10$^+$ cells within the viable CD19$^+$ cell population. (B) Representative flow cytometric profiles of B10 cells (blue) and IL-10$^-$ B cells (purple) and their upregulated expression of activation marker CD27 and co-stimulatory molecules CD80 and CD86, and the corresponding graphical presentation of the mean fluorescence intensity (MFI) of B10 cells compared to IL-10$^-$ B cells by unpaired t-test. *p≤0.05, ****p≤0.0001. Data shown are from three independent experiments.

3.4 Discussion

B cells are a major component of the lymphoid immune system. Their functional promiscuity is due to the variety of B cell subsets that can carry out one or more functions such as antibody production, antigen presentation, or secretion of modulatory cytokines. Historically, B cells in pregnancy and early life have been poorly studied due to their scarcity in the pregnancy microenvironment. However, studies over the past decade have only strengthened the need to explore the role of B cells in gestation as links between B cell abnormalities and obstetric complications have been repeatedly shown (Jensen et al., 2012; Shigeta et al., 2018; Marron and Harrity, 2019).

Variations in frequency and modification in phenotype and function have been demonstrated in maternal B cells from the bone marrow and spleen during murine pregnancy, but examination of the B cell compartment within the pregnancy microenvironment remains limited. As the molecular architecture and function of B cells are heavily influenced by the extracellular environment and their exposure to foreign antigens (LeBien and Tedder, 2008), we hypothesized that B cells within uterine tissue in close proximity to the implanting semi-allogeneic embryo undergo changes in phenotype and function to support the development of maternal-fetal tolerance. In this study, we investigated the modifications in B cells particularly within the uterine mucosa in a mouse allogenic pregnancy model to provide a clearer picture of the role they play during early pregnancy.

Assessment of B cell proportions in the spleen over the gestational period recapitulate the B cell lymphopenia previously reported at mid-gestation (Muzzio et al., 2014). However, we also show a significant increase in $B220^{+}$ B cells in the uterus during the peri-implantation period (days 2.5 to 8.5pc (Figure 3.1). This expansion at the time points approaching, during, and post-implantation, suggests their involvement in the control of the inflammation required for successful decidualization and implantation. At the peri-implantation period, the morphologic and functional changes in the endometrium are accompanied by major changes in the proportion and number of endometrial immune cells (Lee et al., 2011;Robertson and Moldenhauer, 2014). Uterine dendritic cells, decidual macrophages, and regulatory T cells are some of the immune cells recruited into the endometrium to accumulate around the embryo at implantation (Plaks et al., 2008; Nagamatsu and Schust, 2010; Shima et al., 2010). Alteration in the number and function of these cells in the local pregnancy milieu has been demonstrated to be instrumental in the recognition of fetal

alloantigens, decidual differentiation, and the establishment of maternal immune tolerance (Lee et al., 2011; Robertson and Moldenhauer, 2014). Recently, we have shown *in vitro* that human trophoblasts can educate B cells to acquire regulatory properties that protect against T cell-mediated inflammation that may otherwise be detrimental to implantation (Guzman-Genuino et al., 2019). Our results herein showing the expansion B cells in the murine pregnancy microenvironment further suggest an intimate involvement of B cells in facilitating induction of immune tolerance during the peri-implantation phase of pregnancy.

Focusing on the peri-implantation time point at day 5.5pc (Figure 3.2), we showed the pregnant uterus harbors a significantly lower population of $CD138^+$ plasma cells compared to unmated controls. The transmembrane marker CD138 is a syndecan specifically found in plasma cells that has long been correlated with the occurrence of chronic endometritis, an inflammatory condition of the uterine lining often associated with negative reproductive outcomes such as recurrent pregnancy loss, implantation failure, and infertility (Chen et al., 2016; Song et al., 2018). The decrease in the plasma cell population in the uterus, but not in the spleen nor lymph nodes, gives credence to clinical evidence that plasma cell numbers should be at a tolerable level in the pregnancy microenvironment (Matsushita and Tedder, 2011). Thus, the lower plasma cell count is likely suggestive of a normal implantation event.

The increased population of uterine B cells bearing $CD80^+$ and $CD86^+$ at day 5.5pc is particularly interesting, as it implies increased co-stimulatory function that may direct the T cell-mediated immune response (Sahoo et al., 2002). Upregulation of CD80 and CD86 expression on B cells is obtained by triggering and activating the BCR through a variety of stimuli such as ligands and cytokines (Mongini et al., 2002; Suvas et al., 2002). Subsequently, the crosstalk facilitated by the enhanced expression of these two co-stimulatory molecules on activated B cells is capable of skewing T cell activity (Sahoo et al., 2002). In pregnancy, it has been demonstrated that increased CD86 expression in murine peritoneal cavity B1-a B cells is associated with perturbations to pregnancy in a CBA/J mice model, an effect attributed to the increased capacity for Th1 and Th17 differentiation (Muzzio et al., 2014). It may however be a different case within the pregnancy microenvironment as investigated here, where the presence of the blastocyst and associated alterations in cytokine production within the microenvironment may serve as triggers for BCR activation and lead to enhanced expression of both CD86 and CD80 on uterine B cells. Moreover, previous studies demonstrated the upregulated expression of CD80 and CD86 on murine splenic antigen presenting cells such as dendritic cells and macrophages at the pre-

implantation period (day 3.5 pc). This was shown to be crucial in modulating T regulatory cell (Treg) abundance, cytokine production, and ultimately pregnancy outcome (Slawek et al., 2013; Maj et al., 2014). A follow up study examining splenic B cells showed similar results, with CD86 protein expression significantly increased in normal pregnant mice compared to abortion-prone mice at pre-implantation (Lorek et al., 2019). Thus, we postulate that the differential expression of CD80 and CD86 in uterine B cells may be predisposing them to act in a similar manner, that is elevated expression of these molecules on uterine B cells may contribute to establishment of maternal tolerance and therefore appropriate implantation. These findings are certainly intriguing and warrant further investigation that was unfortunately, out of scope of this current study.

Amongst the B cell subsets, regulatory B cells or Bregs have drawn attention as potentially critical players in pregnancy wellbeing. Previous murine studies have shown that splenic Bregs can rescue pregnancies in abortion-prone models (Jensen et al., 2013) and a clinical study has revealed that deficiency in circulating IL-10-producing Bregs is correlated with higher risk of spontaneous abortions (Rolle et al., 2013). Furthermore, it was recently shown that the lack of IL-10^+ B cells in pregnancies led to diminished embryo sizes and stagnated Treg pools, as well as increased susceptibility to LPS which caused intrauterine fetal death (Busse et al., 2019). With the expansion of uterine B cells at the peri-implantation phase, we speculated whether these cells possessed regulatory properties, and indeed demonstrated that uterine B cells from day 5.5 pc pregnant mice effectively suppressed syngeneic $CD4^+$ T cell proliferation and diminished expression of CD25 activation marker in a cell contact dependent manner (Figure 3.3).

Bregs are defined as any B cell carrying out negative regulation of immune response and as such, a plethora of studies have attributed this ability to various subsets of B cells including TIM-1^+ B cells, PD-$L1^+$ B cells, $CD5^+CD1d^{hi}$ B cells, and IL-10^+ B cells (Guzman-Genuino and Diener, 2017). Among these subsets, only the IL-10^+ B cells were significantly expanded in the uterus at the peri-implantation stage (day 5.5 pc) of pregnancy, with more IL-10 produced by B cells compared to non-pregnant controls and splenic B cells (Figure 3.5). In pregnancy, IL-10 is found in high amounts in placental and decidual tissues at early gestation in pre-clinical and clinical studies and is well-established as a vital cytokine for optimal pregnancy outcomes (Thaxton and Sharma, 2010). Mainly thought to be produced by trophoblasts, uterine natural killer cells, monocytes, dendritic cells, and Tregs, IL-10 has been shown to be a key cytokine for the balance of pro and anti-inflammatory signals that

establish maternal immune tolerance during the implantation process (White et al., 2004; Robertson et al., 2007; Robertson et al., 2011). While it was desirable to assess the suppressive capacity of these uterine IL-10$^+$ B cells on CD4$^+$ T cells specifically, the low percentage of IL-10$^+$ B cells (2-5%) within an already scarce population of uterine B cells from an individual pregnant mouse proved to be a major limitation to conducting these experiments.

The molecular surface architecture of uterine IL-10$^+$ B cells is summarized in Figure 3.8 and illustrates how these cells may facilitate their suppressive function. We determined that the majority of uterine B cells isolated from pregnant mice at the peri-implantation time point take on a phenotype descriptive of mature and antigen-driven differentiated B cells: they bear both IgM and IgD, antigen presenting cell marker MHC class II, differentiation markers CD24 and CD21, and activation marker CD38 (Figure 3.6). The dual expression of IgM and IgD is particularly interesting, as IgD co-expression is suggested to indicate a 'tolerogenic' role via regulating autoreactivity brought about by chronic exposure to antigen (Noviski et al., 2018). Likewise, the retention of differentiation markers CD24 and CD21 as well as expression of activation marker CD38 suggested that uterine B cells are mature, but yet to have differentiated into antibody-producing plasma cells (Oliver et al., 1997; Benitez et al., 2014). Furthermore, ~20 and 50% of IL-10 negative uterine B cells express the ligands CD80 and CD86, respectively (Figure 3.7B) which suggests that a significant proportion of the uterine B cells have the capacity for co-stimulating T cell activation, potentially leading to negative regulation by way of Treg expansion and modification of cytokine production (Slawek et al. 2013; Maj et al. 2014). Interestingly, the small proportion observed as IL-10$^+$ B cells within the uterine B cell population exhibited higher expression of co-stimulatory molecules CD80 and CD86 as well as CD27, an activation marker of memory and plasmablast lineages in murine B cells. In this study, these cell surface molecules, together with the cells' capacity for producing anti-inflammatory IL-10, is likely to have contributed to the regulatory phenotype that uterine B cells from pregnant mice exhibit. The differential expression of these surface markers indicates that IL-10$^+$ B cells may be involved in facilitating changes in the maternal immune network as it adjusts to the presence of fetal allo-antigens in early pregnancy. The increased capacity and engagement of these activated IL-10$^+$ B cells for T cell co-stimulation potentially contributes to the range of the T cell repertoire generated in early pregnancy, a process critical for setting the conditions for implantation.

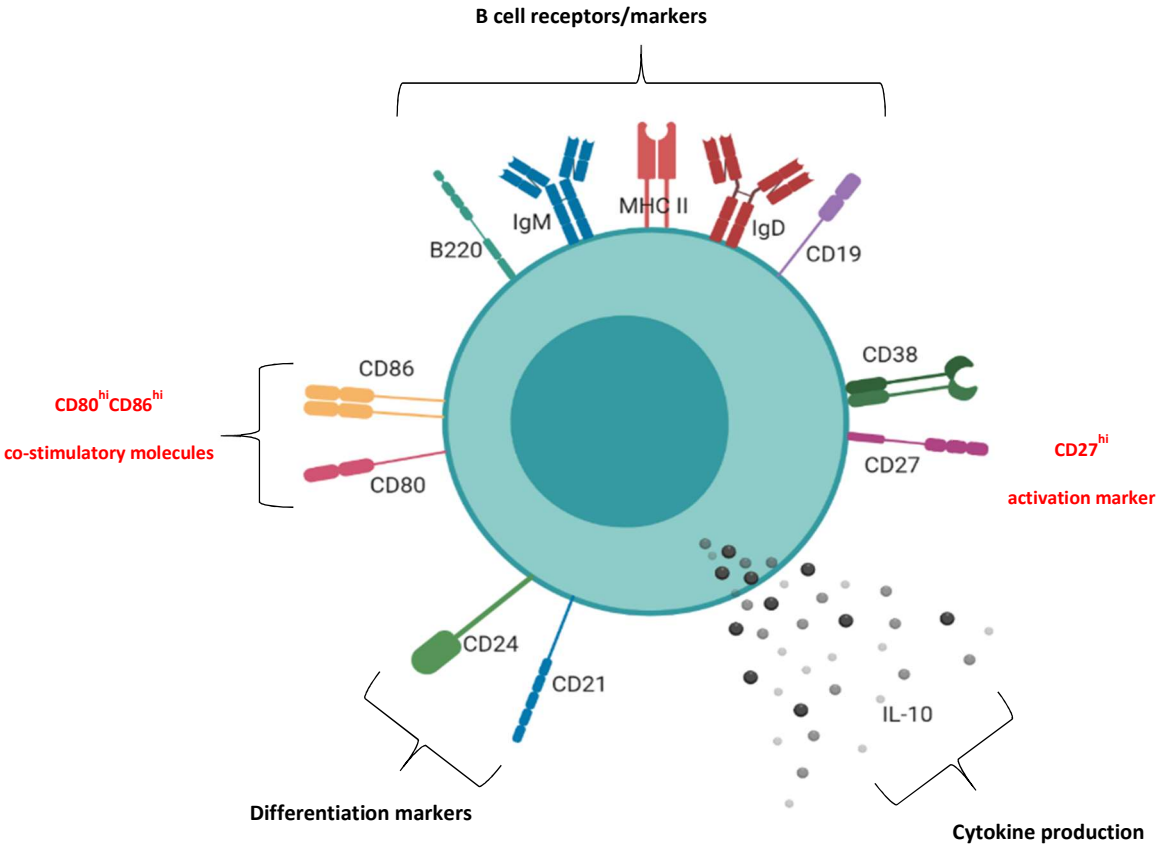


Figure 3.8. Phenotypic structure of an IL-10^{+} uterine B cell at peri-implantation. Schematic diagram of the phenotypic markers expressed by uterine IL-10^{+} B cells at peri-implantation, including B cell receptors and markers (CD19, B220, IgM, IgD, MHC II), activation markers (CD27, CD38), differentiation markers (CD21, CD24), co-stimulatory molecules (CD80, CD86), and IL-10 cytokine production.

3.5 Conclusion

To our knowledge, this is the first comprehensive study on the phenotype and functional characteristics of uterine B cells in early murine allogeneic pregnancy. In this study, we demonstrate an expanded pool of uterine B cells at the time points associated with embryo implantation, which harbor expanded subpopulations of $CD80^{+}CD86^{+}$ B cells and IL-10^{+} B cells, which collectively suppresses $CD4^{+}$ T cell proliferation and activation. Thus, we propose that at this crucial time point, B cells within the uterine microenvironment undergo changes in both population frequency and phenotype that supports a regulatory role and contributes to the immune changes necessary for the progression of a successful pregnancy.

Chapter Four

TROPHOBLASTS PROMOTE INDUCTION OF A REGULATORY PHENOTYPE IN B CELLS THAT CAN PROTECT AGAINST DETRIMENTAL T CELL-MEDIATED INFLAMMATION

This is a modified version of the following publication:

Guzman-Genuino RM, Dimova T, You Y, Aldo P, Hayball JD, Mor G, Diener KR. Trophoblasts promote induction of a regulatory phenotype in B cells that can protect against detrimental T cell-mediated inflammation. *Am J Reprod Immunol 82(6): e13187; 2019*

Chapter 4: Trophoblasts promote induction of a regulatory phenotype in B cells that can protect against detrimental T cell-mediated inflammation

4.1 Abstract

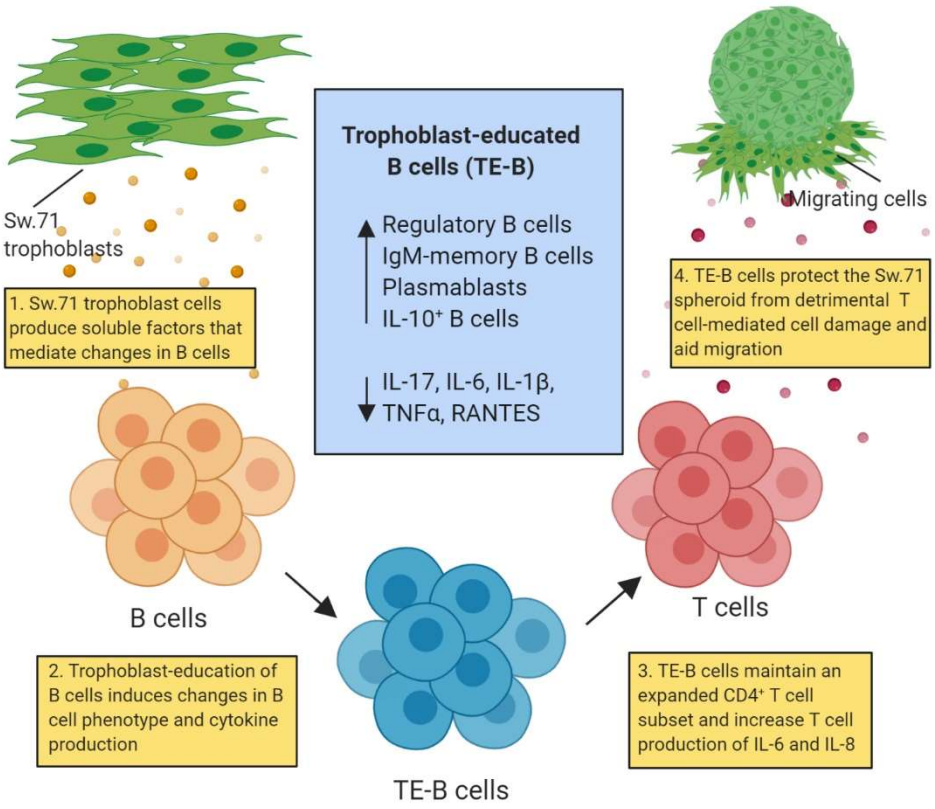
A successful outcome to pregnancy is critically dependent on the initiation of maternal immune tolerance before embryo implantation. Cells of embryonic origin that come in contact with the uterine microenvironment can exert influence over the phenotype and function of immune cells to facilitate robust implantation, however what influence they may have on B cells remains unknown. In this study, we investigate the effect of human trophoblast cells on B cell phenotype and the subsequent effect on peri-implantation events.

We cultured purified human B cells with the first trimester human trophoblast cell line Swan-71 to investigate trophoblast-B cell interactions and utilized trophoblast spheroids in an *in vitro* implantation model of migration and invasion.

Trophoblast-educated B cells or TE-B cells were found to consist of B cells in committed lineages such as plasmablasts and memory B cells, as well as increased proportions in subsets of $CD24^{hi}CD27^{+}$ regulatory B cells and $CD19^{+}IL\text{-}10^{+}$ B cells. Conditioned media from the TE-B cells showed reduced production of pro-inflammatory cytokines that influenced the T cell proliferation and cytokine production. Using trophoblast spheroids, we assessed the role of TE-B cells in trophoblast invasion and migration. Our results demonstrate a protective effect of TE-B conditioned media against deleterious inflammation as evidenced by survival of the trophoblast spheroid in the presence of an immune assault and promotion of a migratory phenotype.

We posit that trophoblast-mediated education of B cells leads to their acquisition of properties capable of modulating inflammation in the uterine environment during the peri-implantation period.

4.1.1 Graphical Abstract



4.2 Introduction

In pregnancy, maternal immune cells in the local uterine micro-environment are critical players in the physiological processes that lead to successful conception, implantation, gestation, and ultimately, parturition (Robertson, 2010). At the outset of pregnancy, the foremost objective is to build and foster a coordinated interplay of immune cells, hormones, and cytokines that is oriented towards securing blastocyst attachment and implantation into the uterine decidua. At peri-implantation, innate and adaptive immune cells including macrophages, natural killer cells, dendritic cells, and T cells accumulate in the endometrium. Interestingly, the phenotypes of endometrial and decidual immune cells in early pregnancy are altered compared with the non-pregnant state; i.e. uterine dendritic cells (uDC) retain an immature tolerogenic state (Du et al., 2014), uterine natural killer cells (uNK) exhibit reduced cytotoxic capability (Acar et al., 2011), and $CD8^+$ T cells in the decidua have reduced expression of perforin and granzyme B (Tilburgs et al., 2010). These modified functional roles are recognized as beneficial and contributory to a receptive uterus and vital for the development of the placenta (Zenclussen and Hammerling, 2015).

The first point of contact between embryonic cells and the maternal uterine micro-environment is via the invading trophoblast cells of the blastocyst. Trophoblast cells present an interesting immunologic occurrence in that they are semi-allogenic and should, in principle, trigger an adverse inflammatory response. Instead, the trophoblast thrives in the tolerant immunological milieu at the implantation site. Previous studies have shown that trophoblast cells can regulate immune cell function through various pathways. First trimester trophoblast cells secrete high levels of TGF-β and thymic stromal lymphopoietin (TSLP), and have been observed to actively recruit and differentiate regulatory T cells (Tregs) (Ramhorst et al., 2012; Du et al., 2014). Exosomes produced by trophoblast cells can act as recruiting agents for monocytes and down-regulate NKG2D receptors on NK, $CD8^+$, and γδ T cells to reduce their cytotoxic capacity (Hedlund et al., 2009; Atay et al., 2011). Trophoblast cells have also been shown to induce a predominantly tolerogenic profile in dendritic cells *in vitro* (Salamone et al., 2012). Trophoblast cells therefore, are suggested to be paramount in shaping the manner in which maternal uterine immune cells respond to invasion of the blastocyst.

So far, studies on lymphocytes within the uterine microenvironment has been largely limited to T cells, which contribute immensely to the controlled inflammatory environment requisite

for uterine receptivity leading to implantation and placentation (Lee et al., 2011). However, there are now a number of studies outlining an emerging role for B cells as being active participants in pregnancy. Recent studies have shown a protective effect of IL-10-producing B cells (B10) in restoring tolerance and rescuing pregnancy. Mouse splenic B10 cells are expanded during normal pregnancy and their adoptive transfer to abortion-prone mice rescued pregnancies (Jensen et al., 2013). Similarly in humans, IL-10 producing $CD19^{+}CD24^{hi}CD27^{+}$ regulatory B cells (Bregs) are significantly higher in women undergoing normal pregnancies in their first trimester as opposed to non-pregnant women, or women who suffered spontaneous abortions (Rolle et al., 2013). These studies suggest that B cells play a role in early pregnancy, thus there is an impetus to investigate events critical at this stage.

In this study, we aimed to investigate the effect of first trimester trophoblast cells on the human B cell phenotype and function. Here, we provide *in vitro* evidence that trophoblast cells can promote the differentiation of regulatory B cells, transitional B cells, IgM-memory B cells, and plasmablasts, which subsequently produced a dampened inflammatory cytokine profile. We also show that these trophoblast-educated B (TE-B) cells can regulate T cell proliferation and cytokine production. Lastly, using trophoblast spheroids as an *in vitro* implantation model, we demonstrate that the addition of conditioned media from TE-B cells protect the spheroid from damage and aid trophoblast migration, despite the presence of deleterious cytokines from highly activated T cells. Thus, we propose that first trimester trophoblast cells can educate B cells to acquire phenotypes and functional properties that contribute in calibrating inflammation that otherwise could be detrimental to a successful implantation event.

4.3 Results

4.3.1 Trophoblast cells modify the phenotype of B cells and reduce pro-inflammatory cytokine production

Our first objective was to determine whether trophoblast cells could influence B cell differentiation. To test this, we cultured purified peripheral blood B cells with either immortalized first trimester trophoblast cells, trophoblast conditioned medium, or normal medium as controls for 48 hours. Cells were subsequently collected, and the B cell phenotype determined by flow cytometry. Figure 1A shows representative flow cytometry

panels for the evaluated $CD19^+$ B cells subsets: $CD24^{hi}CD27^+$ regulatory B cells, $CD24^{hi}CD38^{hi}$ transitional B cells, $CD27^+IgM^+$ memory B cells, and $CD38^{hi}CD27^{hi}$ plasmablasts. Our results indicate that trophoblast cells can directly, or indirectly by secreted factors, induce B cells to undergo differentiation characterized by a significant increase in the proportion of B regulatory, transitional, IgM-memory, and plasmablast subpopulations (Figure 1B). The fact that culture with trophoblast conditioned medium or trophoblasts directly yielded similar alterations in phenotypes suggested that the greater part of the observed phenotypic changes can be incurred through soluble factors present in the conditioned medium rather than cognate interactions with the trophoblast cells. For this reason, in all following experiments we focused on using B cells cultured with trophoblast conditioned medium and refer to them henceforth as trophoblast-educated B cells or TE-B cells.

Next, we characterized the cytokine/chemokine profile of TE-B cells by multiplex assay analysis (Figure 2). Interestingly, B cells cultured in trophoblast conditioned medium showed major differences in their secreted cytokine profile, with concentrations of the pro-inflammatory cytokines IL-17, IL-1β, IL-6, TNFα, and the chemokine RANTES, all significantly reduced compared to that of control medium. All other analytes were not significantly different between cultures. This indicated that secreted factors found in the conditioned medium from trophoblast cell cultures dampened the basal inflammatory cytokine profile in B cells.

The modification in phenotype and the general reduction in pro-inflammatory cytokines alluded to the induction of regulatory properties in these TE-B cells. To verify, we looked specifically at the capacity of these cells to generate the regulatory cytokine IL-10. Expression of IL-10 mRNA was significantly increased in B cells cultured in trophoblast conditioned medium compared to control medium (Figure 3A). Similarly, assessment of intracellular IL-10 indicated that the percentage of IL-10-producing B cells increased with culture in trophoblast conditioned medium. A further increase was shown in B cells cultured with trophoblast cells directly, which yielded similar percentages of $IL\text{-}10^+$ cells to that observed with CpG (TLR9 agonist), a known factor responsible for the expansion of $IL\text{-}10^+$ B cell frequencies in humans (Iwata et al., 2011) (Figure 3B). Lastly, analysis of surface markers showed that $IL\text{-}10^+$ B cells have increased expression of CD24, CD38, and IgM, and intermediate expression of CD27, similar to that of CpG-generated $IL\text{-}10^+$ B cells. The surface marker expression profile indicates that $IL\text{-}10^+$ B cells are found in the suppressive

B cell subsets, namely within the groups of regulatory B cells ($CD19^+CD24^+CD27^+$), transitional B cells ($CD19^+CD24^{hi}CD38^{hi}$), and IgM-memory B cells ($CD19^+IgM^+CD27^+$) (Figure 3C).

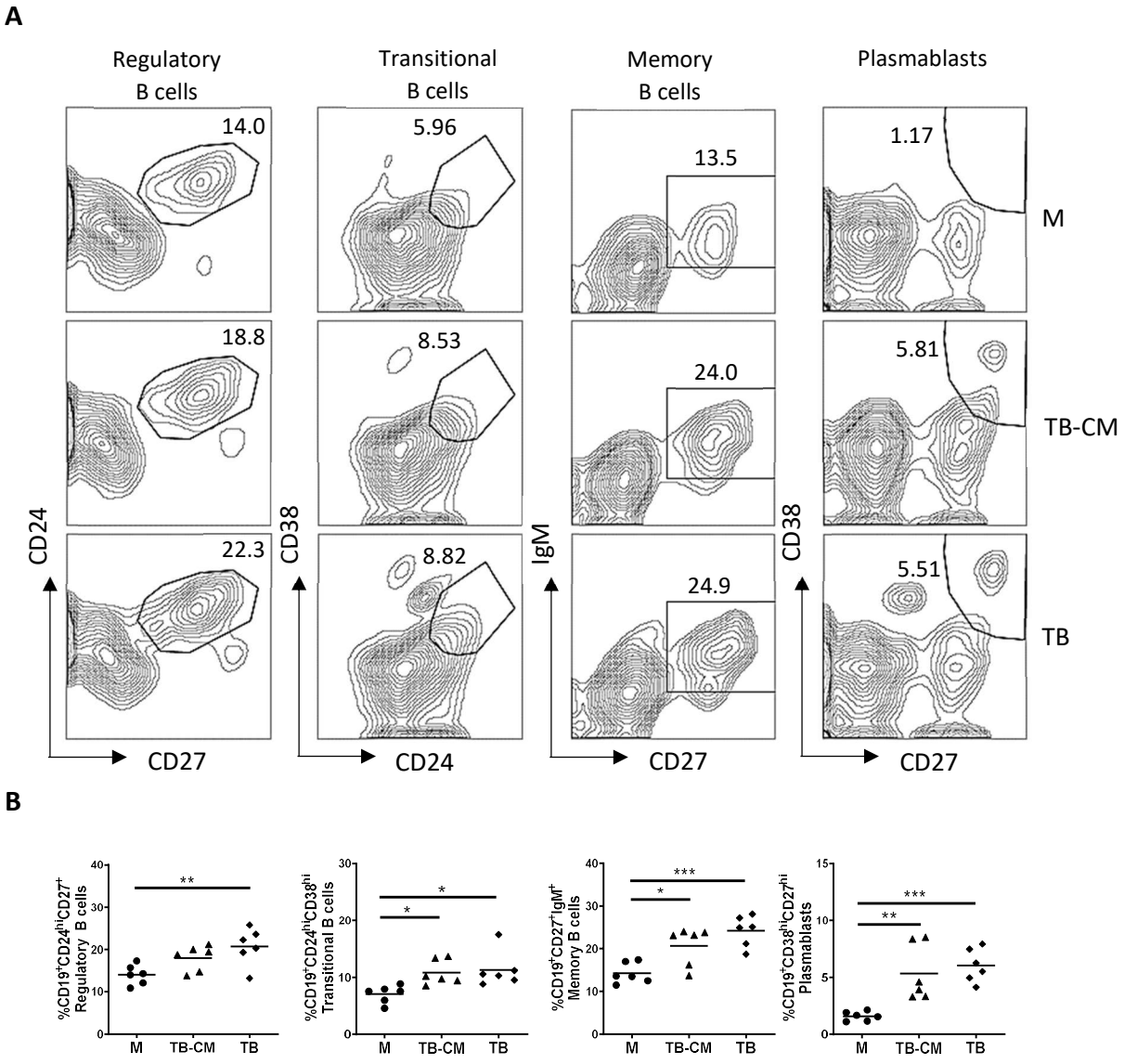


Figure 4.1. **Trophoblast cells modify B cell phenotype**. Purified B cells (10^5 cells/100µL medium) were cultured in normal medium (M), trophoblast conditioned medium (TB-CM), or directly co-cultured with trophoblast (TB) cells. After 48 hrs, recovered B cells were stained with B cell lineage surface markers CD19, CD24, CD27, CD38, and IgM to discriminate between the Breg, transitional, IgM-memory, and plasmablast subpopulations. Representative flow cytometry panels indicating gating strategy shown in (A), with combined results from three independent experiments performed in duplicate shown in (B). *p<0.05, **p<0.01, ***p<0.001.

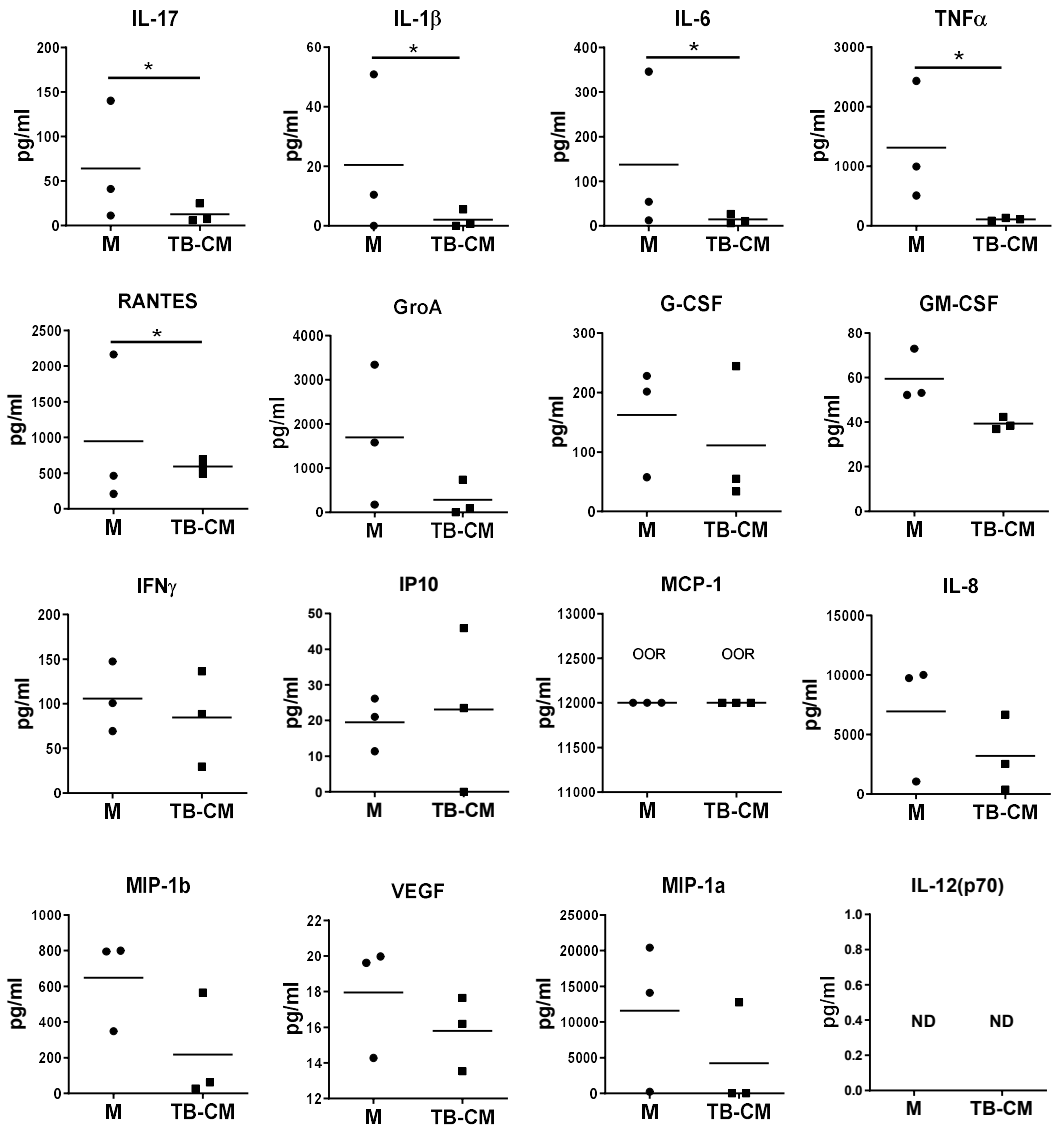


Figure 4.2. **Trophoblast-educated B cells have diminished pro-inflammatory cytokine production.** Cell-free supernatant from cultures of purified B cells incubated in trophoblast conditioned medium (TB-CM) or control medium (M) for 48hrs were quantified against TB-CM alone using Bio-Plex® multiplex assay. Results were expressed in pg/ml +/- SEM with data presented as concentration minus concentrations found in TB-CM alone. IL-12 was not detected (ND) and MCP-1 readouts were out of range (OOR) and could not be extrapolated in the assay. Data represent pooled supernatants of duplicate wells from three independent experiments. *$p<0.05$

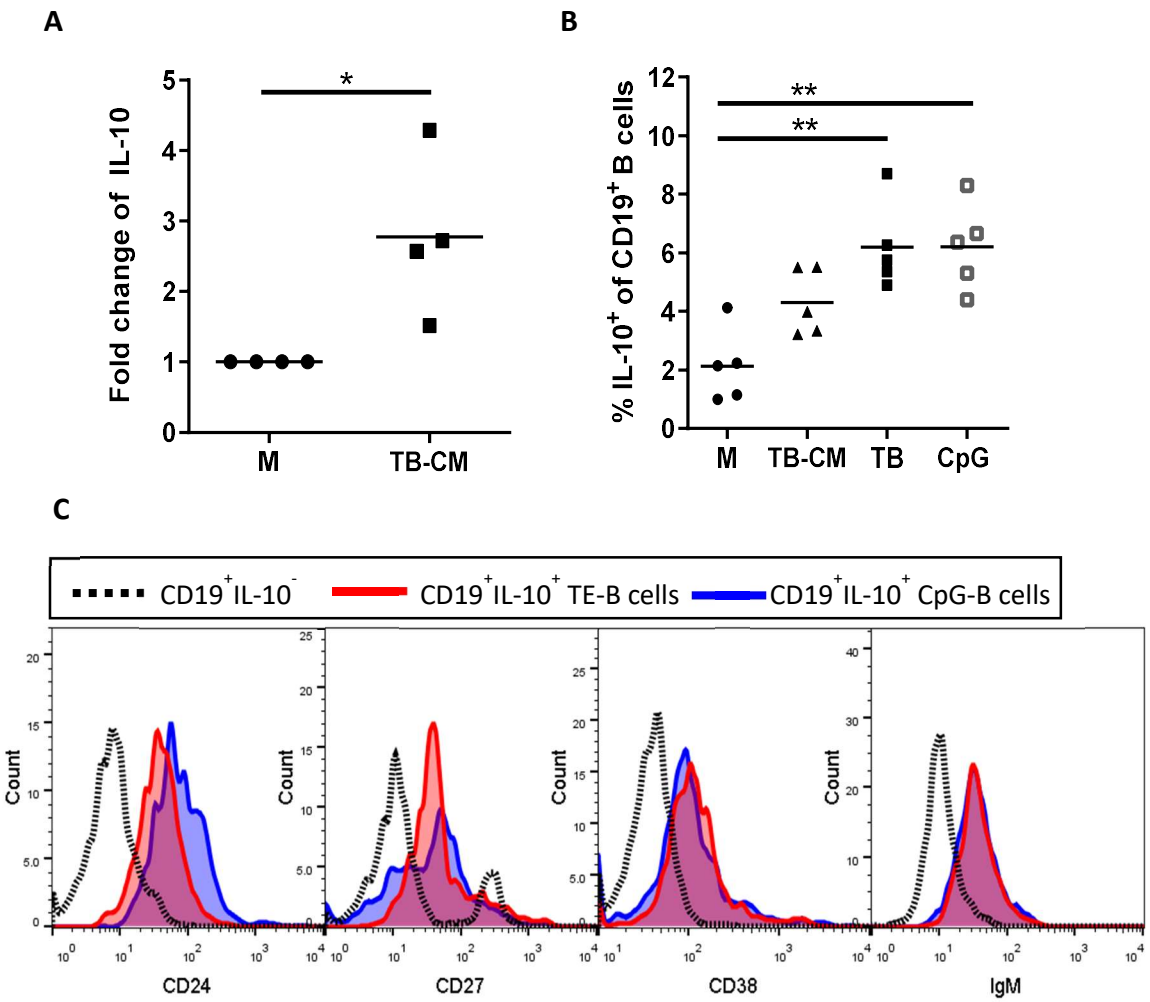


Figure 4.3. Trophoblast cells secrete factors that upregulate IL-10 expression in B cells. (A) Purified B cells were cultured with trophoblast conditioned medium (TB-CM) or normal medium (M) for 48hrs after which B cells were processed for IL-10 mRNA expression by RT-qPCR analysis. Results are shown as fold increase over controls. (B) Production of IL-10 by B cells cultured with M, TB-CM, or trophoblast cell (TB) cells directly were assessed by intracellular flow cytometric methods against positive control cultures stimulated with CpG. Results shown represent data collected from 4-5 independent experiments. (C) Expression of surface markers CD38, CD24, CD27, and IgM on $CD19^{+}IL\text{-}10^{+}$ B cells after trophoblast-education (red) and CpG treatment (blue) was compared to non-IL-10 producing B cells (dotted line). *$p<0.05$, **$p<0.01$

4.3.2 TE-B cells regulate T cell proliferation and cytokine production

B cells perform a range of immunological functions, some of which require close interactions with T cells. At the implantation stage, various T cell subsets participate in the development of a receptive endometrium. Critical events such as generation of Th1/Th2/Th17 cells, development of memory T cells, and induction of Treg cells require an expanded $CD4^+$ T cell subset to generate these lineages (Polese et al., 2014; Feyaerts et al., 2017; Kieffer et al., 2017). Post-implantation, decidual $CD4^+$ T cells are in much lower proportions due to the general predominance of $CD8^+$ T cells (Tilburgs et al., 2010). Here, we explored whether B cells differentiated under the influence of trophoblast secreted factors are functional and able to influence T cell subset composition and T cell cytokine production.

To test this hypothesis, we non-specifically stimulated purified T cells into proliferation with anti-CD3 and anti-CD28 antibodies alone, or in the presence of conditioned medium from cultures of normal B cells, trophoblast cells, and TE-B cells. Cells were then left to rest after which CD4 and CD8 T cell subset composition and cytokine expression was analysed (Figure 4A). Representative flow cytometric panels and gating strategy for the $CD4^+$ and $CD8^+$ T-cell subsets is shown in Figure 4B, as well as the tabulated $CD4^+/CD8^+$ ratios following differentiation. Our data indicated that exposure of proliferating T cells to trophoblast or TE-B cell conditioned media increased the proportion of $CD4^+$ T cells compared to proliferating T cells cultured in medium from B cells alone (Figure 4C). We next examined whether the expansion in $CD4^+$ T cells was as a result of an increase in the Treg population, however whilst TGFβ, the positive control for $CD4^+Foxp3^+$ Treg expansion, generated the highest percentage of $CD4^+Foxp3^+$ Tregs, no such response was observed in our expanded populations suggesting that conditioned medium from TE-B cells are not responsible for Treg differentiation (Figure 4C).

However, the presence of TE-B cell conditioned medium during TCR stimulation had an independent effect on the cytokine profile of proliferating T cells. We observed a significant increase in the expression of the pro-inflammatory cytokines IL-6 and IL-8 by T cells exposed to secreted factors from TE-B cell cultures compared to conditioned medium from cultures of trophoblasts and normal B cells (Figure 4D). Both IL-6 and IL-8 are cytokines that have been repeatedly shown as important factors in implantation as reduced levels have been correlated with a reduction in trophoblast invasion resulting in early miscarriages (von Wolff et al., 2000; Pitman et al., 2013). These findings demonstrate a network of cell

communication where trophoblast cells promote B cell differentiation into unique B cell subtypes with the capacity to regulate T cell proliferation and cytokine production.

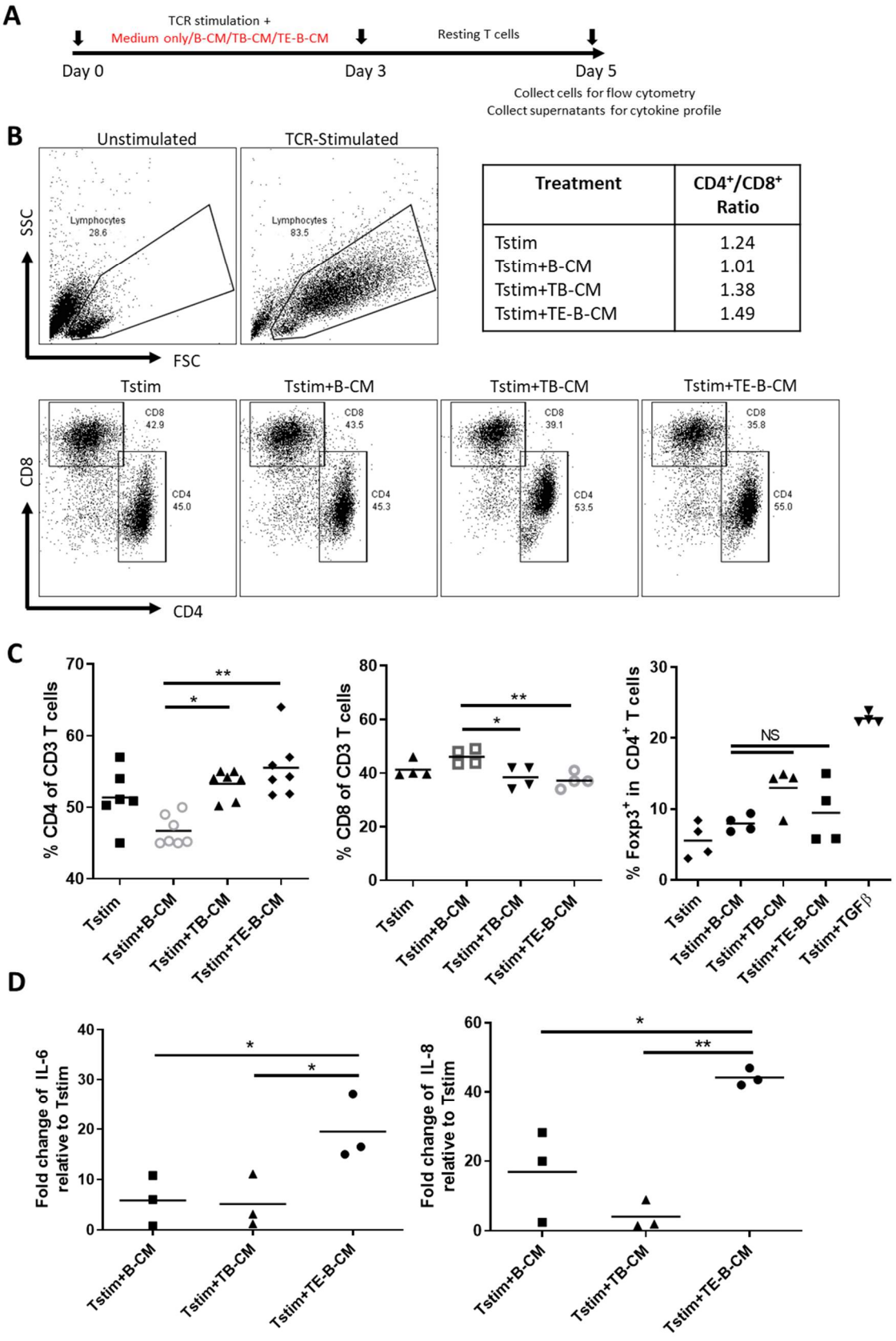


Figure 4.4. **TE-B cells impact on T cell proliferation and cytokine production**. Purified T cells were (A) TCR-stimulated with anti-CD3 and anti-CD28 antibodies in normal medium alone (Tstim), or with addition of conditioned medium obtained from cultures of normal B cells (B-CM), trophoblast cells (TB-CM), or TE-B cells (TE-B-CM). (B) Representative flow cytometry plots comparing side and forward scatter of unstimulated and TCR-stimulated T cells (upper panels), the gating strategy for $CD4^+$ and $CD8^+$ subsets (lower panels), and the summarized $CD4^+/CD8^+$ ratios. (C) Graphical presentation of data combined from a minimum of 4 independent experiments showing $CD4^+$ and $CD8^+$ T cells as a percent of all $CD3^+$ T cells and expression of transcription factor Foxp3 in $CD4^+$ T cells. (D) Fold change increase of IL-6 and IL-8 cytokine production by proliferating T cells cultured in indicated conditioned medium over normal medium controls. *$p<0.05$, **$p<0.01$

4.3.2 TE-B cells contribute to the control of inflammation at implantation

To investigate the potential functional role of TE-B cells at implantation, we utilized a novel 3D *in vitro* model to evaluate trophoblast migration and invasion (You et al., 2019). Trophoblast cells were grown as blastocyst-like spheroids and transferred onto matrigel-coated wells and assessed for evidence of cellular protrusions indicative of invasion into the matrigel matrix. Whilst stromal cell conditioned medium clearly induced prominent protrusions as expected, conditioned medium from TE-B cells or control B cells elicited effects similar to that of media controls (Figure 5A and 5B, left panel). Similarly, stromal cell conditioned medium induced rapid attachment and migration of trophoblast cells (from 12 hours), an effect not replicated with conditioned medium from TE-B cell cultures. Furthermore, TE-B cells did not impact on trophoblast attachment and migration induced by stromal cell conditioned medium (Figure 5C and 5B, right panel) indicating that TE-B cells did not secrete factors that directly or indirectly affected trophoblast attachment, migration, or invasion.

We then examined if TE-B cells might have an indirect effect on trophoblast invasion and migration via modification of T cell activity. We collected the cell-free supernatant from rested T cells non-specifically stimulated in the presence of normal medium or conditioned medium from TE-B or normal B cell cultures (Figure 6A). These supernatants were combined with matrigel and plated into individual wells for trophoblast invasion assays. Conditioned media from stimulated T cells alone or in combination with TE-B cell or B cell conditioned mediums, we were able to induce protrusions from trophoblast cells with varying consistency (Figure 6B and 6C, left panel). However, no conditioned media could induce migration of trophoblast cells (Figure 6D and 6C, right panel). Taken together, these results suggest that soluble factors produced by T cells in a non-inflammatory state have a limited effect on the functional activity of trophoblast cells.

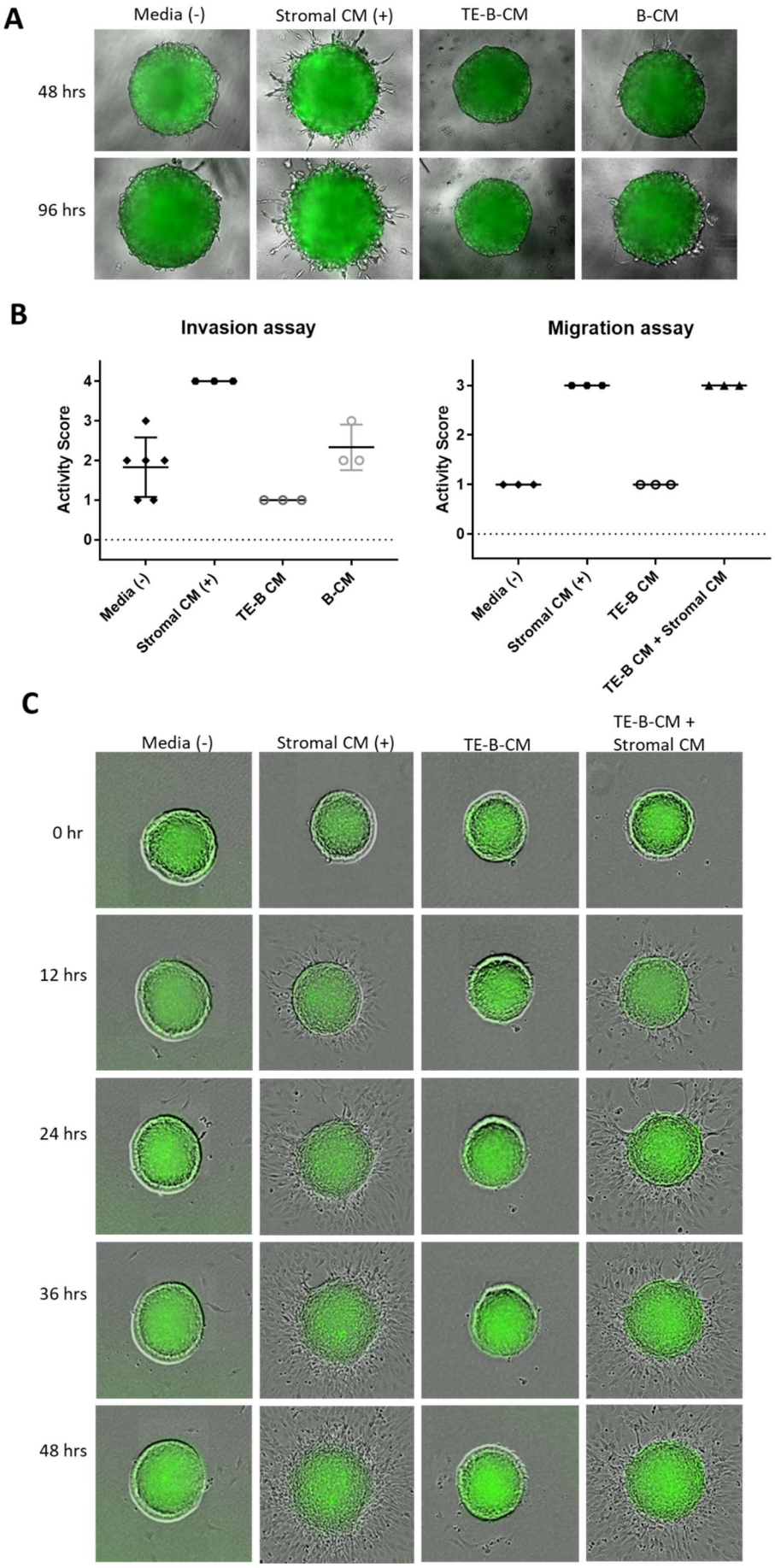

A
Media (-)
Stromal CM (+)
TE-B-CM
B-CM
48 hrs
96 hrs
B
Invasion assay
Migration assay
Activity Score
Media (-)
Stromal CM (+)
TE-B CM
B-CM
TE-B CM + Stromal CM
C
Media (-)
Stromal CM (+)
TE-B-CM
TE-B-CM + Stromal CM
0 hr
12 hrs
24 hrs
36 hrs
48 hrs

Figure 4.5. TE-B cells do not express soluble factors that affect the invasion and migration activity of trophoblast spheroids. (A) For invasion assays, trophoblast spheroids were cultured on matrigel solution combined with normal medium (negative control), or conditioned medium from decidualized stromal cell cultures (stromal CM) as a positive control, or cultures of TE-B cells (TE-B-CM) or normal B cells (B-CM). The formation of trophoblast cell protrusions indicative of invasion into the matrigel matrix was monitored for up to 96 hrs, with protrusions clearly generated by stromal CM as expected, but not from any other conditioned or control medium. Representative photos at 48- and 96 hr-timepoints of the trophoblast spheroids in the invasion assay are shown. (B) Independent invasion (left panel) and migration (right panel) experiments were assessed and given an activity score. Combined data from at least 3 biologically independent experiments are shown. (C) For migration assays, trophoblast spheroids were individually seeded into 96 well plate wells with attachment and migration of trophoblast cells monitored for 48hrs. Stromal CM induced cell attachment and migration, however no such effect was seen with control or TE-B cell conditioned media. TE-B-CM was also not capable of inhibiting migration induced by stromal CM. Representative photos of trophoblast spheroids at 0-, 12-, 24-, 36-, and 48-hr timepoints in the migration assay are shown. All photos were taken using the Revolve Pro microscope in (A), or the IncuCyte ZOOM System in (B) at 200x magnification.

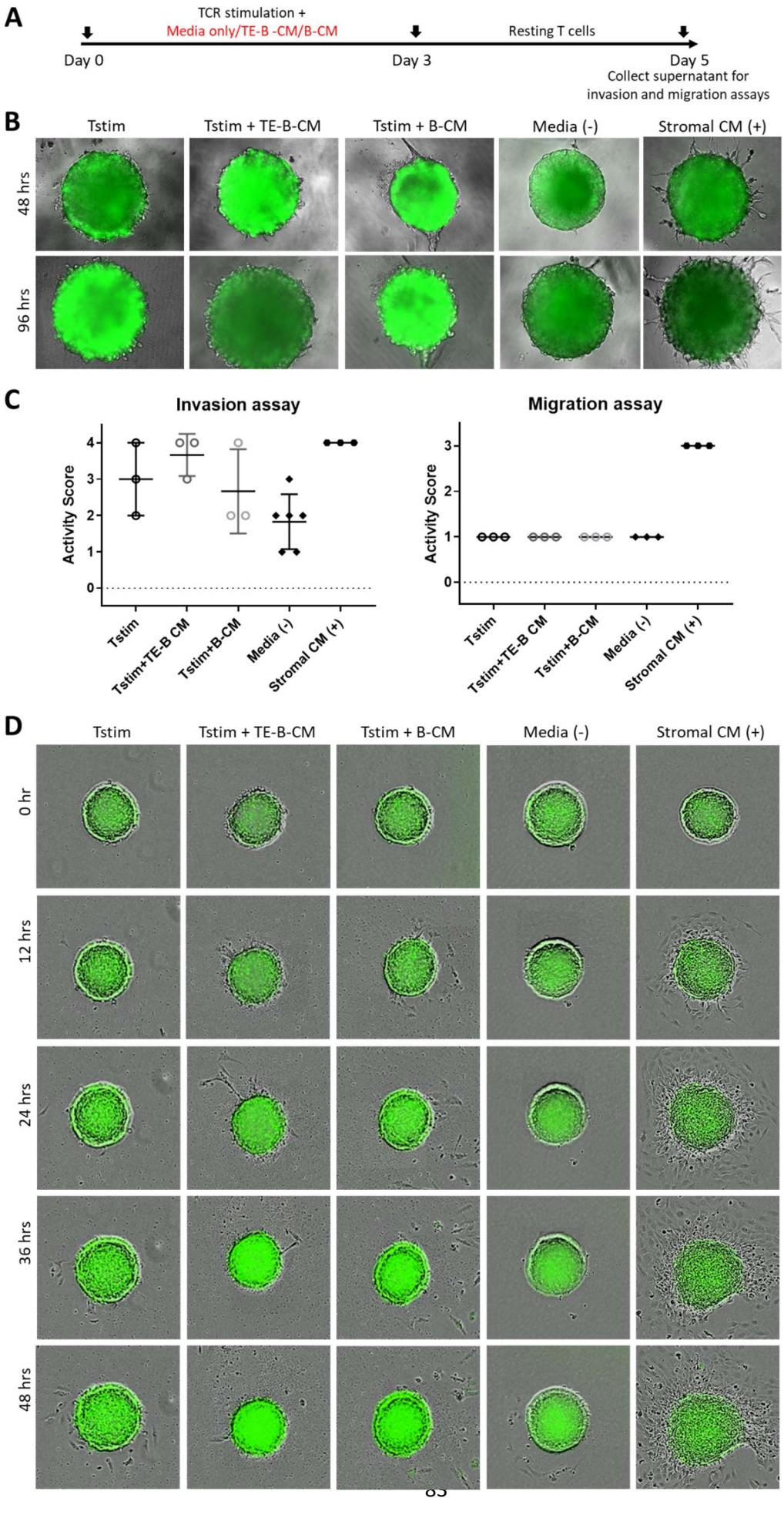

A
TCR stimulation +
Media only/TE-B -CM/B-CM
Resting T cells
Day 0
Day 3
Day 5
Collect supernatant for
invasion and migration assays
B
Tstim
Tstim + TE-B-CM
Tstim + B-CM
Media (-)
Stromal CM (+)
48 hrs
96 hrs
C
Invasion assay
Migration assay
Activity Score
Tstim
Tstim+TE-B CM
Tstim+B-CM
Media (-)
Stromal CM (+)
D
Tstim
Tstim + TE-B-CM
Tstim + B-CM
Media (-)
Stromal CM (+)
0 hr
12 hrs
24 hrs
36 hrs
48 hrs

Figure 4.6. **TE-B cells do not promote trophoblast invasion or migration by indirect effect on T cells.** (A) Timeline of T cell stimulation, resting period, and collection of media is shown. (B) For invasion assays, trophoblast spheroids were incubated on matrigel combined with medium from TCR-stimulated T cells alone (Tstim) or in the presence of conditioned medium from TE-B cells (TE-B_CM) or normal B cells (B-CM). Medium alone or stromal cell medium were used as negative and positive controls respectively. No effect on invasion by any of the conditioned media was observed Representative photos at 48- and 96-hrs timepoints of the trophoblast spheroids in the matrigel invasion assay are shown. (C) Independent invasion (left panel) and migration (right panel) experiments were assessed and given an activity score. Combined data from at least 3 biologically independent experiments are shown. (D) For migration assays, trophoblast spheroids were individually monitored for attachment and migration of trophoblast cells for 48hrs. No migration was induced by stimulated T cell culture media with or without conditioned media from TE-B or normal B cells. Representative photos of trophoblast spheroids at 0-, 12-, 24-, 36-, and 48-hr timepoints in the migration assay are shown. All photos were taken using the Revolve Pro microscope or the IncuCyte ZOOM System at 200x magnification, and are representative of 3 other independent experiments performed in duplicate.

Th_1 inflammation is essential for implantation; however the level of inflammation should fall within a range that is not detrimental to the embryo. In these experiments, we investigated whether TE-B cells could modulate the level of inflammation in a novel trophoblast model of deleterious inflammation where conditioned medium from cultures of PMA/ionomycin activated pre-primed T cells are added to trophoblast spheroids. By combining TCR-stimulation by anti-CD3/CD28 antibodies with PMA/ionomycin stimulation which activates the cells through the $Ca2^{+}$/calmodulin-dependent and PKC signalling pathways, we were able to induce heightened activation in T cells. The resultant conditioned medium derived from these supra-activated cells are predominantly comprised of pro-inflammatory cytokines. Incubation with the activated T cell conditioned medium resulted in restricted spheroid growth and active disintegration of peripheral cells as visualized by a fuzzy outermost layer of dead trophoblast cells clearly identifiable by 96 hours in invasion assays and 48 hours in migration assays (Figure 7). This cell death was confirmed by CellTox green dye binding (Dimova T. et al, unpublished data). To determine whether TE-B cells could dampen the deleterious inflammatory response, conditioned medium from TE-B cell or normal B cell cultures was added to rested TCR-stimulated T cells prior to PMA/ionomycin activation, with resultant supernatants added to wells containing trophoblast spheroids and observed for invasion and migration capacity (Figure 7A). Soluble factors from these activated T cells clearly inhibited generation of cellular protrusions; rather death of the outer spheroid cells is evident (Figure 7B and 7C, left panel). However, activation of T cells in the presence of conditioned medium from TE-B cells could negate this effect, as indicated by increased survival of these trophoblast cells. This effect was also seen when conditioned medium from normal B cells was used. In a similar manner, medium from activated T cells inhibited spheroid growth, attachment and migration, rather death of peripheral trophoblast cells was observed (Figure 7D and 7C, right panel). This outcome could be inhibited by prior addition of conditioned medium from TE-B cells or normal B cell cultures, although conditioned medium from TE-B cells was also able to induce partial trophoblast migration. Together these results suggest that soluble factors produced by TE-B cells may play a role in the regulation of inflammation such that it can protect against embryo cell death and contribute to maintaining the cytokine environment necessary for progression of implantation.

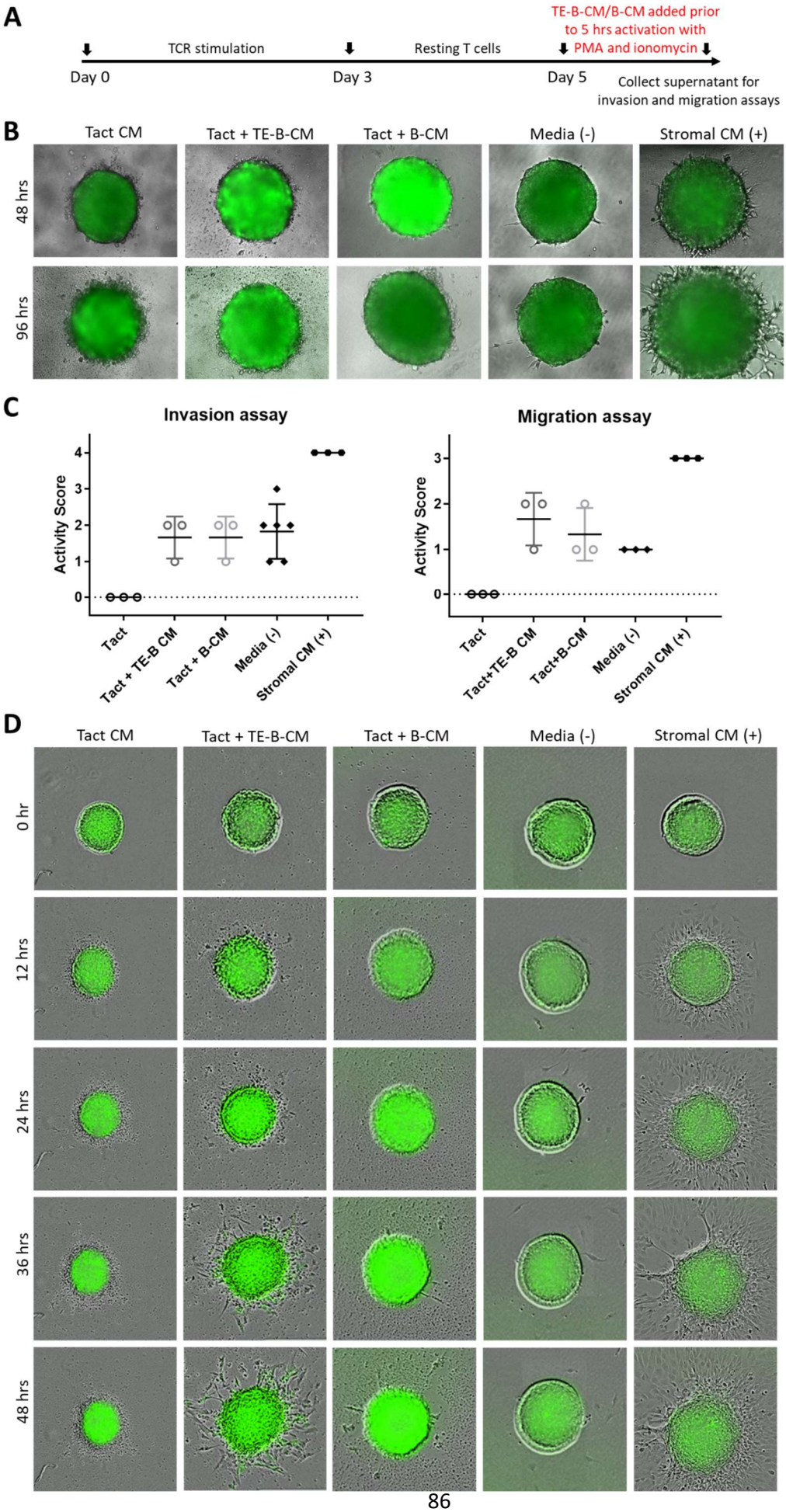

A
TE-B-CM/B-CM added prior
to 5 hrs activation with
PMA and ionomycin
TCR stimulation
Resting T cells
Day 0
Day 3
Day 5
Collect supernatant for
invasion and migration assays
B
Tact CM
Tact + TE-B-CM
Tact + B-CM
Media (-)
Stromal CM (+)
48 hrs
96 hrs
C
Invasion assay
Migration assay
Activity Score
Tact
Tact + TE-B CM
Tact + B-CM
Media (-)
Stromal CM (+)
Tact+TE-B CM
Tact+B-CM
D
Tact CM
Tact + TE-B-CM
Tact + B-CM
Media (-)
Stromal CM (+)
0 hr
12 hrs
24 hrs
36 hrs
48 hrs

Figure 4.7. **Soluble factors from TE-B cells can protect the trophoblast spheroid from cell damage and aid migration in conditions of deleterious inflammation**. (A) Timeline of non-specific T cell stimulation, resting period, and T cell activation by PMA/ionomycin in normal or conditioned media from cultures of TE-B cells (TE-B-CM) or normal B cells (B-CM). (B) Trophoblast spheroid invasion assays. Trophoblast spheroids were plated onto matrigel matrix solutions containing conditioned medium from activated T cells (Tact CM), T cells activated in the presence of conditioned medium from TE-B cells (Tact + TE-B-CM) or normal B cells (Tact + B-CM) and assessed at 48 and 96 hours. Normal medium and stromal cell medium acted as negative and positive controls respectively. (C) Independent invasion (left panel) and migration (right panel) experiments were assessed and given an activity score. Combined data from at least 3 biologically independent experiments are shown. (D) Trophoblast migration assays. Individual trophoblasts were plated in the presence of conditioned medium from activated T cells as above, and assessed every 12 hrs for 2 days. Photos are representative of three independent experiments. All photos were taken using the Revolve Pro microscope or the IncuCyte ZOOM System at 200x magnification.

4.4 Discussion

Pregnancy is a delicate condition requiring a complex and coordinated interplay of immune cells, endometrial cells, and endocrine factors. From the outset, the maternal immune system is tasked with protecting the mother against foreign assault as well as protecting the conceptus from damage. The conceptus on the other hand, imposes an influence on the maternal immune system such that it is accepted into the uterine microenvironment despite its semi-allogeneic nature. At implantation, feto-maternal immune cross-talk is initiated by the trophoblast cells. Trophoblast-immune interactions are categorized into three stages: (1) recruitment – where the trophoblast transmits signals to encourage influx of immune cells in the implantation site, (2) education – where the trophoblast influences the phenotype and function of immune cells, and (3) response – where the immune cells educated by the trophoblast cells carry out functions that support implantation and placental formation (Dekel et al., 2014). The trophoblast's role in the recruitment and phenotype modification of some immune cells has been studied over the years, including the trophoblasts impact on the form and functions of monocytes, NK cells, regulatory T cells, macrophages, and dendritic cells (Atay et al., 2011; Abumaree et al., 2012; Ramhorst et al., 2012; Du et al., 2014; Sotnikova et al., 2014). Interestingly, the B cells' role in implantation has not been extensively explored. In this study, we focused on the effects of trophoblast cells on B cell phenotype and function, and the subsequent role of these trophoblast-educated B cells (TE-B cells) in the context of implantation.

B cell lymphopoiesis generates a diverse repertoire of peripheral B cells, starting from B cells exiting the bone marrow and entering peripheral circulation as immature transitional B cells and naïve B cells. Upon acquiring cognate antigens in secondary lymphoid locations, B cells become activated and differentiate into memory B cells, plasmablasts, or fully differentiated plasma cells bearing combinations of surface markers including CD21, CD24, CD27, CD38, and IgM (Kaminski et al., 2012). In recent years, B cells have been attributed a role in pregnancy due to their production of protective antibodies against paternal antigens as well as the production of damaging auto-antibodies that have been implicated in some pregnancy pathologies (Marie-Cardine et al., 2008). IL-10-producing regulatory B cells have also been identified as important cells in pregnancy well-being (Guzman-Genuino and Diener, 2017). In our study, we show that trophoblast cells have a profound effect on the phenotype and cytokine production of B cells, mainly by inducing the differentiation of B

cells into transitional B cells and committed B cell lineage subsets such as regulatory B cells, IgM-only memory B cells, and plasmablasts. We hypothesize here that the Sw.71 trophoblast cells preferentially differentiate and expand B cells that can potentially function for immune regulation (regulatory B cells and IL-10^{+} transitional B cells) as well as protection against foreign antigen assaults (memory B cells and plasmablasts).

It is tempting to speculate that the observed effect on B cell phenotype and cytokine profiles may stem from the polarizing effect of Sw.71 trophoblast-derived exosomes, a phenomenon that has been previously reported in monocytes wherein their recruitment and differentiation were modified to support trophoblast function (Atay et al., 2011). In the same study, trophoblast-derived exosomes also decreased the production of pro-inflammatory cytokine IL-17 and chemokines CCL5 and macrophage migration inhibitory factor (MIF). Our results emulate their findings of cell-contact-independent modification of immune cells by Sw.71 cells, with B cells undertaking differentiation into specific B cell lineages and production of reduced levels of pro-inflammatory cytokines IL-17, IL-1β, IL-6, and TNF-α. Furthermore, Sw.71 cells are characterized as representing extravillous cytotrophoblasts that express fetal fibronectin secretin, cytokeratin 7, vimentin, and HLA-G, as well as low levels of human chorionic gonadotrophin (hCG) (Straszewski-Chavez et al., 2009). It is possible that hCG from the Sw.71 trophoblast cells may have also influenced the observed phenotypic changes in B cells. There is growing evidence that hCG can mediate immune modulatory effects that promote tolerance at the maternal-fetal interface specifically in early pregnancy and recent studies have demonstrated the capacity of hCG to expand the population of $CD24^{hi}CD27^{+}$ regulatory B cells as well as boost IL-10 production in $CD19^{+}$ B cells (Rolle et al., 2013; Dauven et al., 2016; Fettke et al., 2016; Schumacher, 2017). Additionally, hCG has been shown to alter the secretory profile of dendritic cells and human endometrial epithelial cells to support endometrial receptivity and blastocyst implantation (Wan et al., 2008; Paiva et al., 2011).

Feto-maternal cross-talk in early pregnancy is essential for the establishment of maternal immune tolerance, regulation of trophoblast activity, and remodelling of the uterine spiral arteries (Lash, 2015). Local immune cells in the endometrium are hypothesized to possess embryotrophic properties by providing a cytokine network that controls the immune and endocrine systems, as well as trophoblast function at implantation. The expression of cytokines mediate the embryo-maternal paracrine dialogue necessary for the early stages of implantation and trophoblast invasion (Saito, 2000; Orsi and Tribe, 2008). Our findings

demonstrate a modified cytokine profile of TE-B cells, suggesting their involvement in the balance of cytokines in the maternal-fetal interface which may contribute to implantation success. The decreased production of pro-inflammatory cytokines IL-17, IL-1β, IL-6, and TNF-α of TE-B cells can potentially down-regulate inflammatory reactions and provide cytokine regulation in a predominantly pro-inflammatory environment required during implantation. Moreover, the increased mRNA expression of regulatory cytokine IL-10 indicates that the TE-B cells may also have the capacity to counteract inflammation in the microenvironment when needed. Recently, IL-10 produced by B cells have been demonstrated to provide protection against fetal death in murine pregnancies jeopardized by the presence of lipopolysaccharide (LPS) (Busse et al., 2019), further highlighting the influence of B cells in the immune balance at the maternal-fetal interface. The findings on B cell phenotype demonstrate that trophoblast education of B cells helps set the stage for a balanced immune condition that is conducive for implantation.

Although considered as scarce populations in the uterine microenvironment, B cells are present and active in the endometrium and decidua (Blois et al., 2004; Feyaerts et al., 2017; Rinaldi et al., 2017). In this study, we focused on the interaction of B cells and T cells, specifically the effect of TE-B cells on T cell subset distribution and cytokine production. In most studies of pregnancy, the $CD4^+/CD8^+$ T cell ratio has been reported to be approximately 1 or slightly decreased as the number of $CD8^+$ T cells has been found to be higher than $CD4^+$ T cells (Watanabe et al., 1996). However, in pre-pregnancy endometrium, which is arguably the best representation of the endometrium at the onset of pregnancy, there is a predominance of $CD4^+$ T cells and an increased $CD4^+/CD8^+$ ratio compared to term decidua and peripheral blood (Sabbaj et al., 2011; Feyaerts et al., 2017) presumably in preparation for differentiation into effector T cells necessary for inflammation. Treatment with TE-B conditioned media alongside TCR stimulation maintained the high $CD4^+/CD8^+$ T cell ratio. However comparison with other controls such as trophoblast conditioned medium treatment showed that this is likely a consequence of trophoblast factors and TE-B conditioned media only enhances that effect. The increase in IL-6 and IL-8 production by TE-B treated T cells is interesting as these cytokines play critical roles in implantation events, particularly in spiral artery remodelling. Inadequate levels of IL-6 and IL-8 have been associated with reduced trophoblast invasion translating to spontaneous abortions (Champion et al., 2012; Pitman et al., 2013). IL-8 in particular has been shown to stimulate extravillous trophoblast invasion and is involved in uterine natural killer cell-mediated stimulation of trophoblast invasion (De Oliveira et al., 2010; Jovanovic et al., 2010).The amplified production as per TE-B treatment

is indicative of cytokine production being orchestrated to accommodate the invading trophoblasts. Lastly, we determined if TE-B cells can contribute towards expansion of the $CD4^+Foxp3^+$ regulatory T cells via induction of Foxp3 expression in $CD4^+$ T cells. The results showed levels of Foxp3 expression similar to the treatment with just the trophoblast conditioned medium, supporting the idea that Treg recruitment at implantation is achieved by fetal antigens (Ramhorst et al., 2012), and so at this point there is no indication that soluble factors produced by TE-B cells are involved in Treg induction.

Uterine receptivity pertinent for embryo implantation is brought about by a Th1 inflammation in the endometrium, with the process of trophoblast migration and invasion dependent on the chemotactic gradients and cytokines conceived in the implantation milieu by resident immune cells. This inflammatory activity conditions the uterine microenvironment to receive the invading trophoblast and permit cells of embryonic origin to migrate into maternal tissue (Mor et al., 2011). The various regulatory mechanisms by both immune and decidualized endometrial cells to keep the pro-inflammatory cytokines and chemokines in check indicate that this inflammation is a tightly controlled process. Previous studies as well as the results thus far in this study provide evidence for a significant role of trophoblast cells in orchestrating control of inflammation. In this study, TE-B cells in a non-inflammatory, non-dangerous state by themselves do not have any significant effect on the activity of trophoblast spheroid cells (Figure 5), although combined with conditioned medium from TCR-stimulated T cells, TE-B cells may promote invasion of trophoblast cells (Figure 6). However, they do demonstrate functional activity within a system with prevailing inflammatory conditions (Figure 7). In our activated T cell-mediated inflammation model, we demonstrated a deleterious condition where the inflammation is elevated to toxic levels for the spheroid. This detrimental effect was negated upon the addition of TE-B conditioned medium prior to PMA/ionomycin activation of the stimulated T cells. Furthermore, TE-B conditioned medium treatment in the migration assay resulted in trophoblast cells migrating out of the spheroid in 2 out of 3 independent experiments, as opposed to the activated T cell treatment alone or that with B cell conditioned medium, suggesting that soluble factors from TE-B cells may have prevented and interfered with the normal expression and function of the activated T cells cytokine profiles to a more tolerable level such that spheroid survival and subsequent activity was permitted. This may be due directly to increased levels of IL-6 and IL-8 cytokines found within the TE-B conditioned medium, or indirectly by changes in the proportions of cytokines produced by activating T cells in the presence of TE-B conditioned medium, thus creating a more favourable chemotactic gradient and prompting

migration to occur. Whilst these effects demand further investigation in primary trophoblast cell lines and more physiological settings such as in vivo mouse models, the in vitro models used here imply that B cells may play a specific regulatory role in maintaining an environment conducive for trophoblast invasion and migration, a hallmark event in implantation.

The confirmation that TE-B cells can only partially restore normal migration activity is a testament that the process of implantation is regulated by multiple cellular players working together. The results gathered from the invasion and migration assays emphasize two things: (1) that controlled inflammation is necessary for implantation events to proceed, and that (2) trophoblast-educated B cells contribute in calibrating the inflammation to a tolerable level.

4.5 Conclusion

In toto, we demonstrate in this study that the interactions and interplay of Sw.71 trophoblast cells and B cells influence the generation of a tolerable inflammatory environment that is favourable for trophoblast health and activity. Successful implantation is dependent on a network of immune cells and endometrial cells working together for a balanced inflammatory response necessary in achieving a receptive uterine microenvironment. Cells of embryonic origin, primarily trophoblasts, have been previously reported as significant modifiers of immune function in order to facilitate acceptance of the invading embryonic cells as well as prime cells to ensue immune reactions against foreign antigens should the need arise. In this study, we describe the effect of first trimester trophoblast cells on B cells *in vitro* by showing the phenotypic and cytokine profile changes. TE-B cells are comprised of higher percentages of regulatory cells, IgM-memory cells, plasmablasts, and IL-10-producing immature/transitional cells that exhibit reduced cytokine/chemokine production. These TE-B cells were shown to help maintain an expanded $CD4^+$ T cell subset and stimulate the production of vital cytokines IL-6 and IL-8. Lastly, we demonstrate the role of B cells in counterbalancing inflammation due to activated T cells, enabling our *in vitro* implantation model of trophoblast spheroids to survive and undertake implantation hallmarks. Collectively, our results imply that trophoblast education of B cells may be one of the regulatory mechanisms in place ensuring the success of implantation in pregnancy.

Chapter Five

INVESTIGATING THE THERAPEUTIC POTENTIAL OF REGULATORY B CELLS IN PREGNANCY

Chapter 5: Investigating the therapeutic potential of regulatory B cells in pregnancy

5.1 Introduction

During pregnancy, the maternal immune system undergoes unique adaptations in order to establish and maintain immunological tolerance towards the semi-allogeneic fetus. Ongoing efforts to define the immune network during pregnancy are focused on understanding the functional role of the various immune, endometrial, and fetal cells as well as elucidating the cross talk between these cells. It is now well-accepted that changes in the immune profile occur to optimise the conditions for gestation, i.e. suppression of inflammation mounted against fetal antigens via the dampening of T effector cells, generation of regulatory T cells (Tregs), and modulation of natural killer (NK) and dendritic cell (DC) functions (Mor and Cardenas, 2010; Arck and Hecher, 2013). However, in the event this immune recalibration during pregnancy does not occur, it may potentially lead to disorders and pathologies such as recurrent pregnancy loss, preeclampsia, and immune-based infertility.

Treatment modalities for pregnancy disorders and pathologies are extremely limited due to insufficient knowledge of the immune mechanisms at play as well as the complexity of pregnancy factors which involve multiple biological systems. For example, prescription for treatment for recurrent miscarriages rely solely on the general immunomodulatory effects of certain substances; aspirin for its anti-inflammatory effect, anticoagulants for their anti-inflammatory and anticoagulant effect, and steroids for the anti-inflammatory effect (Carp, 2019). Early attempts at a more advanced version of immunotherapy for addressing recurrent pregnancy loss include paternal leukocyte immunisation wherein the paternal parent's mononuclear cells are injected into the mother in an attempt to induce a change in the balance between Th1 and Th2 cytokines and suppress T cell activity (Ober et al., 1999; Yokoo et al., 2006). Other forms of immunotherapy that have gone through clinical trials include donor white cell immunisation, trophoblast membrane immunisation, and intravenous immunoglobulin immunisation. However, the latest report shows that none of these immunotherapy methods lower the risk of future miscarriage in women who have undergone multiple pregnancy losses (Wong et al., 2014). The failure of these treatment modalities that have taken the blanket approach to addressing pregnancy pathologies only highlight the need to investigate the specific details of the underlying pathophysiology. To be able to effectively manipulate the maternal immune system is critically dependent on understanding the intricate network and cellular interplay that dictate the health and well-being of pregnancy.

In this current study, we focus on investigating the potential of regulatory cells within the maternal immune system as candidates for a therapeutic approach to address pregnancy disorders and pathologies. Regulatory cells are present at the feto-maternal interface and they have demonstrated to be potently immunosuppressive during pregnancy. Current literature describes $CD4^+$ regulatory T cells as essential in maintaining an active state of maternal immune tolerance. Treg cells inhibit effector immunity, downregulate inflammation, and mediate vascular adaptations in early pregnancy that promotes trophoblast invasion and efficient maternal blood supply (Robertson et al., 2018). Through their potent anti-inflammatory activities, Tregs are paramount in controlling inflammation in early pregnancy and establishing a receptive environment for implantation and placental development. Moreover, deficiency in Treg numbers and/or functional competency are associated with infertility and recurrent miscarriages (Aluvihare et al., 2004; Shima et al., 2010; Care et al., 2018). Tregs are particularly important in the peri-implantation period as demonstrated by studies showing a high rate of spontaneous fetal loss in mated mice possessing low percentages of thymic Tregs and elevated decidual Th1 cells, and the subsequent rescue of fetal viability upon adoptive transfer of Tregs (Zenclussen et al., 2005).

In the therapeutic context, Treg cell maldistribution and functional impairment in pregnancy may be one of the etiologies of recurrent pregnancy loss and infertility. Foxp3 mRNA expression in the uterus is decreased in primary unexplained infertility (Jasper et al., 2006) and reduction of Treg cells in the peripheral blood in the late follicular phase leads to failure of artificial insemination by the donor sperm (Lu et al., 2013). This creates an impetus to investigate the factors that affect Treg distribution and function during pregnancy.

In our previous studies, we have identified the presence of another regulatory cell subset from the B cell lineage. These ‘regulatory B cells’ or Bregs possess the capacity to down-regulate immune responses via the secretion of IL-10. We have previously shown that these IL-10-producing Bregs expand in early pregnancy, particularly at the peri-implantation period, and that they contribute to T cell distribution and control of function. In the fields of autoimmunity and transplantation, Bregs are actively being explored as a therapeutic strategy due to their capacity of promoting Foxp3 expression in T cells as well as generating and expanding Tregs in various models of autoimmune diseases and transplantation (Mauri and Menon, 2017; Peng et al., 2018). Drawing from these studies, we hypothesise that Bregs can promote Treg generation and expansion during pregnancy and therefore, can induce

maternal tolerance via control of Treg numbers and function. If proven, the next step is to harness Bregs as a therapeutic agent for addressing Treg-related immune dysfunction during pregnancy. In this study, we focus on defining the interplay of Bregs and Tregs during pregnancy and whether Bregs can restore homestasis in a pregnancy model lacking Tregs.

5.2 Results

5.2.1 Percentages of IL-10$^+$ regulatory B cells are increased in early pregnancy

We first determined whether there was a correlation in the kinetics of Bregs and Tregs during murine pregnancy. Tregs are known to accumulate in the uterine microenvironment and the draining lymph nodes from the estrus stage up to the peri-implantation period of pregnancy (Kallikourdis and Betz, 2007; Shima et al., 2015). Bregs, on the other hand, have been reported to have increased percentages during normal pregnancy compared to pathologic pregnancies in mice, as well as in the first trimester of human pregnancy (Jensen et al., 2013; Rolle et al., 2013). To verify if Bregs numbers were augmented within the same timeframe as Tregs, we conducted a kinetic study to document the percentages of both Bregs and Tregs during the progression of murine pregnancy. The gating strategy for the identification of Bregs and Tregs is shown in Figure 5.1.

Pregnant mice were humanely killed at set time points during the progression of gestation and their spleen, para-aortic lymph nodes, and uterus harvested. Isolated cells from these organs were analysed for Breg and Treg percentages via their production of anti-inflammatory IL-10 (B220$^+$IL-10$^+$) and both activation marker CD25 and transcription factor Foxp3 (CD4$^+$CD25$^+$Foxp3$^+$), respectively. As shown in Figure 5.2A, no observable trend in the proportions of the Breg population in the spleen and the para-aortic lymph nodes over the course of pregnancy. However, in the uterus, Breg levels were observed to increase in early pregnancy (day 0.5 pc to day 8.5 pc), with the peak at day 5.5 pc significantly different from the estrus time point. In contrast, the proportions of Treg cells in these tissues did not exhibit any significant trend over the course of pregnancy, with almost all time points showing a large degree of variance between individual mice. (Figure 5.2B).

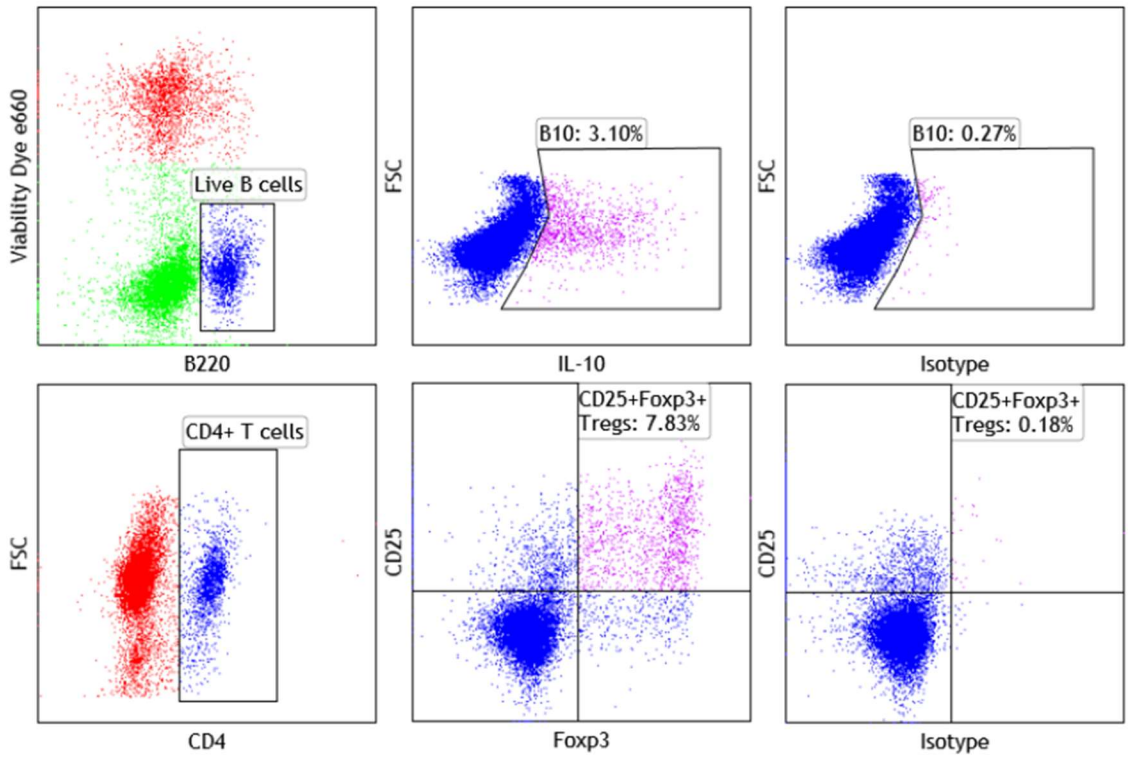


Figure 5.1: Gating strategy for the identification of IL-10-producing B220$^+$ Bregs and CD4$^+$CD25$^+$Foxp3$^+$ Tregs. IL-10 production by viable B220$^+$ B cells was detected by incubating isolated cells from the spleen, para-aortic lymph nodes, and the uterus for 5 hours with a stimulation cocktail of lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA), and ionomycin supplemented with a protein transport inhibitor Brefeldin A. CD4$^+$ Tregs were identified as per their dual expression of the activation marker CD25 and transcription factor Foxp3. Setting of gates was determined using FMO and isotype controls.

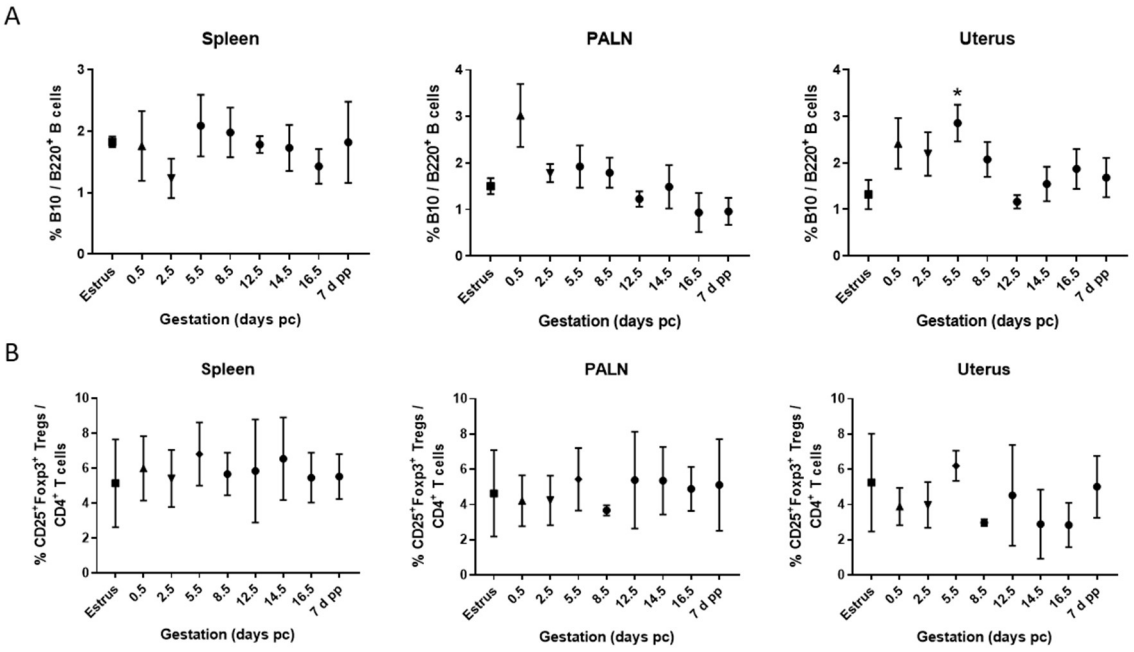


Figure 5.2: Kinetics of Breg and Treg cell populations during murine pregnancy. Graphical summary of the percentages of (A) $B220^{+}IL\text{-}10^{+}$ Bregs and (B) $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs as a proportion of the parental population. Cells were obtained from the spleen, para-aortic lymph nodes, and uterus of estrus and pregnant mice at various days pc (n=3-6 per time point). Data is shown as mean ± SEM and assessed by ordinary one-way ANOVA followed by Dunnett's *post-hoc* multiple comparisons test. *$p \leq 0.05$

5.2.2 Splenic B cells induce Foxp3 expression in $CD4^+$ T cells

The next objective was to evaluate whether B cells possess an inherent tolerogenic capacity capable of expanding allogeneic Tregs *in vitro.* We set up a mixed lymphocyte reaction (MLR) by co-culturing purified C57Bl6/J splenic B cells with proliferation dye-labelled purified allogeneic BALB/c splenic responder T cells. Splenic B cells were co-cultured with the T cells in increasing T:B ratios (1:1, 1:2, 1:4) for 5 days without any cytokines added to the mix. Thereafter, assessment of T cell parameters, specifically proliferation and expression of CD25 and Foxp3, were determined by flow cytometry.

For CD25 expression, the results show that responder T cells markedly expressed the activation marker in the presence of splenic B cells in the co-culture, whereas a culture of T cells alone had only minimal CD25 expression; T cells alone in culture expressed minimal levels of CD25 (2-10%) whereas T cells co-cultured with B cells possessed a significant proportion of $CD25^+$ T cells (20-60%) (Figure 5.3A,B). Moreover, the ratio of B cells to responder T cells was not a factor in the activating capacity as percentages of $CD25^+$ T cells remained in the range of 15 to 60% in all three treatment ratios.

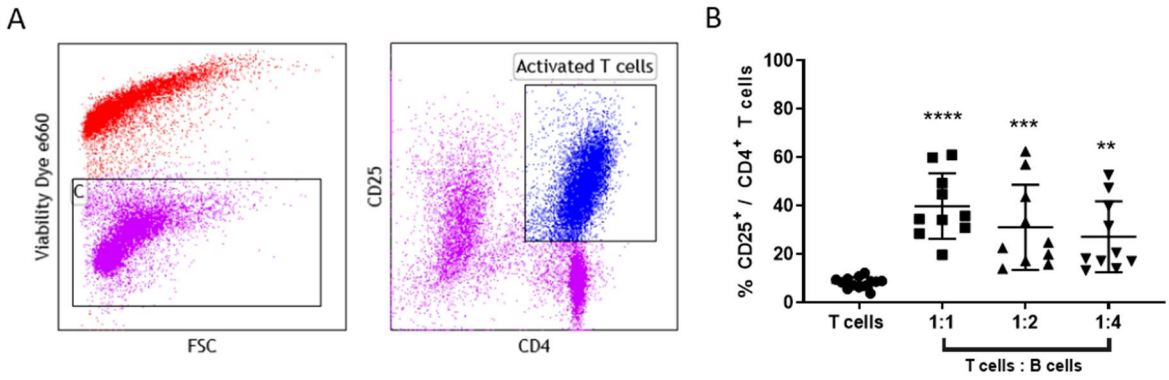


Figure 5.3: MLR co-culture with splenic B cells induces $CD25^+$ activation in responder $CD4^+$ T cells. Purified splenic B cells were incubated in different ratios with purified CD4+ T cells in a mixed lymphocyte reaction. After 5 days of co-culture, cells were harvested and stained for viability and CD4 and CD25 expression. (A) Representative plots showing viable cells gated as per exclusion of the viability dye and analysed for expression of T cell marker CD4 and activation marker CD25 (1:1 ratio). (B) Graphical summary of the percentages of resultant CD25-activated $CD4^+$ T cells. Data shown is combined from 5 independent experiments performed in duplicate. Statistical analysis based on one-way ANOVA and

Dunnett's multiple comparisons test against T cell control. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001

Our results also demonstrate that co-culturing with B cells led to proliferation of the responder T cells, a feat not observed in the T cells alone culture. Proliferating cells comprised 30 to 90% of the harvested cells in the co-culture treatments, regardless of the B cell to responder T cell ratio (Figure 5.4A,B). We then determined if splenic B cells were able to promote Treg expansion by assessing the $Foxp3^+$ expression on co-cultured responder T cells by flow cytometry. We investigated the expansion of Tregs such that if B cells can indeed expand Tregs, this should be aptly reflected by the proliferation of Foxp3-expressing $CD4^+$ T cells. After 5 days in co-culture, there was a significant increase in the percentages of $Foxp3^+$ cells in the proliferating pool of $CD4^+$ T cells (Figure 5.4C,D, right panel). Moreover, proliferating cells included $Foxp3^+$ T cells which had increased proportions commensurate to the increase in ratio of B cells to T cells in the co-culture. The results herein suggest that B cells, without the addition of exogenous antigens, are able to induce $Foxp3^+$ T cells to undergo cell division which leads to an expansion in Treg proportions (Figure 5.4C,D).

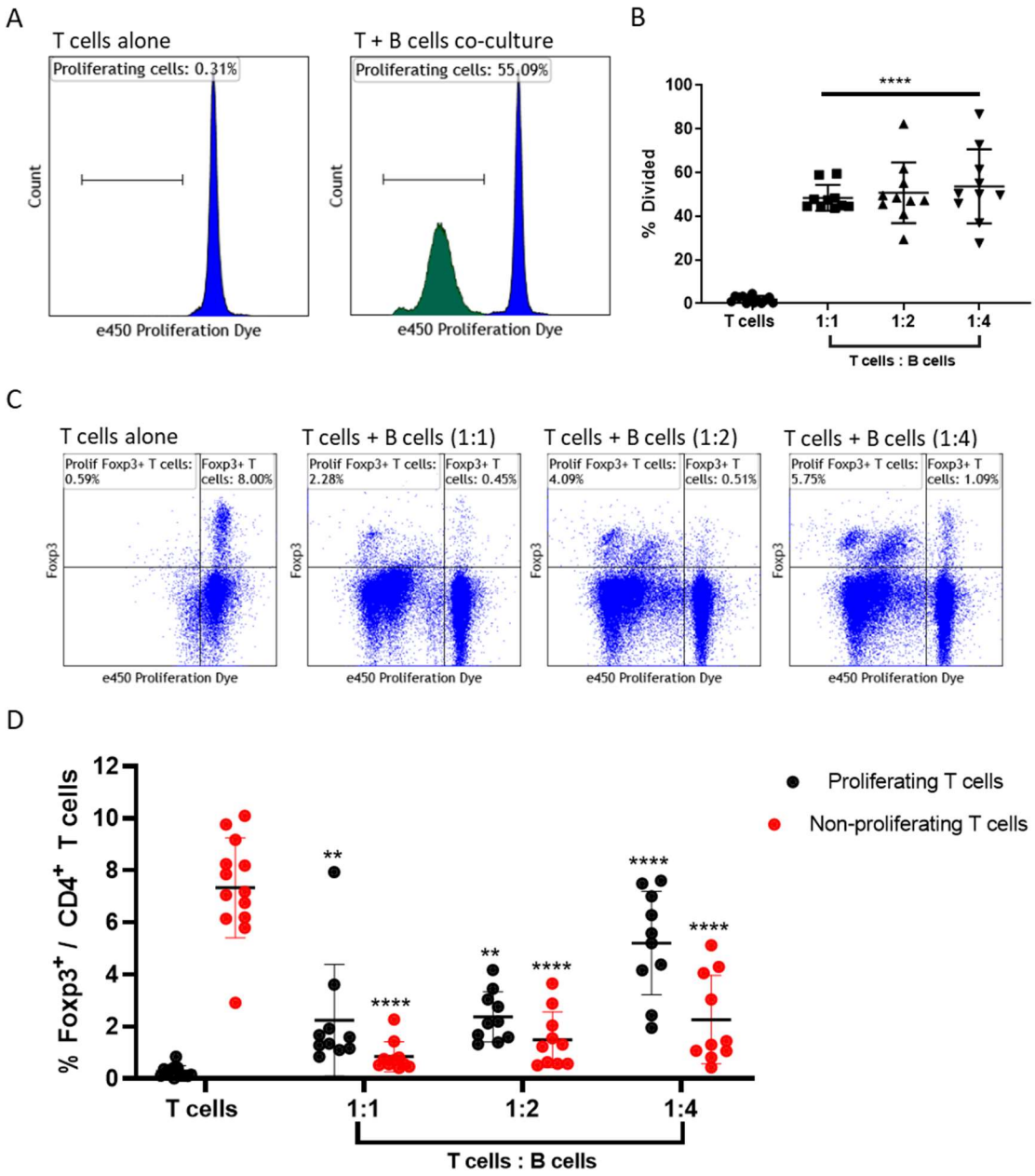


Figure 5.4: MLR co-culture with splenic B cells stimulate proliferation of Foxp3$^+$ responder T cells. Responder CD4$^+$ T cells labelled with e450 Proliferation Dye were assessed after 5 days alone, or in co-culture with splenic B cells. Harvested cells were then fixed and permeabilised for Foxp3$^+$ transcription factor detection. (A) Representative histogram illustrating the proliferation of CD4$^+$ T cells co-cultured with B cells compared to the control with CD4$^+$ T cells alone. (B) Graphical summary of percent divided cells in co-cultures of increasing T:B cell ratios. (C) Representative flow cytometric panels illustrating the increase in Foxp3$^+$ expressing cells within the proliferating population as the ratio of T:B increases. (D) Graphical summary of Foxp3-expressing CD4$^+$ T cells within the proliferating (black) and non-proliferating (red) populations. Data shown is combined from 5 independent

experiments performed in duplicate. Statistical analysis based on one-way ANOVA and Dunnett's multiple comparisons test to T cell control. **p≤0.01, ****p≤0.0001

5.2.3 Induced Bregs promote Foxp3 expression in $CD4^+$ T cells

The previous results highlighted the capacity of naïve B cells for tolerance induction by expanding the $Foxp3^+$ Treg pool *in vitro*. We next sought to investigate if this effect can be augmented by increasing the proportion of IL-10^+ Bregs in the B cell co-culture. Purified splenic B cells were stimulated for 48 hours with agonistic anti-CD40 mAb to induce an increase in IL-10-producing cells (Yanaba et al., 2009). At the end of the 48-hour activation period, the cells were analysed for their capacity to produce IL-10, as determined by intracellular cytokine staining after restimulation; culture supernatant was analysed for production of IL-10. Flow cytometric analysis (Figure 5.5A,B) indicated a significantly higher percentage of anti-CD40-stimulated B cells possessed the capacity to produce IL-10 compared to unstimulated B cells. This was further verified by assessment of IL-10 secreted into the supernatant. A significant increase in production of this cytokine by the anti-CD40 stimulated B cells was apparent (Figure 5.5C). The anti-CD40 stimulated B cells will henceforth be referred to here as CD40-induced Bregs, or simply iBregs.

Using CD40-induced Bregs, we repeated the same MLR experiment as described above to determine whether iBregs promoted $Foxp3^+$ expression in T cells. We found that co-culturing iBregs with responder T cells resulted in a significant increase in $CD25^+$ CD4 T cells compared to unstimulated T cell only controls (Figure 5.6A). Furthermore, iBregs induced a higher percentage of $CD25^+$ activated T cells (50-80%) compared to that of naïve B cells (15-60%; Figure 5.3B). Co-culturing with iBregs also led to higher survival and proliferation in the responder T cells with dividing cells making up 50 to 90% of the collected cells (Figure 5.6B). In both parameters, the number of iBregs added to the co-culture had an additive effect; the higher the ratio of iBregs to T cells, the more activated and proliferative the responder T cells become. T cells alone in culture did not upregulate the CD25 activation marker nor underwent cell division, as opposed to responder T cells co-cultured with iBregs. Moreover, there was a profound increase in the percentage of proliferating $Foxp3^+$ T cells induced by iBregs. 10 to 30% of the total responder T cells (Figure 5.6C,D), compared to less than 10% induced by B cells alone (Figure 5.4C,D).

Our results herein provide evidence that B cells stimulated with agonistic anti-CD40 mAb contain a larger proportion of IL-10-producing Bregs and that co-culturing responder T cells with these cells significantly boosts T cell activation, proliferation, and Foxp3$^+$ expression.

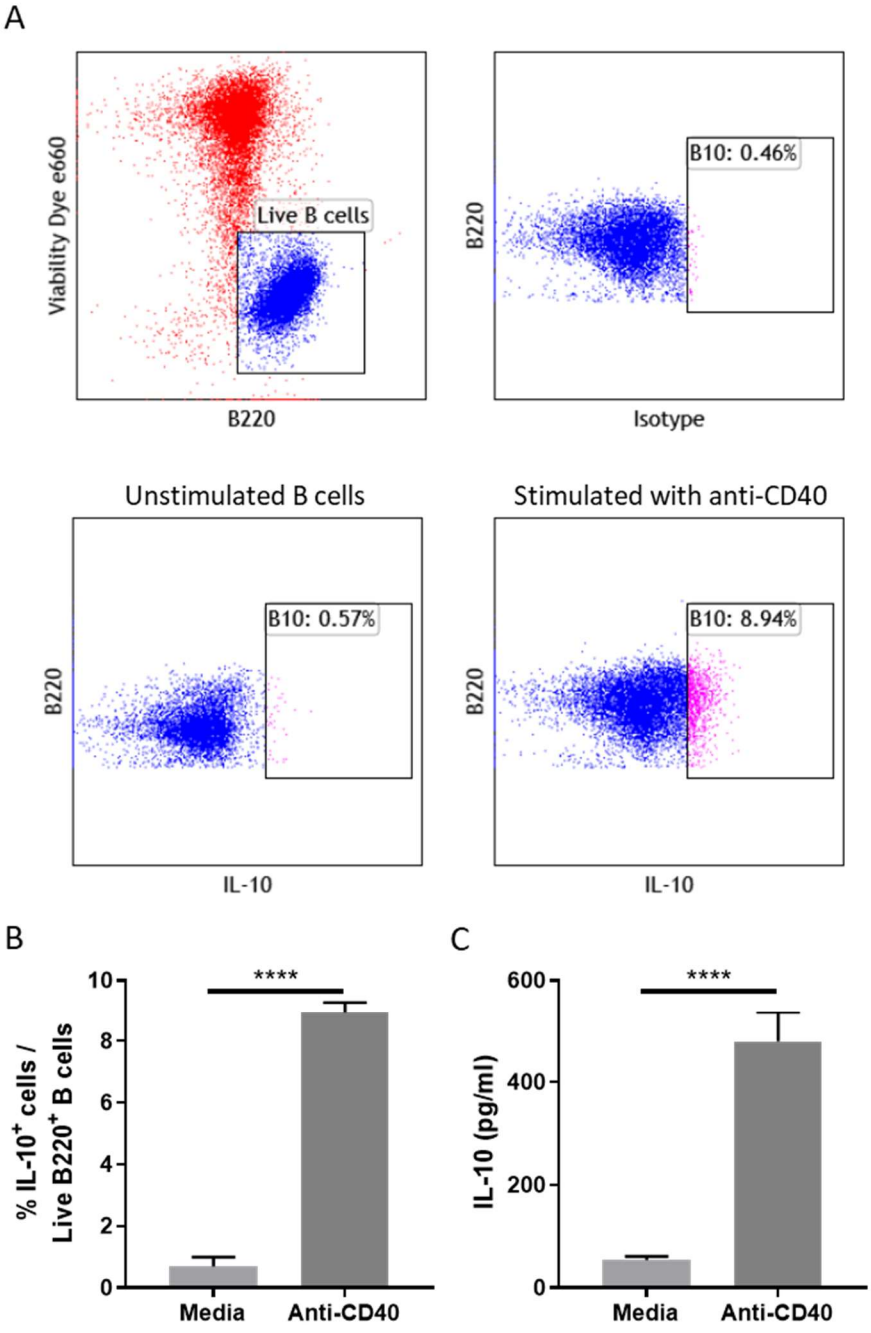


Figure 5.5: Stimulation of B cells with anti-CD40 increases the proportion of IL-10$^+$ Bregs. Splenic B cells were isolated and stimulated with agonistic anti-CD40 mAb (1 ug/mL) for 48 hours. 5 hours prior to end of stimulation period, the cultures were restimulated with a cocktail of PMA, ionomycin, LPS, and Brefeldin A for IL-10 intracellular staining, or PMA, ionomycin, and LPS for measuring IL-10 secreted into the supernatant. (A) Representative flow cytometric panels of live B cells gated and analysed for expression of intracellular IL-10. (B) Comparison of percentage IL-10$^+$ B cells under

unstimulated and anti-CD40 mAb-stimulated conditions. (C) IL-10 production by unstimulated and stimulated B cells assessed by ELISA. Data is represented as mean ± SEM and compared by unpaired *t*-test. Data shown are from three independent experiments. ****p≤0.0001

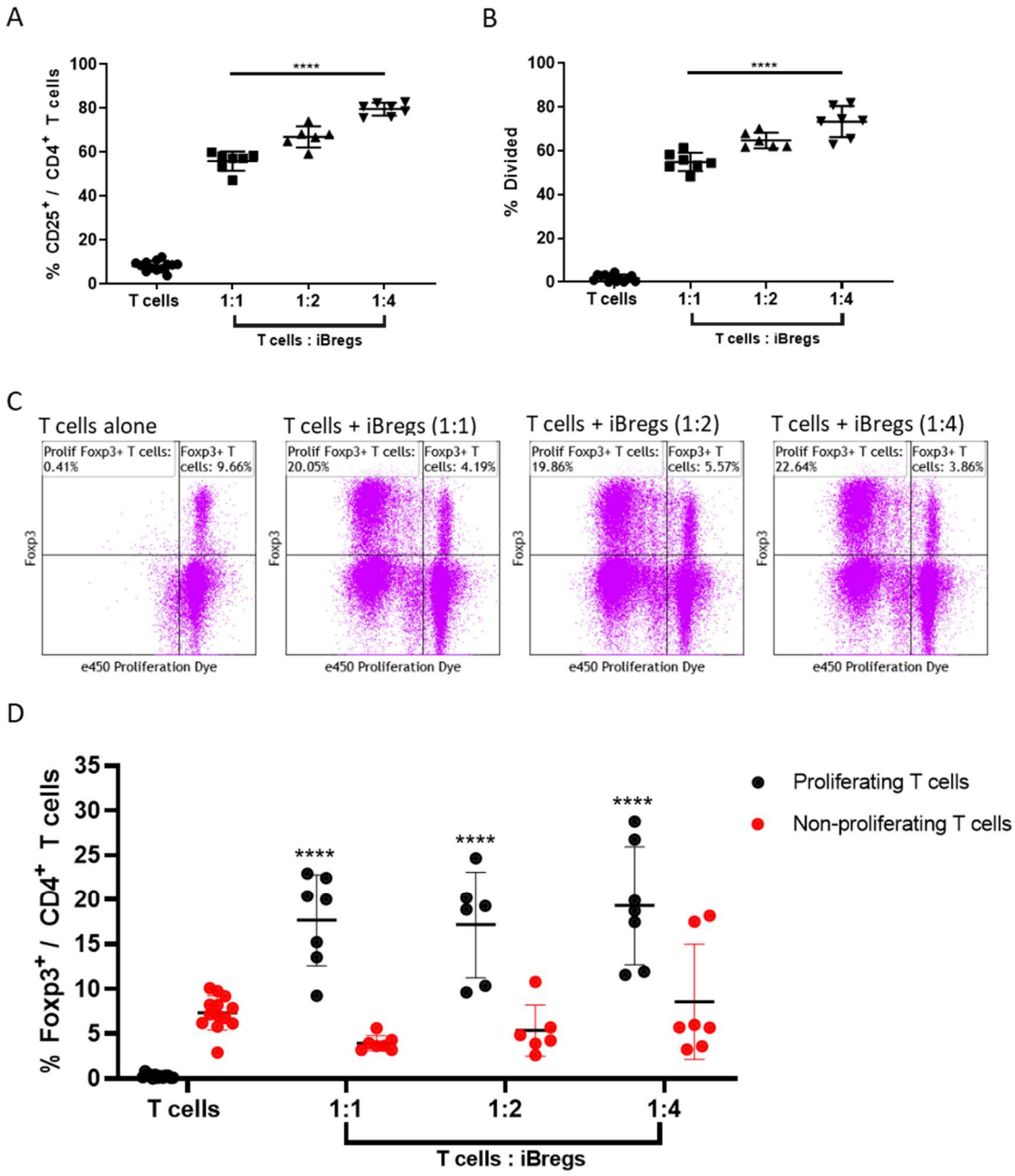


Figure 5.6: CD40-induced iBregs significantly increase the proliferation of Foxp3$^+$ responder T cells in MLR co-culture. Responder CD4+ T cells labelled with e450 Proliferation Dye were assessed after 5 days alone, or in co-culture with CD40-induced Bregs. Harvested cells were then fixed and permeabilized for Foxp3$^+$ transcription factor detection. (A) Graphical summary of percentage CD25-activated CD4$^+$ T cells after co-

incubation with increasing ratios of iBregs. (B) Graphical summary of percent divided cells in co-cultures of increasing T:iBreg cell ratios. (C) Representative flow cytometric panels illustrating the increase in Foxp3$^+$ expressing cells within the proliferating population as the ratio of T:iBreg increases. (D) Graphical summary of Foxp3-expressing CD4$^+$ T cells within the proliferating (black) and non-proliferating (red) populations. Data shown is collected from 3 independent experiments. Statistical analysis based on one-way ANOVA and Dunnett's multiple comparisons test to T cell control. ****p≤0.0001

5.2.4 Tregs induced by iBreg suppress T cell proliferation and activation

To determine if the iBreg-induced Tregs are functional as regulatory cells, we analysed their ability to suppress lymphocyte proliferation and cytokine production *in vitro* as outlined in Figure 5.7.

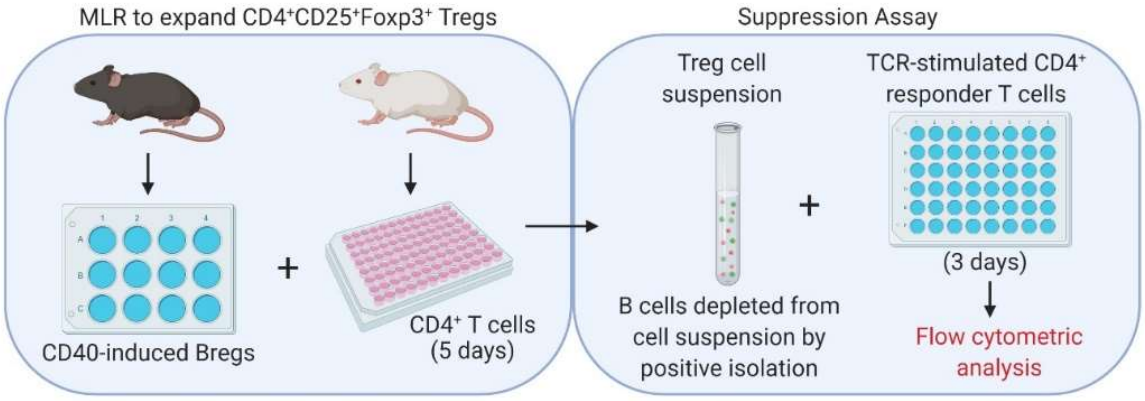


Figure 5.7: Process flow of assessing Treg suppressive capacity on T effector cells. Tregs were generated from an MLR co-culture with CD40-induced Bregs for 5 days. B cells were then depleted by positive selection. The resultant Tregs were transferred to a new plate where they were co-cultured at different ratios with fluorescently-labelled CD3/CD28 -stimulated CD4$^+$ responder T cells for 3 days. Proliferation of T effector cells were then assessed by flow cytometry.

Results shown in Figure 5.8 indicated that Treg cells generated from the MLR co-culture could suppress proliferation of the responder T effector cells as demonstrated by an observed decrease in proliferation indices (PI). Unstimulated responder T cells, with minimal cells undergoing cell division, recorded the lowest PI whilst TCR-stimulated effector T cells exhibited the highest PI (Figure 5.8A,C). Further, as the ratios of Treg to T cells were increased, suppression of proliferation became increasingly evident; i.e. addition of Tregs

into the cell culture subsequently reduced the proliferation profiles of T effector cells in a dose-dependent manner (Figure 5.8B,C). Inhibition was confirmed by assessment of percent divided effector T cells and percentage of $CD25^+$ activated T cells within the fluorescently-labelled responder population (Figure 5.8D,E). Collectively, the data shows that Tregs generated in the presence of CD40-induced Bregs are functionally suppressive.

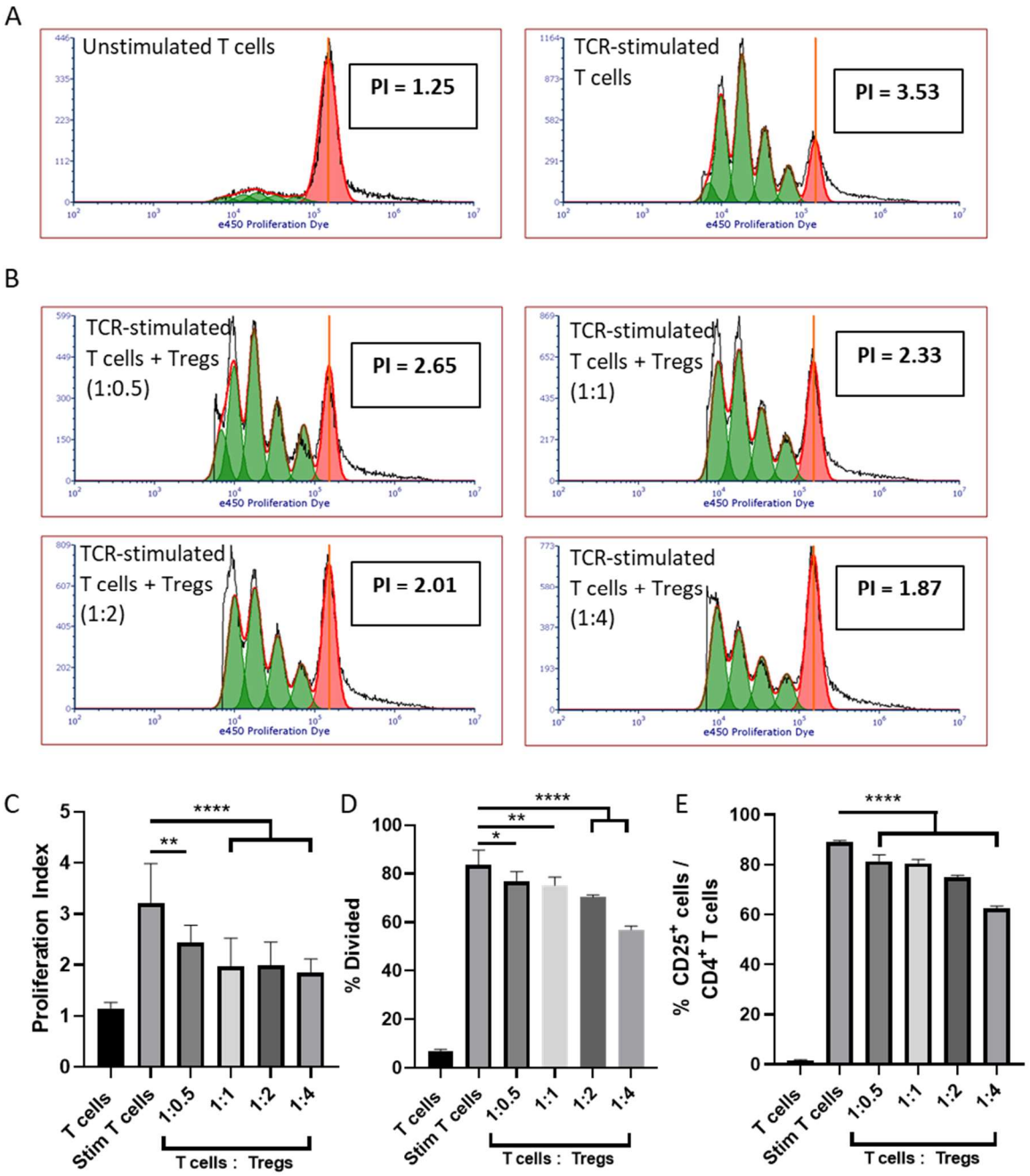


Figure 5.8: Proliferation and activation of T effector cells was inhibited by Tregs generated from MLR co-culture with CD40-induced Bregs. Representative flow cytometric panels illustrate the proliferation of $CD4^+$ responder T cells in (A) experimental controls and (B) treatment groups with increasing ratio of T:Tregs in co-culture. The bottom

panels present the graphical summaries of (C) proliferation indices, (D) percent divided, and (E) percentage of CD25-activated responder T cells of the different treatment groups. Data shown is mean ± SEM collected from three independent experiments. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001

5.2.5 Adoptive transfer of iBregs in pregnant C57Bl/6J mice increased unconventional Treg levels in the uterus

The *in vitro* data collected strongly suggested that iBregs are capable of expanding functional Foxp3^{+} Tregs. We next sought to determine if this expansion was observed *in vivo*. CD40-induced Bregs were adoptively transferred into wild type (WT) female C57Bl/6J mice that were either in the estrus phase of the estrous cycle, or prior mated with a BALB/c stud (day 0.5 post-coitus; Figure 5.9).

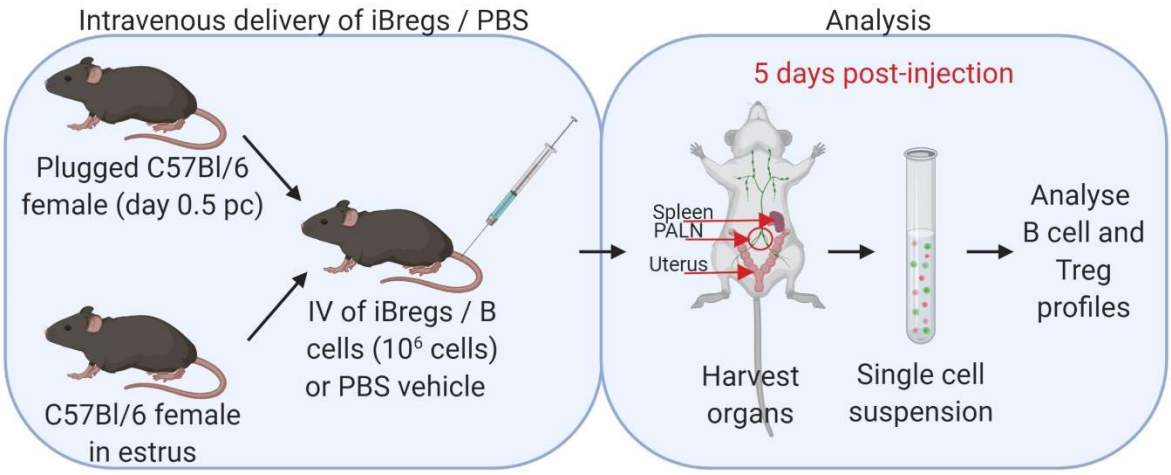


Figure 5.9: Process flow for evaluation of the impact of iBreg adoptive transfer in estrus and pregnant mice *in vivo*. Female C57Bl/6J mice in estrus stage (identified as per microscopic assessment of vaginal flushing) or mated (day 0.5 pc) were intravenously injected with 1x 10^{6} of CD40-induced Bregs or unstimulated B cells. After 5 days, mice were humanely killed and organs harvested for analysis of B cell and Treg profiles by flow cytometric methods.

No noticeable morphological changes were noted at 5 days post-injection. In all pregnant mice (plug to pregnancy rate was 75%), implantations were observed as normal and healthy. The number of implantations did not change between groups, regardless of prior iBreg or B cell transfer (Figure 5.10).

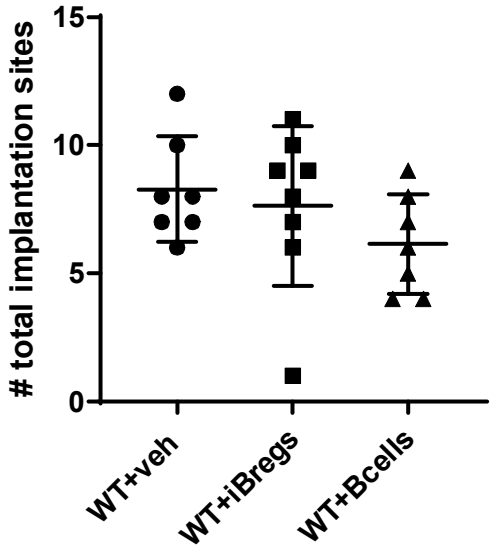


Figure 5.10: Adoptive transfer of iBregs or unstimulated B cells did not affect implantation in pregnant mice. The total number of implantation sites was determined by visual inspection of the uterine horns at dissection on day 5.5pc (n=7-8). Data are presented as the mean ± SEM and analysed using one-way ANOVA (p=0.05). Differences between groups was not significant.

We then investigated whether adoptive transfer of iBregs or B cells altered the proportions of B cells in the spleen, para-aortic lymph nodes, and the uterus. Interestingly, intravenous transfer of B cells into estrus mice led to significantly higher percentages of $CD19^+$ B cells in the spleen and uterus relative to mice treated with iBregs and vehicle control (Figure 5.11A). In contrast, an increased percentage of B cells was detected only in the uterus of the iBreg-treated group compared with those mated mice that received B cells or diluent control (Figure 5.11B).

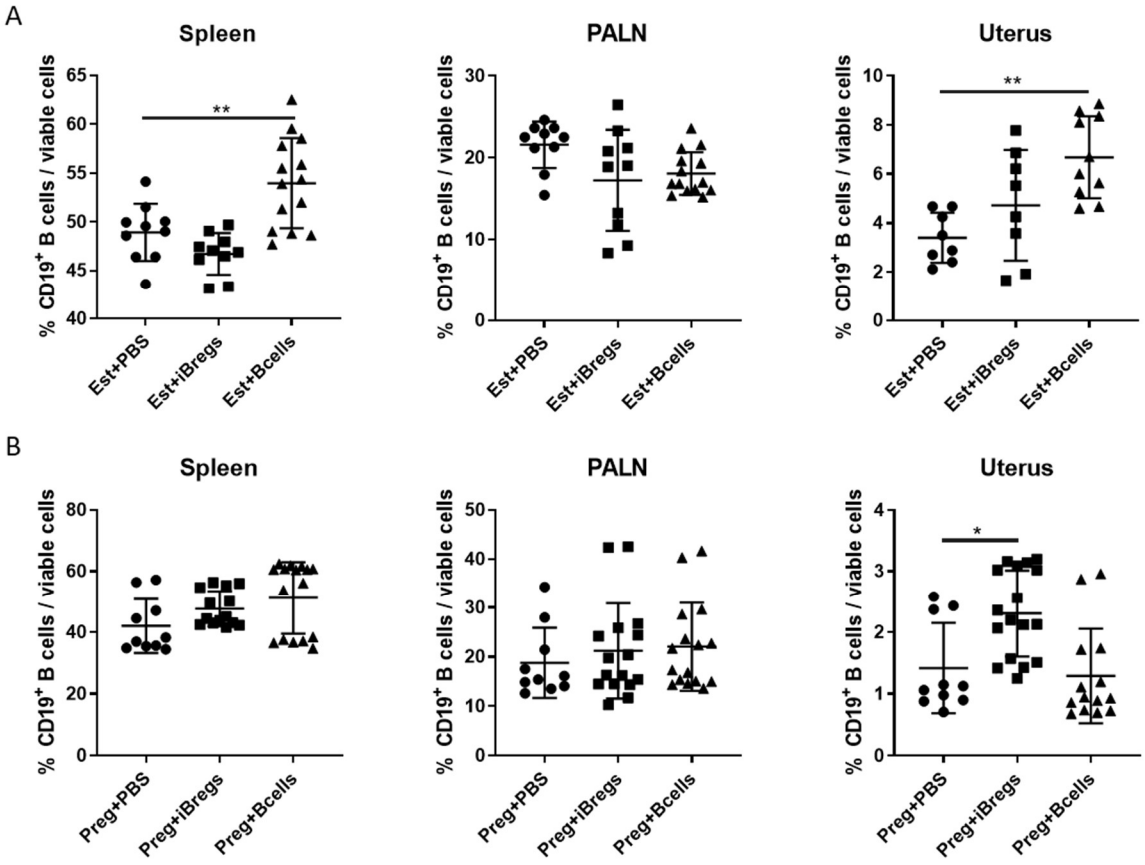


Figure 5.11: CD19$^+$ B cell proportions in the spleen and uterus were altered upon adoptive transfer of B cells/iBregs. Graphical summaries of the percentages of CD19$^+$ B cells among viable cells from spleen, para-aortic (uterine-draining) lymph nodes, and uterine tissue in (A) virgin estrus mice and (B) pregnant mice, that had received PBS (vehicle), iBregs (1 x 10^6 cells), or B cells (1 x 10^6 cells) 5 days earlier. Data are presented as the mean ± SEM and statistically compared using one-way ANOVA, followed by Dunnett's *post-hoc* multiple comparisons test. *p≤0.05, **p≤0.01

Lastly, we evaluated the impact of adoptively transferred B cells or iBregs on the Treg levels in pregnant mice. Addition of iBregs or B cells into the blood circulation of recipient mice expanded the percentages of conventional CD4$^+$CD25$^+$Foxp3$^+$ Tregs in the spleen and para-aortic lymph nodes, but intriguingly, the percentage of these Tregs in the uterus significantly diminished (Figure 5.12A). Instead, iBregs significantly amplified the percentage of unconventional, CD25-negative Tregs (CD4$^+$CD25$^-$Foxp3$^+$) (Figure 5.12B).

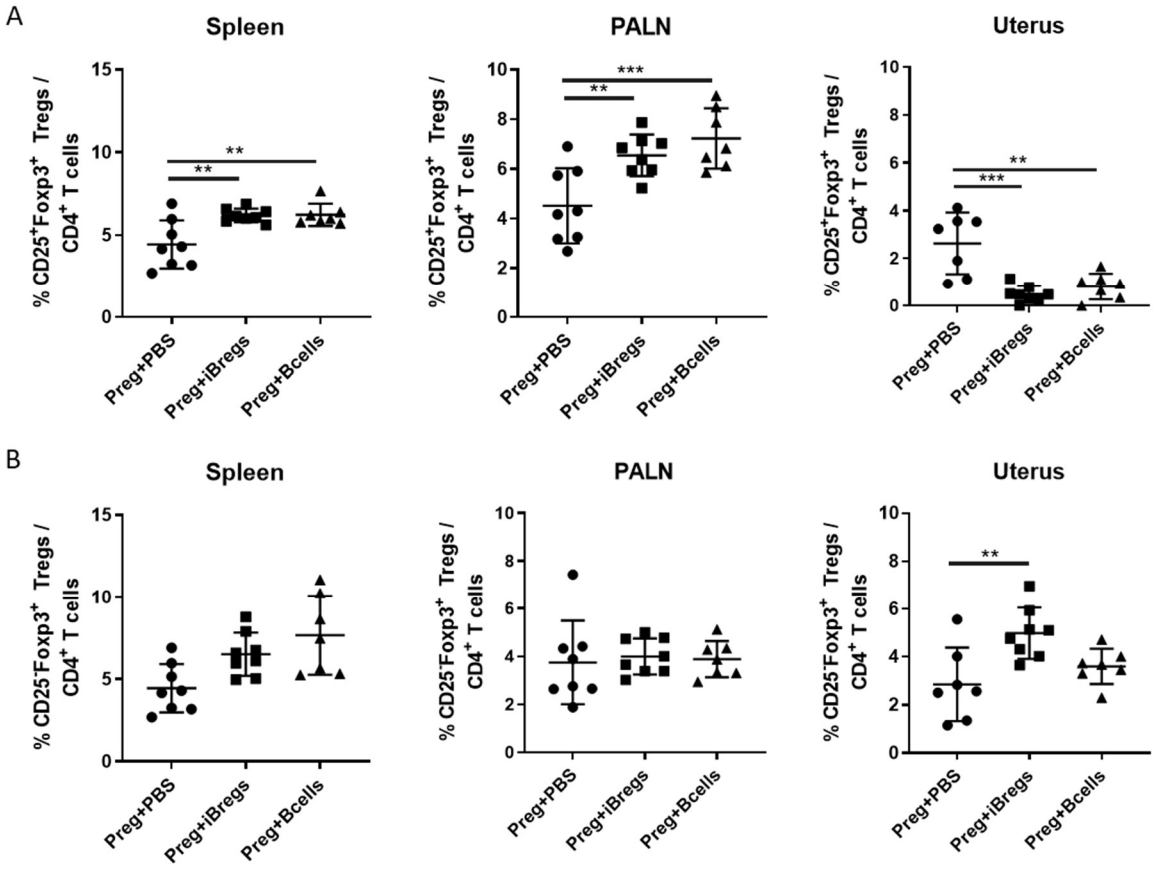


Figure 5.12: Adoptive transfer of iBregs increased the percentage of CD25$^-$Foxp3$^+$ unconventional Tregs. Graphical summaries of the percentages of (A) CD25$^+$Foxp3$^+$ conventional Tregs and (B) CD25$^-$Foxp3$^+$ unconventional Tregs in the spleen, para-aortic lymph nodes, and the uterus. Data are presented as the mean ± SEM and statistically compared using one-way ANOVA, followed by Dunnett's *post-hoc* multiple comparisons test. Each data point represents the average per dam. **p≤0.01, ***p≤0.001

5.2.6 Adoptive transfer of iBregs in Treg cell-depleted Foxp3-DTR mice restored normal embryo distribution in early pregnancy

To examine the effect of iBregs in a pathological condition of pregnancy, we employed a mouse model that allowed for acute Treg cell depletion in early pregnancy (Care et al., 2018). Mated Foxp3-DTR (forkhead box P3-diphtheria toxin receptor) mice received either CD40-induced Bregs or the PBS vehicle control upon detection of a copulation plug (day 0.5 pc), with Treg cells depleted by administration of diphtheria toxin (DT) on days 3.5 and 5.5 pc (Figure 5.13). Confirmation of Treg depletion was assessed by intracellular staining for Foxp3 expression at day 7.5 pc. Viable CD4$^+$ T cells expressed the activation marker CD25

but severely lacked Foxp3 expression (less than 0.5%) (Figure 5.14). Wild-type DT-treated pregnant C57Bl/6J female mice were examined as a control for normal Treg expression at day 7.5 pc.

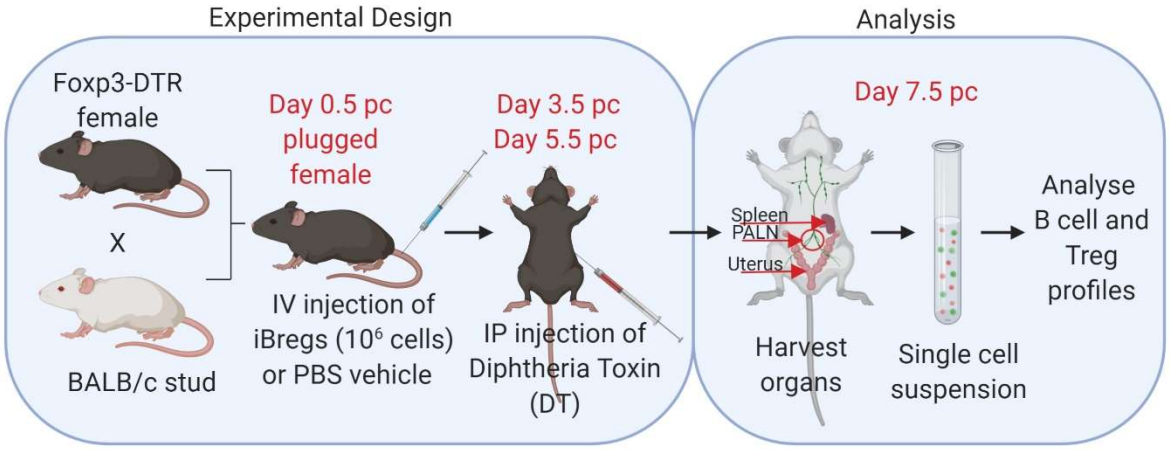


Figure 5.13: Flowchart of conditions for the *in vivo* evaluation of iBreg adoptive transfer in Treg-depleted Foxp3-DTR female mice. Mated and plugged female Foxp3-DTR mice were intravenously injected with 10^6 cells of CD40-induced Bregs or Dulbecco's PBS as the vehicle control. At days 3.5 and 5.5 pc, the mice were injected intraperitoneally with diphtheria toxin (DT) for conditional depletion of $Foxp3^+$ Tregs. At day 7.5 pc, mice were humanely killed and organs harvested for analysis of B cell and Treg profiles.

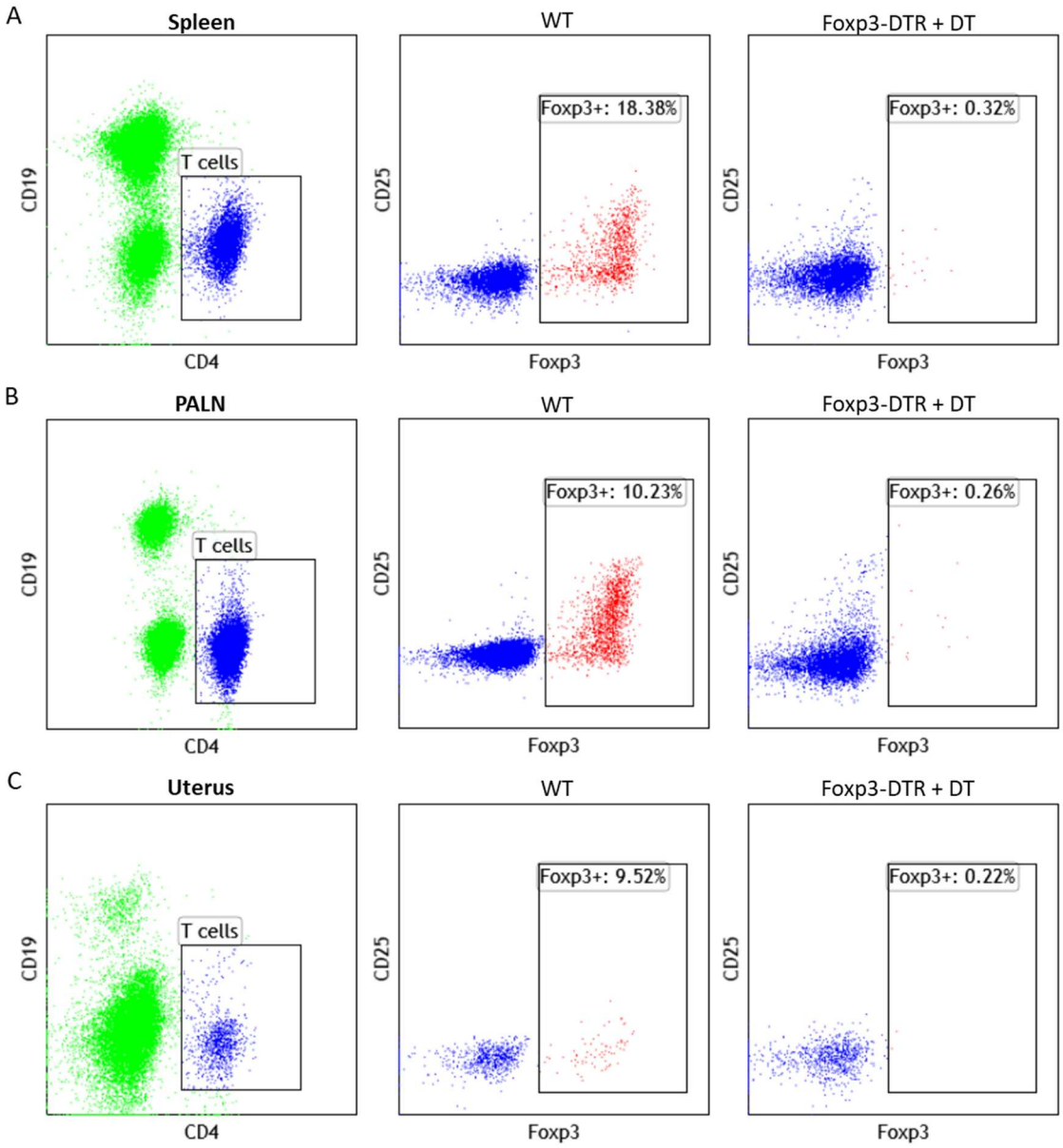


Figure 5.14: Administration of diphtheria toxin (DT) to Foxp3-DTR female mice depleted Foxp3$^+$ expressing CD4$^+$ T cells in the spleen, PALN and uterus. Representative dot plots showing CD4$^+$ T cells gated (left panel) and analysed for CD25 and Foxp3 expression in DT-treated wild-type control mice (middle panel) and Foxp3-DTR mice (right panel). Wild-type C57Bl/6J female mice typically possessed 10 to 20% of CD4$^+$Foxp3$^+$ T cells at day 7.5 pc, whilst depleted Foxp3-DTR female mice routinely possessed less than 0.5% Foxp3$^+$ cells of all T cells from the spleen, PALN, and uterus in DTR mice.

In preliminary experiments, mated female mice that received iBregs or diluent control at d0.5pc were humanely killed at day 7.5pc and dissected for the collection of the organs of interest: the spleen, para-aortic lymph nodes, and the uterus. The uterus was inspected for pregnancy parameters, specifically the number of implantation sites, occurrence of

resorption, and the distance between implantation sites. The number of implantation sites between the PBS-treated group and the iBregs-treated group were statistically similar and no resorptions were observed (Figure 5.15B). Morphological evaluation of the impregnated uterus with respect to distance between implantation sites showed an interesting comparison of the two treatments. Pregnant mice that received diluent control displayed unusual distribution of implantation sites along the horns of the uterus (Figure 5.15A,C). The implantation sites were positioned in close proximity, with a median distance of 0.25 mm. In contrast, the implantation sites of Treg-depleted pregnant mice that received iBregs exhibited a normal punctuated distribution along the uterus with a median distance of 1.75 mm between sites (Figure 5.15C). These preliminary results suggested that lack of Treg cells during early pregnancy may affect normal implantation, manifested in this mouse model as abnormal spacing of the implantation sites.

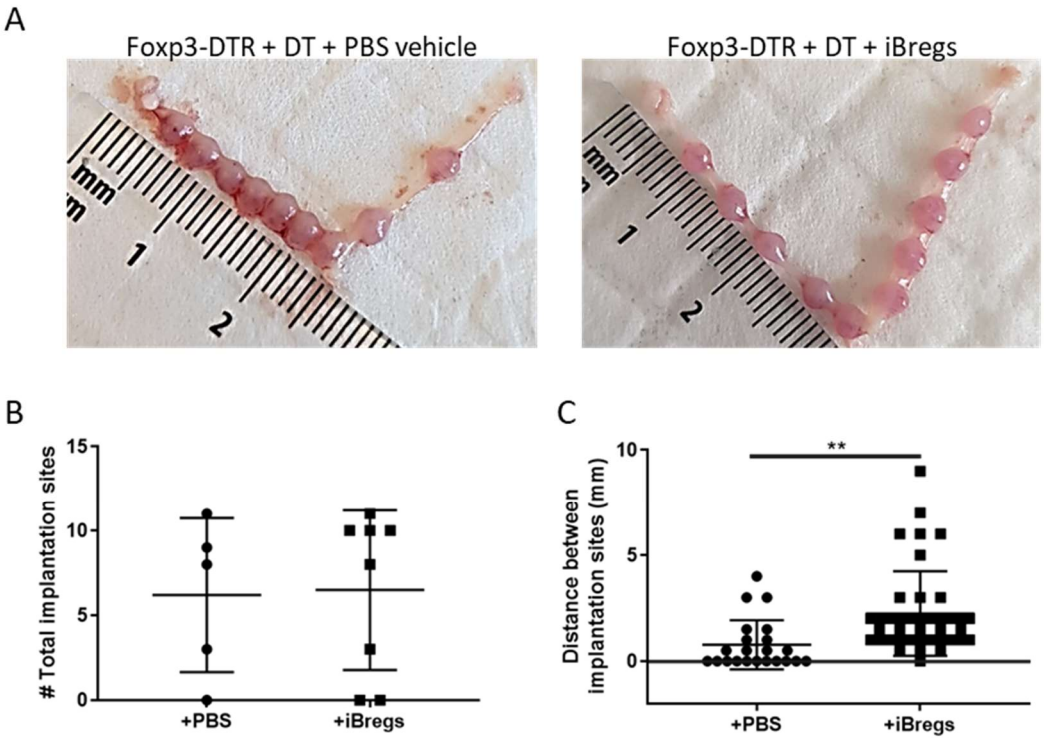


Figure 5.15: Morphological examination of uterine implantations in Treg-depleted pregnant Foxp3-DTR female mice at day 7.5 pc. DT-injected pregnant Foxp3-DTR mice were examined at day 7.5 pc for clinical signs of pathology. (A) Representative images of uterine implantation sites on day 7.5 pc from Foxp3-DTR+DT (left) and Foxp3-DTR+DT+iBregs (right) mice. (B) Total number of implantation sites combined from n=4-6 individual pregnant mice administered PBS or iBregs. (C) Graphical summary of the distance between implantation sites as measured in the distribution of embryos along the

uterine horns of pregnant mice. Data shown is mean ± SEM and statistically analysed using unpaired *t*-test. **p≤0.01

5.2.7 Adoptive transfer of iBregs in Treg-depleted Foxp3-DTR mice alters cytokine production and $CD25^+$ activation in T cells

Finally, we examined the impact of adoptively transferred iBregs to Treg-depleted mice with respect to the serum cytokine profile as well as the lymphocyte profile in the spleen, lymph nodes, and uterus. Blood samples were obtained at termination of the experiment, and subsequently analysed for cytokine/chemokine content using MAGPIX®. The following cytokines/chemokines were examined: IFNγ; IL-12p70; IL-13; IL-1β; IL-2; IL-4; IL-5; IL-6; TNFα; GM-CSF; IL-18; IL-10; IL-17A; IL-22; IL-23; IL-27; and IL-9.

Out of the 17 cytokines/chemokines analysed, only the anti-inflammatory cytokine IL-10 was significantly different between the two cohorts indicating that adoptive transfer of iBregs led to an increased level of circulating IL-10 in the blood serum (Figure 5.16). All other cytokines and chemokines tested showed no significant differences or were below the limit of detection. Although among the cytokines with values beneath detection limits, extrapolated data from the standard curve of pro-inflammatory cytokine IL-1β showed a notable significant increase in the PBS vehicle control cohort, which suggested increased inflammatory activity (data not shown).

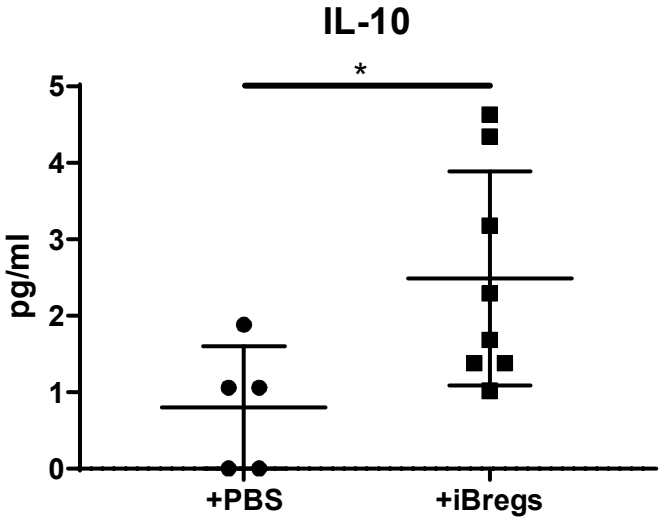


Figure 5.16: Cytokine analysis show increased levels of IL-10 in serum of the iBreg-treated Treg-depleted cohort. Serum was collected from each individual mouse at d7.5 pc

and analysed for cytokine/chemokine content. Data of IL-10 concentrations are shown as pg/ml ± SEM and statistically analysed using an unpaired *t*-test. *p≤0.05

Lastly, the lymphocyte profile of the harvested organs was examined, specifically the proportions of $CD19^+$ B cells, $CD4^+$ T cells, and percentage CD25 activation in $CD4^+$ T cells. As shown in Figure 5.17A and B, the percentages of $CD19^+$ B cells and $CD4^+$ T cells found within the spleen, lymph nodes, and the uterus were not different between the two cohorts. However, expression of the activation marker CD25 on $CD4^+$ T cells was significantly increased in the spleen and para-aortic lymph nodes of mice that received iBregs (Figure 5.17C). No differences in uterine tissue were evident. This suggested increased priming of the $CD4^+$ T cell population in sites where B and T cells regularly interface, possibly as a mechanism for controlling potential DT-induced inflammation.

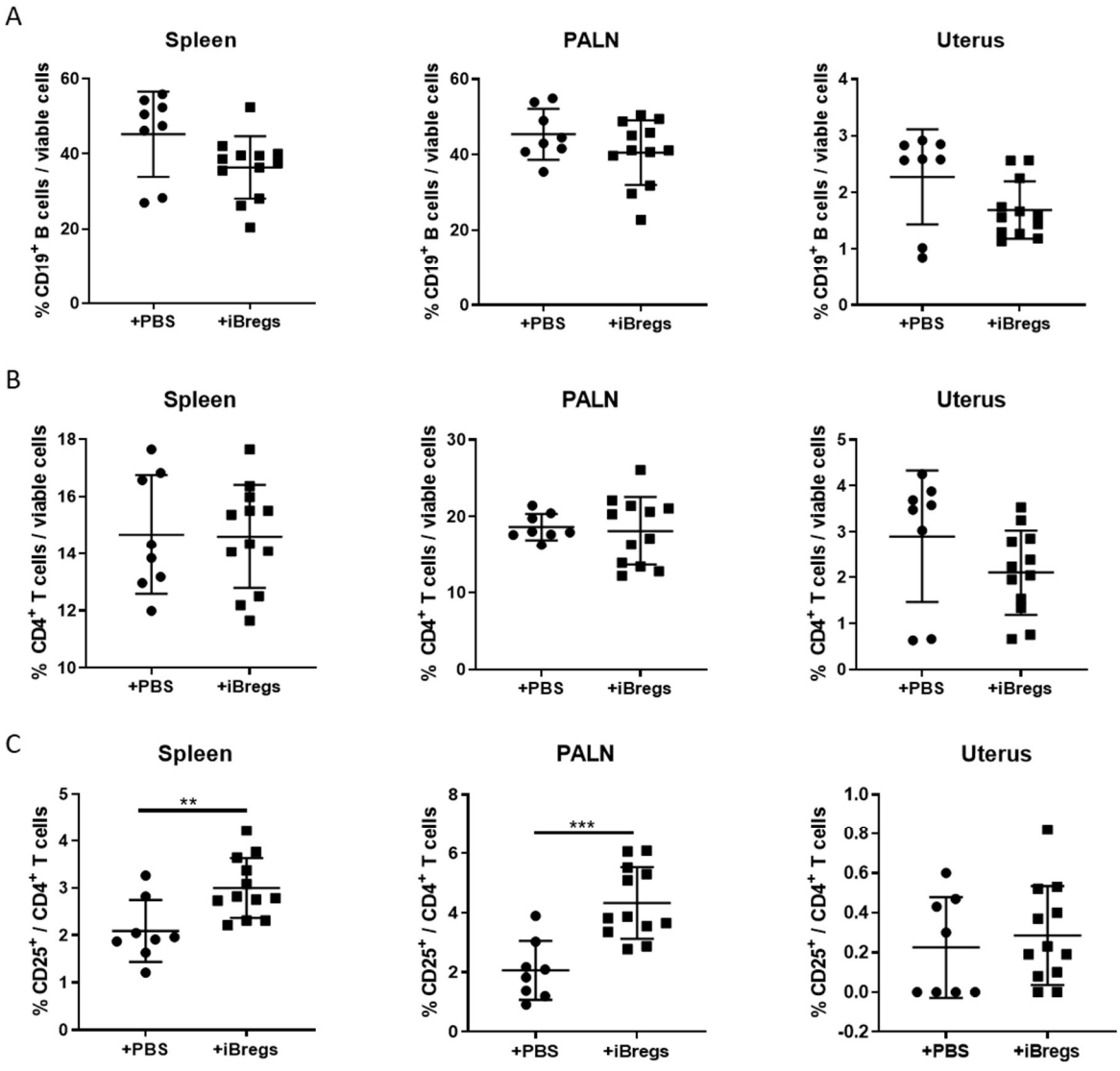


Figure 5.17: Lymphocyte profile in the spleen, para-aortic lymph nodes, and uterus of PBS- and iBregs-treated mice. Graphical summaries of percentages of (A) $CD19^+$ B cells among viable cells, (B) $CD4^+$ T cells among viable cells, and (C) CD25 expression in $CD4^+$ T cells in the spleen, para-aortic lymph nodes, and uterus at day 7.5 pc. Data shown is mean +/- SEM and statistically analysed using unpaired *t*-test. **p≤0.01, ***p≤0.001

5.3 Discussion

It is now widely appreciated that immune cells are key players in uterine development and remodelling during pregnancy. Physiologically, pregnancy brings about many changes in hormone levels as well as cytokine and chemokine signals that consequently facilitate modifications in immune cell phenotype and function. In the previous chapters, we have shown that B cells are subject to these phenotypic and functional alterations during pregnancy. In mouse pregnancy, B cells exhibit population expansion and acquire properties that regulate T cell proliferation while *in vitro* human implantation models suggested a possible mechanism for the acquisition of these regulatory properties. Having established that B cells are part of the local immune network facilitating pregnancy well-being, we proceeded to evaluate whether they can be exploited to manipulate pregnancy outcome. This chapter delved deeper into the functional interplay of Bregs with T cells, specifically how their interaction may pave a way for potential therapeutic strategies tailored for pregnancy pathologies.

The majority of studies on Bregs present their percentages and function *vis a vis* the more studied subset of regulatory cells – the Tregs. A plethora of studies especially in the fields of autoimmunity and transplantation provide evidence of functional interplay between the two regulatory subsets, with an emphasis on Bregs facilitating primary induction of Tregs. *In vitro* studies show that co-culturing $CD4^+CD25^-$ effector T cells with IL-10^+ Breg cells lead to increased contact time and result in suppression of pro-inflammatory cytokine production, enhancement of Treg activity, and conversion of effector T cells into Treg by upregulation of Foxp3 expression (Carter et al., 2011; Lu et al., 2017). *In vivo*, adoptive transfer of different subtypes of Bregs such as transitional-2 marginal zone precursor Bregs and IL-10-competent $CD19^+Foxp3^+$ Bregs likewise increased the number of $Foxp3^+$ Tregs (Carter et al., 2011; Park et al., 2016). Clinically, IL-10 secretion and upregulated expression of co-stimulatory molecules CD80 and CD86 by B cells have been shown to drive naïve T cell differentiation toward $CD4^+Foxp3^+$ Tregs and $CD4^+$IL-10^+ Tr1 cells (Mielle et al., 2018). In the context of pregnancy, Treg functions have been well documented as essential immune players and master regulators in facilitating uterine receptivity and embryo implantation, with a significant expansion in the pregnancy microenvironment commencing during estrus and early pregnancy (Robertson et al., 2018). With Bregs intrinsically linked with expansion in autoimmunity and transplantation, we investigated whether the percentages of $CD4^+CD25^+Foxp3^+$ Tregs and $CD19^+$IL-10^+ Bregs were correlated

throughout gestation (Figure 5.2). Although no significant differences were detected between timepoints for both Tregs and Bregs in the spleen, para-aortic lymph nodes, and the uterus, the higher percentages of both types of regulatory cells in the uterus in early pregnancy suggested their joint involvement in the processes pertinent to embryo implantation.

The pathophysiology of certain unexplained pregnancy disorders such as primary infertility, idiopathic recurrent spontaneous abortion, and pre-eclampsia have been linked to inadequate numbers of Tregs, imbalance of Tregs and effector T cells, or an impairment of Treg function (Jasper et al., 2006; Sasaki et al., 2007; Yang et al., 2008; Lee et al., 2011). Therefore, targeting Tregs to address these obstetric conditions is an appealing prospect. The increase in Breg and Treg numbers in the early pregnancy timeframe provides an imperative to study whether Bregs plays a role in the expansion of Tregs. Thus, we investigated the capacity of Bregs to manipulate Treg number and function in a multi-step proof of concept.

The role of B cells, whether they are tolerogenic or immunogenic, is still contentious although there is increasing number of studies supporting a tolerogenic function by way of Treg expansion. The first direct evidence demonstrated that murine splenic naïve B cells preferentially expanded allogeneic $CD4^+Foxp3^+$ T cells than $CD4^+Foxp3^-$ T cells (Chen and Jensen, 2007). Splenic B cells were also shown to efficiently convert allogeneic naïve T cells into $Foxp3^+$ Tregs when co-cultured in the presence of TGFβ and IL-2 (Zhong et al., 2007). It has also been demonstrated that human Treg cells can be directly expanded using allogeneic B cells and that these Tregs express high levels of Foxp3, maintain an anergic phenotype, and can suppress alloproliferation of $CD4^+CD25^-$ responder T cells in an alloantigen-specific manner (Chen et al., 2009). Interestingly, there was a tissue-resident specific effect as Zhong et al. showed a preferential induction of induced Treg cells with suppressive function upon incubation with follicular B cells or B2 cells, but not B1 cells as the B1 cells strongly activated T cells and induced Th1 cell differentiation. In our own MLR experiment, we demonstrated that co-culturing splenic B cells with $CD4^+$ T cells significantly increased the percentage of T cells expressing the activation marker CD25 compared to a monoculture of $CD4^+$ T cells (Figure 5.3). Moreover, the presence of the B cells stimulated alloproliferation in the responder T cells while responder T cells in monoculture remained in a non-proliferative state (Figure 5.4). Finally, among the proliferating responder T cells, there was an increasing trend of Foxp3 expression as the number of co-cultured splenic B cells increased (Figure 5.4). Our results herein recapitulate

the existing findings by Chen et al (2007) supporting the notion of B cells as tolerogenic immune cells. The MLR experiments successfully demonstrated that exposure of naïve CD4 T cells to allogeneic splenic B cells potentiates Foxp3 expression in thriving and proliferating activated T cells.

After verifying that B cells can indeed augment Treg numbers *in vitro*, it remained that splenic B cells yielded a low percentage of Foxp3-expressing T cells in the MLR assays, ranging from 2% to 8% of the total CD4+ T cell population. In a therapeutic context, a significant expansion of Tregs is needed to acquire the necessary number needed for application. Further, an additional benefit of using *in vitro* expanded Tregs is the superior capability of *in vitro* Tregs in suppressing T cell responses against alloantigens, compared to primary Treg cells (Chai et al., 2008). Previously, Treg expansion can be facilitated via retroviral transduction of $CD4^+$ T cells, polyclonal expansion of naturally occurring Tregs, and induction of Tregs using dendritic cells (Jaeckel et al., 2005; Trenado et al., 2006; Yamazaki et al., 2006). After successful demonstration that B cells can stimulate allogeneic Treg expansion, we then endeavoured to raise the magnitude of expansion by using agonistic anti-CD40 to expand the population of IL-10-producing Bregs within the stimulated B cells.

There are two potent methods of promoting IL-10 production competence in B cells by polyclonal stimulation *in vitro*: incubating with toll-like receptor (TLR)-4 agonist lipopolysaccharide (LPS) or incubating with CD40 monoclonal antibodies (Yanaba et al., 2009). There is prior evidence that stimulating naïve spleen B cells *in vitro* with LPS or agonistic CD40 mAb gives rise to Bregs that inhibit or prevent autoimmunity and reduce inflammation (Tian et al., 2001; Mauri et al., 2003; Matsushita et al., 2010). We compared the efficacy of induction between LPS and CD40 mAbs and our results showed that CD40 ligation is the more potent strategy, with the B cell yield possessing higher percentage viability and higher percentage of IL-10-producing B cells. Further characterization verified that stimulation of B cells with anti-CD40 increased the proportion of $IL\text{-}10^+$ Bregs (Figure 5.5) which was consistent with the findings of multiple studies that demonstrated CD40 engagement increased the regulatory activity of B cells. Cellular profiling revealed that anti-CD40 engagement on B cells led to increased expression of Major Histocompatibility Complex (MHC) Classes I and II and co-stimulatory molecules CD80 (Boldison et al., 2019). The therapeutic potential of using B cells stimulated with anti-CD40 was further expanded in the landmark study of Yanaba et al. 2009 which showed that stimulation with agonistic CD40 monoclonal antibodies for 48 hrs significantly expanded the percentage of

regulatory B cells. Subsequent to CD40 ligation, the maturation and regulatory functions of B cells are altered, with a significant percentage of B cells upregulating CD5 expression and IL-10 competence (Yanaba et al., 2009; Tedder, 2015).

In seminal autoimmunity and transplantation studies, CD40 engagement on B cells likewise leads to enhancement of Treg activity. Anti-CD40-stimulated B cells have been shown to be more potent in inducing and expanding alloantigen-specific $CD4^{hi}CD25^{+}$ Tregs, as well as possessing greater suppressive capacity as compared to immature dendritic cells (Zheng et al., 2010). We therefore sought to provide parallel experimental evidence to demonstrate the increased Treg activity in response to co-culturing with CD40-induced Bregs. Similar to the previous MLR setup, we determined the degree to which Bregs converted T cells to Treg cells by co-culturing titrated doses of CD40-induced Bregs with $CD4^{+}$ responder T cells for 5 days (Figure 5.6). Our results demonstrated that that allostimulation of responder T cells with Bregs resulted in the following significant changes: (1) upregulation of activation molecule CD25, (2) increased expression of transcription factor Foxp3 in proliferating and non-proliferating T cells, and (3) efficient expansion of $Foxp3^{+}$ T cells. Thus, allostimulation with Bregs generated CD25 and Foxp3 expressing T cells – cells possessing high suppressive potential. The subsequent suppression assay conducted showed that these generated T cells possess robust regulatory activity against TCR-stimulated T cells (Figure 5.7, 5.8).

The previous findings support the development of an *ex vivo* culture system as a means for preventing disease initiation and progression, and even reversal of established disease as seen in mice with EAE (Yoshizaki et al., 2012). The ability to generate increased numbers of Bregs from one donor mouse paves the way to develop a therapeutic approach that can significantly alter a physiological or disease state. Various groups have previously shown that the adoptive transfer of Bregs into autoimmune mouse models can lead to durable remission of symptoms and that Breg-T cell cognate interactions are central underlying mechanisms (Matsushita et al., 2008; Rafei et al., 2009; Ray and Basu, 2014; Pennati et al., 2016). These studies put forward the notion that expansion and adoptive transfer of autologous Breg cells may be a plausible therapeutic option. It is thus necessary to fully investigate the impact of adoptively transferred Bregs in our preferred field of application – pregnancy.

In a normal, semi-allogeneic pregnancy, we demonstrated herein that adoptive transfer of B cells or Bregs did not elicit any appreciable impact to pregnancy well-being (Figure 5.10).

A closer look at the percentage of $CD19^+$ B cells in the organs of interest (spleen, para-aortic lymph nodes, and the uterus) provided further insights as per the immunological effects of adoptively transferred autologous B cells or Bregs. The results were interesting in that there appeared to be a marked difference in $CD19^+$ B cell distribution between the non-pregnant and the pregnant cohort. Female mice in estrus had increased percentages of $CD19^+$ B cells in the spleen and the uterus only when injected with non-stimulated B cells whereas no such effect was observed when injected with CD40-induced Bregs (Figure 5.11). The former observation is consistent with a pioneer study showing that intravenous transfer of B cells leads to an accumulation in both the spleen and the lymph nodes (Roth and Mamula, 1997). The lack of accumulation in these regions in the CD40-induced Breg cohort suggested that the homing capacities of these cells vary. Remarkably, in the pregnant cohort, the addition of CD40-induced Bregs significantly raised the percentage of $CD19^+$ B cells exclusively within the uterus. We speculate this is due to the perceived ability of CD40-induced Bregs to localize in regions rich in chemokines, cytokines, and cognate ligands, a phenomenon that has been observed in cancer studies where *ex vivo*-generated CD40-B cells act as cellular adjuvants for chemotherapy (von Bergwelt-Baildon et al., 2006; Kondo et al., 2009). The uterus during pregnancy undergoes numerous changes that require significant cytokine and chemokine changes as well as an influx of fetal antigens and immunological molecules that potentially serves as the main draw for CD40-induced Breg homing.

Next, we assessed whether adoptive transfer of B cells or CD40-induced Bregs had any impact on the percentage of Tregs in the organs of interest. It was intriguing to observe a significant expansion of $CD4^+CD25^+Foxp3^+$ Tregs in the spleen and the para-aortic lymph nodes, yet the proportion of these cells substantially declined within the uterus. Instead of the conventional Treg phenotype, adoptive transfer of CD40-induced Bregs led to an expanded population of $CD4^+CD25^-Foxp3^+$ T cells – a population lacking the activation marker CD25. These are still considered Tregs by virtue of their Foxp3 expression (Figure 5.12). There are currently different schools of thought regarding the functional characteristics of these 'naïve' Tregs, with some authors proposing that these cells are likely immature regulatory cells and some suggesting that they are effector $CD4^+$ T cells with a transient expression of Foxp3 due to T cell activation (Liang et al., 2005; Wang et al., 2007; Angerami et al., 2017). Nevertheless, these studies have consistently demonstrated that these $CD4^+CD25^-Foxp3^+$ T cells have the potential to convert into $CD4^+CD25^+Foxp3^+$ cells and acquire the conventional Treg phenotype and function. Interestingly, $CD4^+CD25^-Foxp3^+$ T cells found in HIV-TB co-infection patients exhibited a more differentiated phenotype with

a higher expression of CD27 and CD45RA which rendered them a higher effector memory phenotype compared to healthy controls. In this study, these naïve or unconventional Tregs were characterized as a Treg population with preserved inhibitory capacity, with a distinct phenotype with higher expression of surface marker CD39 and PD1 and a more differentiated memory or effector profile (Angerami et al., 2017). Moreover, Dimova et al. identified an exclusive accumulation of $CD4^+CD25^-Foxp3^+$ cells in human normal early pregnancy decidua, at about 10 times higher in numbers compared to peripheral blood. The enrichment in the decidua is proposed to be an additional reservoir of $Foxp3^+$ naïve Treg cells that can be converted to conventional Treg cells in the uterus upon activation/homeostatic expansion (Zelenay et al., 2005; Dimova et al., 2011). It is plausible that transferred Bregs that localised in the uterus induced these $CD4^+CD25^-Foxp3^+$ Tregs, in line with their role as regulatory gatekeepers during pregnancy.

Having described the impact of Breg adoptive transfer in a normal pregnancy, we then proceeded to evaluate any effect of injecting these cells in a Foxp3-DTR pregnancy model, where fetal loss is attributed to the DT-mediated depletion of Tregs (Kim et al., 2007; Care et al., 2018). The objective of this experiment was to determine whether Bregs could compensate for the lack of Tregs in the pregnancy microenvironment. We confirmed that DT administration to plugged mice at d 0.5 pc caused Treg cells to be depleted in the spleen, para-aortic lymph nodes, and in the uterus at d 7.5 pc (Figure 5.14). We then assessed the implantation site number, location, and spacing of Foxp3-DTR pregnant mice injected with PBS (control) or CD40-induced Bregs at d 0.5 pc. At d 7.5 pc, the number of implantations per pregnancy and the location of implantations were comparable between the two cohorts. Although fetal loss was not seen in the control Foxp3-DTR pregnant mice as expected (Care et al., 2018), morphological aberrations were observed in the form of clustering implantation sites (Figure 5.15). In a normal and healthy pregnancy, the spacing of embryos along the uterine horn is evenly distributed along the entire horn, thus the clustering of implantation sites indicate abnormal embryo implantation distribution (O'Grady and Heald, 1969; Yoshinaga, 2013). Minimal spacing of implantation sites result in uterine overcrowding which has been shown to negatively affect the survival and development of fetuses due to poor blood supply reaching each implantation site and inadequate surface area for the development of the placenta (Argente et al., 2008). Interestingly, the clustering was not evident in Foxp3-DTR pregnant mice adoptively transferred with CD40-induced Bregs. Currently, the mechanism governing embryo spacing in the uterine horns is unclear, however from our preliminary results it is tempting to speculate that Treg depletion provided the

conditions leading to this occurrence and that the presence of Bregs protected the health of the pregnancy.

With respect to mechanism, we profiled and compared a broad range of circulating pro- and anti-inflammatory cytokines and distribution of B cells and T cells in the organs of interest for both control and treated mice cohorts. Only one out of the 17 cytokines were shown to be significantly different between the two groups, the anti-inflammatory cytokine IL-10 (Figure 5.16). Although limited information can be gleaned from the cytokine profile of peripheral blood, the difference in IL-10 levels suggested that the Foxp3-DTR control mice may have a Th1 bias that could explain the abnormal morphological characteristics of the pregnancy. On the other hand, the cohort that received CD40-induced Bregs and exhibited higher IL-10 serum levels had likely a higher Th2 cytokine secretion characteristic of regulated cytokine control during the peri-implantation phase of pregnancy (Robertson et al., 2011). The percentages of $CD19^+$ B cells and $CD4^+$ B cells were statistically similar for the two cohorts, however the percentage of $CD4^+$ T cells that were positive for the activation marker CD25 were significantly higher in the spleens and lymph nodes of the CD40-induced Breg cohort compared to the control. It is interesting to note that the same trend was not observed for the uterus, which supports our hypothesis that Breg leads to induction of naïve Tregs ($CD4^+CD25^-Foxp3^+$ T cells) rather than the conventional phenotype. These interesting results fit the proposed protective effect of Bregs on Treg-depleted pregnancies; however, further studies will be needed to elucidate the mechanism by which this may occur.

Although more replicates and higher sample number and DT-treated controls are needed to verify and validate the findings herein, these preliminary experiments on adoptive transfer of Bregs into the Treg-depleted Foxp3-DTR mice indicate that Bregs may be capable of compensating for the systemic lack of Tregs during pregnancy. Our data suggests that Treg depletion may lead to an abnormal, possibly pathological, pregnancy phenotype and that this condition may be circumvented by delivering Bregs at the outset of pregnancy. Therefore, these results provide initial evidence for a possible therapeutic application of *ex vivo* expanded CD40-induced Bregs for immune-based pregnancy pathologies.

5.4 Conclusion

In this chapter, we investigated the functional interplay of Bregs and Tregs – a necessary step towards evaluating whether Bregs have a therapeutic potential to mediate Treg-related

immune dysfunction during a pregnancy. Herein we have shown that CD40-induced Bregs, which contain an expanded population of IL-10-producing B cells, can convert T cells into functional Tregs as well as promote T cell activation and proliferation. Furthermore, results of the adoptive transfer studies indicate that Bregs could localise within the uterus and expand the pool of unconventional $CD4^{+}CD25^{-}Foxp3^{+}$ Tregs. Lastly, adoptive transfer of Bregs in the Foxp3-DTR mouse model prevented morphological aberrations that could potentially affect pregnancy well-being, thereby suggesting that Tregs are not actually *sine qua non* in the implantation process and that Bregs may engage in a compensatory role to ensure a healthy pregnancy.

Chapter Six

CONCLUSIONS AND FUTURE PERSPECTIVES

Chapter 6: Concluding remarks and future perspectives

6.1 Conclusions

Pregnancy presents a unique immunological paradox that requires a shift in form and function among immune cells involved in the pregnancy cellular network. Until recently, B cells were deemed irrelevant within the uterine microenvironment during pregnancy due to their scarcity. However, the B cell subset Bregs presented an appealing case. Bregs have been highlighted repeatedly as the 'new kid on the immunosuppressive block', based on seminal studies in autoimmune diseases, transplantation tolerance induction, cancer, infectious diseases, and allergy (Rosser et al., 2015; van de Veen et al., 2016; Dai et al., 2017; Mauri et al., 2017; Sarvaria et al., 2017; Wortel and Heidt 2017; Sokolov et al. 2018; Wasik et al., 2018). Drawing inspiration from the research insights in these fields, we hypothesised that Bregs could contribute in the induction of maternal immunological tolerance that is essential in establishing a healthy pregnancy. The structure of this thesis reflects our logical approach towards elucidating the role of Bregs in early pregnancy. We first endeavoured to gain a deeper understanding about the nature of Bregs found specifically in the uterine microenvironment, then examined the interactions that are crucial to their development, and finally evaluated if these cells can be potentially harnessed as a therapeutic strategy for improved management of immune-mediated pregnancy pathologies.

In this thesis, we achieved the following goals: (1) defined the phenotype and functional characteristics of uterine B cells in early murine allogeneic pregnancy (Chapter 3); (2) showed that trophoblast cells influence the generation of regulatory B cells *in vitro* (Chapter 4); and (3) demonstrated the therapeutic potential of Bregs in addressing Treg-related immune dysfunction in pregnancy (Chapter 5).

The discoveries arising from these studies indicated that tolerance mechanisms involving Bregs are in place during pregnancy. The knowledge gained in Chapter 3 and Chapter 4 were crucial for development of an integrated understanding of Breg function within the context of the pregnancy immune network within the uterine microenvironment. This information is critical in establishing how Bregs work during pregnancy and has direct translational implications to maternal-fetal health. Thereupon, the results in Chapter 5 substantiates the potential use of Bregs as a therapeutic agent to address pregnancy pathology.

The work described in Chapter 3 is notable in that it highlights the plasticity of B cells in response to pregnancy. Previously, B cells in pregnancy were often examined within the limitations of producing 'good' protective antibodies and 'bad' autoantibodies that were correlated with pregnancy complications. The results obtained in this study demonstrated the range of form and function B cells can take on at homeostasis and the corresponding shift to a regulatory role during pregnancy. Interestingly, this regulatory modification only peaks during the peri-implantation period when it is likely needed the most; further proof that functional promiscuity is a salient feature in B cells. It is worth noting that this study is the first to comprehensively describe murine uterine B cells and Bregs during pregnancy, in the process filling a major knowledge gap needed for the basic understanding of the pregnancy immune network within the local microenvironment.

Chapter 4 confirmed that fetal factors, specifically trophoblast cells, can educate B cells to undertake modifications in their phenotype and subsequently, their function. Trophoblast-educated B (TE-B) cells had an expanded proportion of Breg subsets ($CD24^{hi}CD27^{+}$ Bregs and $CD19^{+}IL\text{-}10^{+}$ Bregs) which likely contributed to the capacity of TE-B cells to produce a resultant supernatant that could protect an embryo surrogate (trophoblast spheroid) against deleterious inflammation. In this study, the emphasis is placed on fetal-maternal cross-talk as the prevailing mechanism that facilitates many changes in the uterine microenvironment during early pregnancy – and now the list includes alteration of the B cell immune profile as well.

Lastly, in Chapter 5 we investigated the therapeutic potential of Bregs by exploiting their functional interplay with Tregs. As previously demonstrated, the prevalent Breg phenotype during the peri-implantation period is marked by IL-10 production and/or expression of co-stimulatory molecules. In principle, both conditions predispose Bregs to act as promoters of Foxp3 expression in T cells. We successfully recapitulated Treg induction by B cells *in vitro* and discovered that we could amplify the induction of Tregs by using CD40-induced Bregs. Interestingly, subsequent *in vivo* analysis revealed that CD40-induced Bregs delivered intravenously led to the induction of naïve Tregs instead of the conventional phenotype and that adoptively transferred CD40-induced Bregs into Foxp3-DTR mice restored homeostasis in early pregnancy which resulted in normal implantations. This chapter provides a closer look at the therapeutic potential of Bregs in the context of an immune-based pregnancy pathology; our findings suggest that Bregs can indeed act as a compensatory tool to protect pregnancy well-being when needed.

6.2 Future perspectives

The novel discoveries discussed herein contribute immensely to the fundamental biology of maternal-fetal interactions during pregnancy. The current state of knowledge of regulatory B cells specifically within the context of reproductive immunology is extremely limited, and so these chapters benefit the field by providing a foundational account of the forms and functions of Bregs during pregnancy. The immediate gains of these discoveries will be realised in their use as a springboard for future relevant research, specifically in uncovering how immunity against extended-self and foreign antigen are regulated. However, the emphasis is on long-term gains, as establishing how Bregs work within the pregnancy immune network has direct translational implications in terms of development of therapeutic interventions for the improvement of maternal-fetal health.

Approximately half of early pregnancy pathologies that result in recurrent pregnancy loss are classified as idiopathic – not attributed to fetal chromosome abnormalities, infections, endocrinological causes, uterine abnormalities, antiphospholipid antibody syndrome or other autoimmune diseases. In these cases, immunological dysregulation remains to be the likely etiological candidate as evidenced by accompanying abnormal immune conditions such as elevated NK cell levels, elevated NK activity in peripheral blood or in the intrauterine environment, dysregulated cytokines, imbalanced Th1 and Th2 cell reaction, elevated Th1/Th2 cell ratio, and sharing of human leukocyte antigen alleles between partners (Beaman et al., 2012; Zarnani 2015). Within this context, immunotherapy directed towards improving uterine receptivity and enhancing implantation should be the most effective treatment intervention to improve pregnancy outcome. However, the effectiveness of immunotherapies in pregnancy remains contentious mainly due to the lack of clinical evidence showing a benefit in prevention of early miscarriages. Current immunotherapy methods include the use of intravenous immunoglobulin (IVIG), lymphocyte immunotherapy (LIT), intrauterine infusion of granulocyte colony-stimulating factor (GCSF) and PBMCs, subcutaneous administration of TNF-α inhibitors, leukemia inhibitory factor, and oral administration of glucocorticoids (Achilli et al., 2018). All these methods use a blanket approach to treatment, hence generating a multitude of questions for the underlying mechanisms and their possible side effects such as genetic abnormalities. More targeted approaches like using uterus-like NK cells or Treg cells to modify the local pregnancy microenvironment are gathering momentum in terms of scientific support but are still in the early stages of development (Moffett and Shreeve, 2015; Bulmer and Lash, 2019;

Robertson et al., 2019; Tsuda et al., 2019). Taking together the current knowledge and the findings of this thesis, we propose that Bregs may well be considered also as a potential immunotherapeutic agent for reducing the risk of pregnancy complications. Additionally, the rationale for considering Bregs for immunotherapy is made stronger by the scientific indications here and elsewhere that Bregs have earlier involvement in immune processes compared to Tregs. Thus, the therapeutic effect of Bregs may be broader than that of Tregs.

From a clinical perspective, the role of Bregs as vital contributors to clinical immune homeostasis has been acknowledged and appreciated, most especially in the field of autoimmunity where it was first demonstrated that pan-B cell depletion by Rituximab had limited effectiveness due to the concomitant depletion of beneficial Bregs (Jamin et al., 2008). However, because the Breg field is still relatively new, clinical applications have been few and far between. The clinical trials involving Bregs over the past ten years have focused primarily on determining Bregs' potential as a prognostic or diagnostic marker of disease. Moreover, clinical trials have been limited to autoimmune diseases such as chronic immune thrombocytopenia, inflammatory rheumatisms, and systemic lupus erythematosus (clinicaltrials.gov). Of note, a patent is currently granted to the Tedder group, Duke University (2018), where the invention is described as IL-10-producing B cells to be used as tools for manipulating autoimmune or inflammatory diseases and conditions, as well as for treating infectious disease and/or cancer. There are also patents pending for methods of producing IL-10-producing B cells but other than that, no significant clinical applications for a Breg-based therapy as intervention for any kind of condition has commenced.

In the context of pregnancy, for now we can only speculate on the possible details of a Breg-based therapy. What would be the possible target application for Breg-based therapy? What would be the best clinical implementation approach for Breg-based therapy? What are the possible modalities of treatment – personalized therapy and off-the shelf therapy?

One of the key discoveries in this thesis is the plasticity of the B cell population at the peri-implantation period in murine pregnancy. The upward shift of maternal Bregs during this time and their corresponding functionality establish their underlying importance at this particular pregnancy phase. Therefore, the most logical focus should be on interventions applied early in pregnancy, to have a reasonable chance of addressing disease etiology. Perhaps one of the most studied immune cells that have been investigated for clinical application against gynecologic malignancy are natural killer (NK) cells. A recent study (Fu et al. 2017) demonstrated that adoptive transfer of uterus-like NK cells reversed impaired fetal growth and rebuilt the appropriate local environment to foster a healthy pregnancy.

Similar to Bregs, uterine NK cells peak only during early pregnancy and fetal cells such as trophoblasts orchestrate the NK cells with desirable functional capabilities such as promotion of placental vascular growth, facilitation of decidualization and trophoblast invasion, appropriating immune balance (Colucci and Kieckbusch, 2015; Eberl et al., 2015; Nagashima et al., 2013; Zhang et al., 2011). We have shown in this thesis that Bregs can modify the pregnancy microenvironment conditions to be favorable for healthy implantation of blastocyst, or a model thereof. Thus, the period of application during pregnancy that would be ideal for Breg immunotherapy is during the peri-implantation period, making it a potential intervention strategy for addressing recurrent pregnancy loss due to repeated implantation failure (RIF).

In a pregnancy condition wherein, the immune balance is compromised at the outset, intravenous infusion of Bregs could serve as a possible remedy. An increase in circulating Bregs to maintain or develop the appropriate local pregnancy microenvironment in early pregnancy therefore is the primary goal. In the clinical setting, the strategy by which this can be effectively done has yet to be established. Hypothetically, expanding the Breg population in humans could take one of two therapeutic approaches, each with corresponding merits and drawbacks: (1) *In vivo* targeting of Bregs for therapy wherein Breg cells are generated *in vivo* through the injection of a drug that can induce the activation signals for Breg cell differentiation and expansion, or (2) Adoptive Breg cell therapy wherein Breg cells or Breg progenitors are generated *ex vivo* (isolation) and stimulated *in vitro*, expanding the IL-10^{+} Breg population (expansion) then subsequently injecting these to the receiver to stimulate efficient regulatory function (infusion) (Figure 6.1).

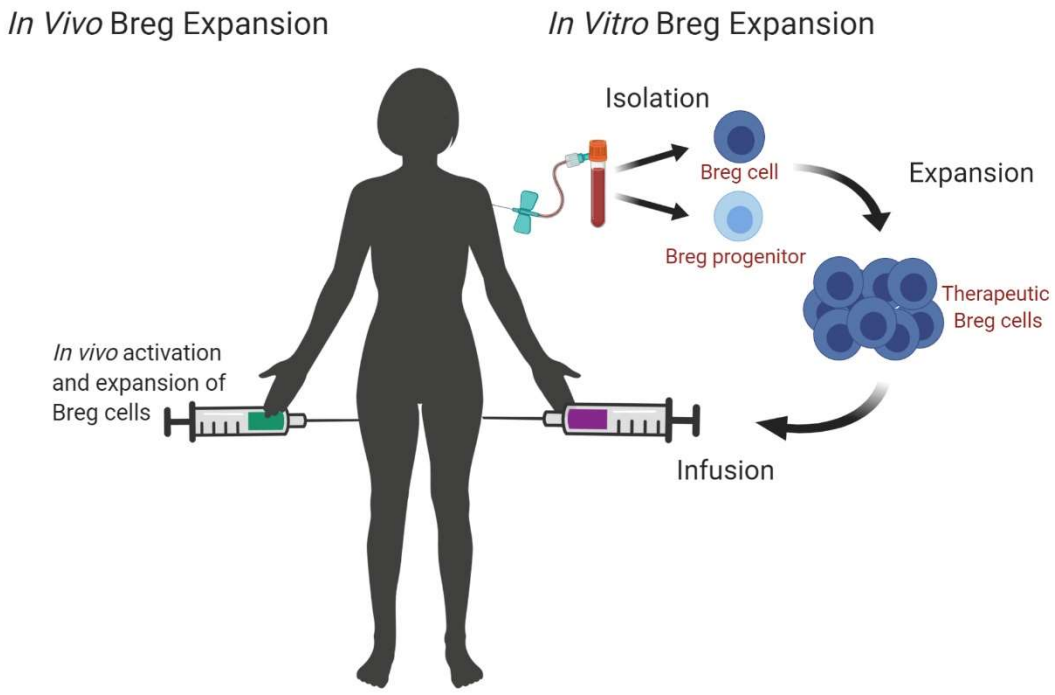


Figure 6.1: Expansion of Breg cells for therapeutic use may be induced *in vivo* or *in vitro*. *In vitro* expansion of Breg cells employs the use of drugs or reagents previously established as Breg inducers, to be administered to the patient for systemic activation and expansion. *In vitro* expansion involves the isolation of Breg cells or progenitors from the patient, expansion of the Breg population, and infusion of the resultant enriched cell culture for therapy.

In vivo generation and expansion of naturally occurring Bregs using drugs offers ease and convenience. Hypothetically, another advantage of *in vivo* generation of Bregs is the ability to amplify autologous cells that have built-in specificity for any foreign antigen that may be present in the system. However, extensive research must be conducted to ensure that the drug of choice to expand Bregs do not elicit undesirable side effects on other cells and tissues. At this point, therapeutic agents that have been shown to be correlated with Breg expansion *in vivo* include cytokine IL-35 that promote the generation of a unique Breg subset that produces IL-35 in a mouse model, and the hormone hCG during pregnancy in mice and humans (Wang et al., 2014; Rolle et al., 2013; Liu et al,. 2019). It is paramount to first define the optimum signal which acts as Breg drivers to effectively design and fine-tune an *in vivo* application, especially if the goal is to orchestrate an immune intervention in a complex biological phenomenon as pregnancy. Unfortunately, this has yet to be realised and the risk remains too high to be considered in such a delicate scenario as pregnancy.

The second method involving *ex vivo* manipulation to generate Bregs has been widely investigated in murine models across different fields. This method uses a three-step approach – isolation, expansion, then infusion (Figure 6.2). B cells could be positively isolated from peripheral blood apheresis units and incubated with reagents known to induce and expand the Breg population. A multitude of studies have demonstrated a number of extrinsic stimulators for inducing and expanding Breg cells by modifying B cells to possess regulatory features. Engagement by agonistic CD40 mAb or CD40 ligand, TLR9 ligand CpG, BAFF, and the GM-CSF and IL-15-derived fusokine GIFT15 have been shown to promote Breg differentiation and expansion *in vitro* (Deng et al. 2012). Genetic reprogramming aimed at producing Bregs has also been explored. Transduction using a retroviral vector encoding an antigen fused to an immunoglobulin heavy chain molecule or a lentiviral vector expressing IL-10 have been shown to enhance B cell inhibitory activity on antigen-specific T and B cells and increase their capacity to induce immune tolerance (Scott, 2010; Calderon-Gomez et al., 2011). Breakthroughs in genetic engineering and editing also pose an exciting and attractive strategy for designing therapeutic Breg cells. Using CRISPR-Cas9 genome editing technology, isolated B cells could be engineered to undergo genome alterations that mediate their functional properties. Reprogramming parts of the BCR thereby modifying antigen/antibody specificity and function has been recently demonstrated (Voss et al. 2019; Greiner et al. 2019). Thus, it is now conceivable to 'make' Bregs from B cells and direct their functionality towards a specific antigen – in the case of a pregnancy-focused immunotherapy, directing the Breg's functionality specifically to fetal antigens. The last step in this method is infusion of the therapeutic immune cells into the patient. A key advantage of an *ex vivo* expansion strategy includes the ability to perform cellular phenotyping of the resultant population after expansion to filter out the desired Breg cells and govern the dose of administered cells. Comparing the *in vivo* and *in vitro* approaches to a Breg-based therapy, the *in vitro* method appears to be the more flexible and feasible option.

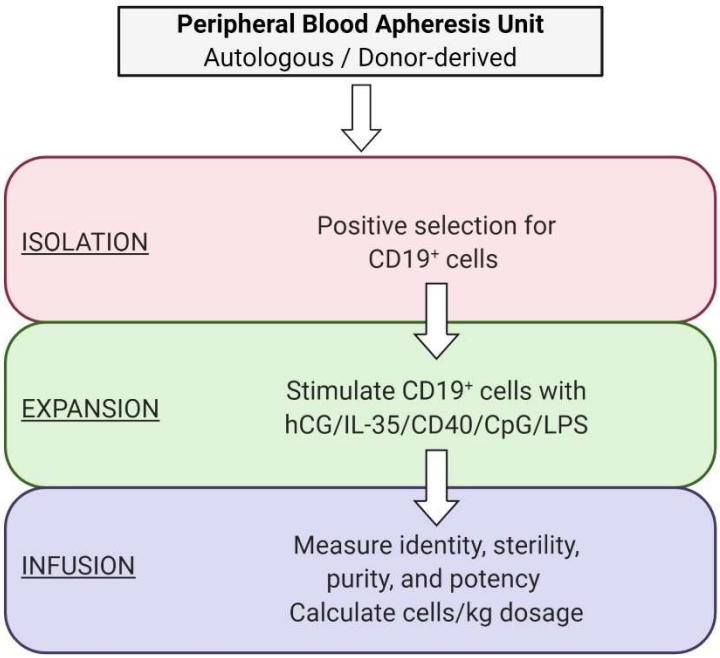


Figure 6.2: Three-step approach of a Breg-based immunotherapy utilising the *in vitro* method of Breg expansion.

Lastly, we examine the possibility of an 'off-the-shelf' Breg-based therapy. Cell therapy can be autologous, where it is derived from a patient's own cells, or allogeneic, where it is derived from donor cells. Moreover, allogeneic therapy enables large numbers of doses to be generated from just one donor to treat multiple patients. Autologous therapy can be time-intensive and expensive due to the complicated process of a personalised treatment whereas the allogeneic approach could offer greater convenience, lower costs, and make treatment available to more patients (Malik et al. 2019). Considering that the cost burden of pregnancy difficulties and complications are generally high, an allogeneic therapy offering the benefits mentioned is a favorable option. Perhaps the biggest challenge associated with allogeneic therapy is the concomitant immune response mounted by the host's immune system to non-self-donor cells that would lead to rejection. However, with the advent of genetic editing, this challenge could be met by engineering cells to be hypoimmunogenic thus eliminating the risk of rejection (Malik et al. 2019). Moreover, the results in Chapter 5 highlight the ability of Breg cells to home in on the cytokine/chemokine-rich uterus during pregnancy. Thus, the possibility of a Breg-based therapy that is directed to increase Breg circulation in an antigen non-specific and tissue-targeted manner is entirely plausible.

As it stands now, research on Bregs and their role in pregnancy is still in its infancy. Although great progress has been achieved over recent years, there are still numerous questions to be answered; insufficient data remains an impediment to further research and

applications. We are hopeful however that in the future, Breg-based cell therapy may be available as therapeutic intervention to ensure a healthy pregnancy.

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APPENDIX

frontiers in Immunology

REVIEW
published: 17 February 2017
doi: 10.3389/fimmu.2017.00172

Regulatory B Cells in Pregnancy: Lessons from Autoimmunity, Graft Tolerance, and Cancer

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The success of pregnancy is contingent on the maternal immune system recognizing and accommodating a growing semi-allogeneic fetus. Specialized subsets of lymphocytes capable of negative regulation are fundamental in this process, and include the regulatory T cells (Tregs) and potentially, regulatory B cells (Bregs). Most of our current understanding of the immune regulatory role of Bregs comes from studies in the fields of autoimmunity, transplantation tolerance, and cancer biology. Bregs control autoimmune diseases and can elicit graft tolerance by inhibiting the differentiation of effector T cells and dendritic cells (DCs), and activating Tregs. Furthermore, in cancer, Bregs are hijacked by neoplastic cells to promote tumorigenesis. Pregnancy therefore represents a condition that reconciles these fields—mechanisms must be in place to ensure maternal immunological tolerance throughout gravidity to allow the semi-allogeneic fetus to grow within. Thus, the mechanisms underlying Breg activities in autoimmune diseases, transplantation tolerance, and cancer may take place during pregnancy as well. In this review, we discuss the potential role of Bregs as guardians of pregnancy and propose an endocrine-modulated feedback loop highlighting the Breg–Treg–tolerogenic DC interface essential for the induction of maternal immune tolerance.

Keywords: regulatory B cells, regulatory T cells, autoimmunity, cancer, pregnancy, tolerance, estrogen, progesterone

OPEN ACCESS

Edited by:
Song Guo Zheng, Penn State Milton S. Hershey Medical Center, USA

Reviewed by:
Abdelhadi Saoudi, Institut national de la santé et de la recherche médicale (INSERM), France
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Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *02 November 2016*
Accepted: *03 February 2017*
Published: *17 February 2017*

Citation:
Guzman-Genuino RM and Diener KR (2017) Regulatory B Cells in Pregnancy: Lessons from Autoimmunity, Graft Tolerance, and Cancer. Front. Immunol. 8:172. doi: 10.3389/fimmu.2017.00172

INTRODUCTION

Pregnancy is a remarkable biological process wherein the mother carries an offspring that is semi-allogenic, that is, half of its genome is derived from the foreign paternal parent. It imposes significant physiological stress, requiring the mother's body to undergo immense functional changes. Pregnancy in mammals represents a unique compromise in the context of immunity—the maternal immune system must accommodate the semi-allogenic fetus by dampening its immune responses to maintain a state of immunological tolerance throughout gravidity, while retaining the capacity to identify pathogens and destroy them with appropriate control of resultant inflammation. This dichotomy necessarily requires a fine and highly regulated balance between immune tolerance and immune activation.

In pregnancy, the immune profiles of dendritic cells (DCs), natural killer cells, macrophages, and T cells are by necessity modified (1–4). Recently, it has been shown that the B cell profile is also modified in response to the immunological needs of the mother during pregnancy (5). The role

of B cells in pregnancy holds much interest, especially in light of the recent identification of a B cell subset capable of negative regulation, that is, can suppress immune inflammation. Due to their unique role amongst the B cell types, this subset is aptly designated as 'regulatory B cells' or 'Bregs.'

Regulatory B cells are being extensively studied in three major fields: autoimmunity, transplantation tolerance, and cancer. Seemingly different, conditions in these fields are in fact outcomes of immune dysfunction. Whereas autoimmune diseases reflect an inadequate ability to control an overactive immune response to self, development of cancer results from the enthusiastic suppression of the immune system thus allowing a mutated self to survive and proliferate. The role of Bregs as negative regulators of the immune system varies in these different contexts. As mediators of immune suppression, they have been implicated in the cessation of autoimmune diseases and graft tolerance as the expansion of the Breg population is highly correlated with amelioration of the disease and maintenance of tolerance (6). On the other hand, Bregs have been tagged as pro-tumorigenic due to their capacity to inhibit the action of effector immune cells whose purpose is to identify and eliminate cancer cells, hence providing a favorable environment for the tumor to grow (7, 8).

As pregnancy requires selective immunological tolerance, this condition may be viewed as a reconciliation of the two extreme paradigms. The mechanisms that inhibit immune flares or chronic inflammation in autoimmune diseases and transplantation may be at play in the induction of maternal immune tolerance during pregnancy. Similarly, the pro-tumorigenic mechanisms that allow cancer development to take place may also be responsible for allowing the growth and development of a semi-allogeneic fetus in the mother's womb. In this review, we investigate the possible mechanisms by which Bregs confer protection to the semi-allogeneic fetus during pregnancy by assessing their role in autoimmune diseases, graft tolerance, and cancer. We also examine how the maternal endocrine system sets the stage for pregnancy, particularly how it endeavors to create an environment conducive for the expansion of Bregs.

REGULATORY B CELLS

B cells are best known to organize immune responses by production of antibodies. Representing the humoral arm of adaptive immunity, they are the primary facilitators of antigen-specific immune responses *via* antibody production and differentiation into memory cells that provide long-lasting immunity. However, reports over the past 40 years indicate that not all B cells function for that purpose. The earliest studies (1974) found that B cells could suppress delayed-type hypersensitivity reactions in guinea pigs, implying an inhibitory effect of B cells on T cell function (9, 10). Further evidence of this B cell regulatory phenotype eventuated more than two decades later, with the observation in a murine autoimmune model that inflammation was exacerbated in the absence of B cells (11). While this suggested that B cells may play a down-modulating role in the inflammatory response, it was only in 2000 that Mizoguchi et al. formally described and reported a subset of B cells that inhibited, rather than promoted, the inflammatory response in a mouse model of inflammatory bowel disease (12). This peculiarly suppressive B cell subset was classified as 'regulatory B cells' or 'Bregs.' Since then, defective Breg function or deficiency in Breg levels have been implicated in conditions involving uncontrolled pro-inflammatory immune responses; most extensively in autoimmune diseases and renal transplantation cases (13–16).

Breg Phenotypic Identification

Defining a specific Breg phenotype has proven to be a difficult as multiple B cell "subsets" have been reported to function as negative regulators of the immune response. While there is no unifying characteristics with respect to cell surface activation and lineage markers as of yet, initial reports indicated that the regulative properties of these unique B cells were attributed exclusively to the production of the anti-inflammatory cytokine interleukin-10 (IL-10) (13, 17, 18). However, more recent studies have revealed B cell subsets with IL-10-independent regulatory functions, indicating that some Bregs employ a multi-mechanistic, and possibly cooperative, approach for regulating immune responses. Given the lack of a unified approach and as IL-10 production is the most reported mechanism of suppressive action; IL-10 production remains the defining feature of Bregs.

Different B-cell subsets that have been attributed with regulatory function in mice include the transitional 2 marginal-zone precursor (T2-MZP) cells, $CD5^+CD1d^{hi}IL\text{-}10^+$ B (B10) cells, follicular (FO) B cells, marginal-zone (MZ) B cells, $CD5^+$B-1a cells, $CD5^+CD178^+$ killer B cells, GIFT-15 B cells, plasma cells, plasmablasts, TIM-1^+ B cells, and PD-$L1^{hi}$ B cells (19, 20). In humans, immature B cells, IL-10^+ B cells (B10), GrB^+ B cells, Br1 cells, and plasmablasts are reported to play immunosuppressive roles (19). Despite the diversity in phenotype, most B cell subsets that carry out negative regulation produce anti-inflammatory cytokines, with the majority of the cell surface marker-defined subsets enriched with IL-10-producing cells. In mice, the suppressive IL-10-producing Bregs, also known as B10 cells are characterized by the $CD1d^{hi}CD5^+$ phenotype (21). Among the splenic B10 cells, both marginal-zone B (MZ B) cells and T2-MZP B cells have been shown to have a protective effect in mouse models of lupus and autoimmune arthritis due to their IL-10 competency (22, 23). The peritoneal cavity contains B-1a cells that are also a major source of IL-10 (24). In humans, $CD19^+CD24^{hi}CD38^{hi}$ B cells isolated from human peripheral blood are classified as Bregs due to their ability to suppress inflammation by a combination of IL-10 production and CD80 and CD86 costimulation (25), while the IL-10-competent $CD24^{hi}CD27^+$ B cells are proposed as the Breg subset analogous to the mouse regulatory B10 cells (26). The heterogeneity of these subsets suggests that Bregs are not derived from one specific lineage; rather they may acquire their regulatory ability through exposure to environmental stimuli.

Since surface markers identifying these subsets are varied, there are currently ongoing attempts to identify a unique global indicator for Bregs, analogous to the transcription factor FOXP3, a defining feature of regulatory T cells (Tregs). Evidence suggests that the T cell immunoglobulin and mucin domain 1 (TIM-1) may be an inclusive marker for Bregs as it identifies about 70% of all IL-10^+ B cells in all subsets mentioned (27). However, CD9 has also been reported as a marker for murine IL-10-competent Bregs

by virtue of transcriptomic analysis which strongly associated its expression with Breg biogenesis and function (28). In principle, identification of a unique marker signifying a direct regulatory function in B cells would reduce the reliance on functional assays and increase our understanding of the role that Bregs may play in different diseases and allow exploration of therapeutic options. To date, elucidating the Breg phenotype and underlying ontology continues to be an active research endeavor.

Bregs' Suppression Mechanisms

Since formal recognition, Bregs have been described mainly in the context of autoimmunity, transplantation tolerance, and cancer. The best studied mechanism for negative regulation of Bregs is their capacity to produce anti-inflammatory cytokines, especially IL-10. The principal function of IL-10 is to repress excessive inflammation and maintain homeostasis through numerous mechanisms, which includes hampering antigen presentation and expression of costimulatory molecules CD80, CD86, and MHC class II by macrophages and DCs, as well as limiting the activation of and cytokine release by T cells in immune responses and promoting the differentiation of Tregs (29).

While IL-10 production remains the hallmark of Breg's negative regulatory function, other independent suppressive mechanisms have come to light in recent years and include transforming growth factor beta (TGF-β), IL-35, and IL-17. Production of TGF-β by B cells leads to promotion of Treg development in diabetes, transplant tolerance, allergic diseases, and colitis (30). Moreover, about 60% of IL-10-producing B cells in mice also express IL-35, a cytokine identified as a key player in the recovery phase of experimental autoimmune encephalomyelitis (EAE), and necessary for improved resistance to *Salmonella* infection (31). Secretion of IL-17 by activated B cells has been reported to occur upon contact with parasite-derived trans-sialidase, a feature shown to be critical in the control and resolution of infection (32). This result emphasizes the importance of Breg function in a disease that has a high potential to develop into chronic inflammation.

Various surface molecules on B cells have also been identified as the main instigators of negative regulation upon cognate interaction with pro-inflammatory cells. Among the most studied are programmed death receptor ligand 1 (PD-L1), granzyme B, and Fas ligand (FasL). Upregulated PD-L1 expression on B cells is critical in down-modulating T cell activity. In EAE, adoptive transfer studies demonstrate that PD-L1$^+$ B cells confer protection and reduce disease severity *via* restriction of helper T (Th) cells inflammatory role and induction of Treg activity (33–35). Furthermore, PD-L1$^+$ B cells have been shown to regulate cytotoxic T cells in both *Salmonella* infection and prostate cancer (36, 37), with high expression shown to directly suppress the FO Th response and differentiation *via* attenuation of downstream signaling pathways concomitant to programmed death receptor 1 (PD-1) ligation (38). Similarly, B cells expressing FasL were found to be critical in preventing graft rejection in mice and modulating the induction of autoimmune disease through a myriad of mechanisms including promotion of apoptosis of autoreactive T cells and the generation of Tregs (39, 40). Lastly, IL-21-induced granzyme B expression in B cells was shown to impart a regulatory function against effector T cells in the tumor microenvironment (41).

Breg Detection and Expansion

Due to the inconsistencies in specific surface markers used to classify Breg subsets, the most common approach for Breg identification remains a combination of surface marker staining and intracellular IL-10 staining. In mice, isolated Bregs do not readily produce IL-10 *ex vivo*, therefore to identify this specific subset of B cells, cells are subjected to acute B cell stimulation—5 h of culture with a cocktail of lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate, and ionomycin to enhance the translation of cytokine genes, and thus detectable IL-10 production (42). In the spleen and other lymphoid tissues, Breg progenitor cells have also been identified (43). These are defined as B cells that do not express IL-10 following the short-term stimulation but may be induced to Breg maturation and IL-10 competency by culture with agonistic CD40 monoclonal antibody for 48 h. This technique has proven beneficial for expanding this rare subset for subsequent *in vivo* applications such as adoptive transfer experiments. Together, active and progenitor Bregs account for 3–8% of basal B cell levels in the murine spleen, of which 1–3% are IL-10-competent Bregs. Lower percentages are found in the blood, lymph nodes, intestinal tissues, Peyer's patches, and the central nervous system (21, 44, 45). In humans, IL-10-producing B cells isolated from the peripheral blood are found to express increased levels of CD19, IgD, CD27, CD48, and/or CD148 at varying proportions and phenotypic combinations. IL-10-competent Bregs have also been identified in peripheral tissues such as spleen, tonsils, and umbilical cord blood at less than 1% of the total B cell population (26).

Bregs IN AUTOIMMUNITY

In the last decade, numerous studies have been conducted to determine the operating mechanisms that impart Breg-mediated suppressive function. The majority of what is currently known stems from the multitude of studies focusing on autoimmune diseases where it has been demonstrated that Bregs interact and regulate the function of T cells, DCs, monocytes, macrophages, and natural killer cells.

The regulatory role of B cells in EAE, a mouse model for multiple sclerosis (MS), was first apparent when exacerbation of disease was observed in B cell-deficient B10.PLμMT mice (11). The current model posits that Bregs regulate EAE severity through IL-10 production and expression of costimulatory molecules CD40, CD80, and CD86, thus limiting the type 1 cytokine response *via* cross talk between B cells and T cells, and recruitment of FOXP3$^+$ Tregs into the central nervous system (6, 14). B cells expressing toll-like receptor (TLR)-2 and TLR-4 also suppressed inflammatory T cell responses (Th1 and Th17) and stimulated the recovery phase of EAE (46). Other mechanisms such as the presence of glucocorticoid-induced TNF ligand and elevated levels of B and T lymphocyte attenuator on B cells also play a role in inducing and maintaining the Treg pool in the central nervous system during EAE, a process which ultimately facilitates the protection against or amelioration of the disease (47, 48). Interestingly, recent evidence indicates that Bregs can also directly access the central nervous

system (CNS). Bone marrow cells transiently stimulated with the TLR-9 agonist CpG generate proB (CpG-proB) cells, which upon transfer to recipient mice at the onset of EAE symptoms differentiate into mature B cells with regulatory function. Some of these mature B cells home to the inflamed CNS for local production of IL-10 thus instigating a switch of the host cytokine profile from inflammatory to immunoregulatory, while others enter reactive lymph nodes and restrain encephalitogenic T cells *via* CCL19 expression. Together these functions cooperatively enhanced amelioration of active EAE (49). Evidence of the regulatory function of B cells in human disease is also accumulating, with B cells from MS patients observed to have diminished capacity for IL-10 production on activation as opposed to healthy controls. This suggests that dysregulation of this pathway contributes to MS pathogenesis (50–52).

Type 1 diabetes (T1D) is characterized by the autoimmune-mediated destruction of pancreatic β cells. Circulating $CD19^{+}CD27^{-}CD24^{hi}CD38^{hi}$ Bregs have been found to decrease with age and as such are hypothesized to be a major contributing factor to the prevalence of T1D in older age groups (53). Patients with T1D also exhibit the lowest frequency of B10 cells compared with patients with type 2 diabetes, latent autoimmune diabetes in adults, and healthy controls (54). These results highlight the importance of B cells in the immunopathogenesis of autoimmune diabetes and suggest that antigen-activated B10 cells may have a role to play in inhibiting autoreactive T cell responses to islet-specific antigen in healthy individuals, as the absence of this population leads to hyperglycemia in T1D patients. In non-obese diabetic (NOD) mice, adoptive transfer of $CD1d^{hi}CD5^{+}IL\text{-}10^{+}$ Bregs prevented T1D development. Further, the addition of tolerogenic DCs to the transferred therapeutic mix reversed the onset of T1D by augmenting the frequency of $IL\text{-}10^{+}$ Bregs (55). In the same manner, adoptive transfer of CpG-proB cells, either directly or indirectly after maturation into mature Bregs, protected against the pathogenesis of T1D in NOD mice *via* suppression of IL-21 and induction of apoptosis in effector T cell populations, including specific diabetogenic T cells (56). The DC- and CpG-mediated suppression of T1D through Breg activity reiterates the notion that B cells have an intrinsic suppressive potential, which is only activated when the environmental cues are appropriate.

In systemic lupus erythematosus (SLE), Bregs are emerging as vital players during disease initiation. Adoptive transfer of splenic $CD5^{+}CD1d^{hi}$ Bregs from wild-type mice extended the survival period of $CD19^{-/-}$ mice in this spontaneous lupus model (57). In humans, SLE patients exhibited similar frequencies of circulating $CD24^{hi}CD38^{hi}$ B cells but had significantly lower $IL\text{-}10^{+}$ percentages compared to normal healthy controls (58). Further, there is evidence of functional impairment in Bregs from SLE patients. Isolated Bregs were unresponsive to CD40 ligation and exhibited lower IL-10 production compared to Bregs obtained from normal patients, which resulted in inadequate suppression of T cell activation and proliferation (59). Compromised cross talk between IL-10-producing $CD24^{+}CD38^{hi}$ Bregs and interferon alpha-producing plasmacytoid dendritic cells has also been documented as a key contributor to the pathogenesis of SLE (60).

Studies of rheumatoid arthritis (RA) suggest that Bregs are responsible for the primary induction of Tregs. Specifically, IL-10 from B cells aids in the establishment of the Treg to inflammatory Th1 and Th17 cell ratio as seen in chimeric ($IL\text{-}10^{-/-}$ B cell) mice which exhibit a significant decrease in FOXP3 expressing $CD4^{+}$ T cells; the low number of Tregs occurred in parallel with an increase in Th1 and Th17 cell populations in the draining lymph nodes of the inflamed joints (61). Furthermore, the adoptive transfer of Bregs into these chimeric mice restored the Treg population to normal levels and decreased inflammation by inhibiting Th1 and Th17 differentiation, thus decreasing IFNγ and IL-17 cytokine levels, and reinstating a balanced Th1/Th2 response (62). This notion of Bregs influencing T cell plasticity was similarly demonstrated by injection of exogenous B10 cells which effectively suppressed the development of arthritis by suppressing Th17 cell generation (63). Clinical studies report decreased frequency of $CD24^{hi}CD38^{hi}$ Bregs in RA patients compared to unaffected individuals; it was shown that normal levels of Bregs effectively inhibited $CD4^{+}CD25^{-}$ T cell differentiation into Th1 and Th17 cells and promoted the differentiation of T cells into Tregs *via* IL-10 production (64). Moreover, it has been demonstrated that many RA patients achieve remission during pregnancy, presumably due to a shift in T cell function from a Th1 to a Th2 phenotype, but relapse postpartum, a phenomenon possibly correlated to the increase of Breg levels during pregnancy and consequent decrease upon parturition (65–67).

The regulatory function of human B cells in autoimmune diseases is realized in case studies where complete B cell depletion therapy with rituximab (anti-CD20 monoclonal antibody) has resulted in aggravation of the disease or onset of new immune-mediated pathologies. For instance, a patient with intractable ulcerative colitis experienced severe clinical aggravation of disease upon treatment with rituximab, a result ascribed to the depletion of both the intestinal and systemic suppressive B cell population (68). Similarly, rituximab was an effective treatment in a patient with Graves' disease; however, development of ulcerative colitis and arthritis was observed shortly thereafter. Again, the depletion of colonic B cells was implicated, with symptoms alleviated upon repopulation (69).

Therefore, in autoimmune disease, the suppressive role of Bregs in the framework of T cell function occurs at two levels: (1) control of T cell plasticity by invoking the production of Tregs, inhibiting activation of naïve T cells, and suppressing Th differentiation; and (2) manipulation of the production of proinflammatory and anti-inflammatory cytokines and their balance thereof.

Bregs IN TRANSPLANTATION TOLERANCE

Graft tolerance has been best studied in liver and kidney transplantations. Postoperative care involves continuous treatment with immunosuppressive drugs to prevent the immune response to the alloantigen, which may lead to organ rejection. Some recipients require immunosuppression medication for their entire lifetime; however there are reports of operationally tolerant recipients maintaining graft function despite immunosuppressive drug withdrawal (70). The containment of the inflammatory response that typically ensues without immunosuppressive drugs

points to the valuable function of regulatory immune cells—the Tregs which have an established role in graft tolerance, and the Bregs, whose role in transplantation has recently emerged.

Initial reports implicating a role for B cells as facilitators of graft tolerance resulted from studies investigating the effect of CD40 blockade on resting B cells. Permanent survival of mouse pancreatic islet allografts was demonstrated upon pretreatment with allogeneic non-T cell small lymphocytes (elutriated from spleen cell suspensions) plus blocking antibody to CD40L (71). Moreover, pretreatment of resting B cells with anti-CD40 monoclonal antibody prolonged fully allogeneic mouse cardiac allografts and augmented the hyporesponsiveness to MHC molecules *in vivo* (72), suggesting a dominant role of the CD40/CD40L pathway in tolerance induction. The mechanism by which anti-CD40 antibodies can elicit a tolerogenic response was elucidated a decade later when it was demonstrated that CD40 ligation resulted in the differentiation of IL-10$^+$ transitional-2 (T2) Bregs, the rescue of B cells, and transitional B cell subsets from apoptosis, as well as prevention of differentiation into mature FO B cells. Specifically, anti-CD40 Ab administration induced the differentiation of T2 and MZ B cells and expanded the population of IL-10$^+$ Bregs *in vitro* and *in vivo*, resulting in significantly improved renal disease and controlled progression of lupus in mice (73). This was also reflected in tolerant kidney and cord blood transplant patients where increased CD40 ligation potentiated IL-10 production by naïve and transitional B cells (74–76).

In a rat model of kidney allografts, intravenous injection of bulk donor B cells to the recipient at the time of transplantation was by itself proven to be effective in inducing long-term acceptance (more than 300 days) of the graft. This was a significantly longer time period than when donor T cells were administered 17 days after transplantation (77). Studies of islet allografts in mice identified TIM-1 as an inclusive marker for IL-10 and IL-4 expressing Bregs in all major B cell subpopulations including the transitional, MZ, FO, and CD5$^+$CD1d^{hi} cells. More importantly, ligation by anti-TIM-1 induced the regulatory function of these B cells constitutively expressing TIM-1 *via* IL-10 production. Adoptive transfer of TIM-1$^+$ B cells from untreated BALB/c allograft recipients into chemically diabetic B cell-deficient allograft recipients prolonged graft survival by activation of transferred IL-10-producing TIM-1$^+$ B cells and augmented the frequency of FOXP3$^+$ Tregs (27). Moreover, the mucin domain of TIM-1 has been identified as being primarily responsible for Breg induction and maintenance in prolonged allograft survival (78). Aside from TIM-1$^+$ Bregs, tolerance in a mouse model of MHC class I mismatched skin transplantation was associated with a transient expansion in T2 B cells in mice tolerized by donor splenocyte transfusion and ligation with CD40L. These tolerized B cells were characterized by expression of downregulated levels of CD86 and upregulated levels of TIM-1, and prolonged skin allograft survival *in vivo*, as well as suppressed T cell activation *in vitro*. Moreover, it was seen in this study that tolerized B cells and graft-specific Tregs worked synergistically in prolonging graft survival, albeit in an IL-10-independent fashion (79). Similarly, the adoptive transfer of TGF-β-producing Bregs induced the expression of FOXP3 in CD4$^+$CD25$^-$ T cells, thus activating and expanding the Treg population which contributed to protracted graft survival (80). These reports reinforce the multi-mechanistic mode of suppression by Bregs in that through IL-10, TGF-β, CD86 costimulation, and other surface molecules, antigen-specific tolerized B cells no longer stimulate allospecific T cell activation, but instead support the induction of Treg function to enact a tolerogenic response.

In humans, research has also indicated a critical role for B cells in regulating alloimmunity. For operationally tolerant renal transplant recipients, renal allograft tolerance was strongly associated with a B cell signature consisting of an increased expression of multiple B cell differentiation and activation genes over that observed in non-tolerant patients receiving immunosuppression and non-transplanted controls. The three genes identified as reliable predictors of operational tolerance—*IGKV4-1*, *IGLL1*, and *IGKV1D-13*—are genes that are upregulated during transition from pre-B to mature B cells and during class switching post-antigen stimulation, suggesting that transitioning B cells may be highly involved in tolerance induction and/or maintenance (15). Moreover, operationally tolerant patients displayed higher transitional (CD24^{hi}CD38^{hi}) and naïve B cell frequencies, lower numbers of memory B cells, and an enriched IL-10$^+$ B cell population—all phenotypes that were strongly associated with reduced allograft rejection rates (81–84). Together these recent findings suggest assigning a prospective biomarker status on transitional B cells. Additionally, operationally tolerant patients retain the capacity to activate the CD40 pathway essential to Breg activation (16), and although IL-10-producing Bregs are higher in these patients, other mechanisms such as granzyme B$^+$ and TGF-β production may also account for the inhibitory effect on T cells essential to maintaining transplantation tolerance (80, 85).

Bregs IN CANCER

Oncogenesis is primarily a battle between the immune system and the cancer stem cells. Tumor development and progression is dependent on the lenience of the microenvironment, to which leukocytes play a critical role. Both anti-tumorigenic and pro-tumorigenic immune mechanisms transpire in the early stages of cancer development, with the net effect dictating whether the tumor develops or not (86). In particular, the induction of immune tolerance within the tumor environment has been shown to curtail immune-enhancing anti-tumorigenic efforts and encourage tumor progression and subsequent metastases. Immunosuppressive cells such as Bregs and Tregs have been implicated in facilitating immune tolerance, and ultimately, cancer escape.

For instance, tumor growth is contingent on the immune profile within the local microenvironment. Strong infiltration of antitumor CD8$^+$ T cells and natural killer cells and decreased levels of regulatory B and T cells and myeloid-derived suppressor cells (MDSCs) all result in tumor destruction, whereas reversed proportions support tumor growth. In the tumor-draining lymph nodes, increased accumulation of B and T cells resulted in lymphangiogenesis and increased lymph flow (87), which contributed to and accelerated the rate of metastasis (88). Moreover, B cell accumulation in lymph nodes of preneoplastic Eμ-*c-myc* mice was positively correlated with expansion of the lymphatic

sinuses and enhanced tumor growth, as opposed to preneoplastic B cell-deficient μMT mice which did not undergo lymphangiogenesis and exhibited minimal tumor growth (89). However, tumor growth was accelerated in the B cell-deficient μMT mice upon adoptive transfer of naïve T2-MZP cells, highlighting the role of transitional and suppressive B cells in cancer escape. Furthermore, B cells bearing downregulated MHC class II and CD86 expression and upregulated Ly6A/E, PD-L1, and CD39 selectively accumulated in the draining lymph nodes of mice with HPV-related cancer. The presence of PD-L1 and CD39 on the B cell surface confers regulative properties *via* direct inhibition of T cell function upon cognate interaction. The depletion of B cells in this model resulted in a robust Th1 cytokine response and decline in recruitment of Tregs, which was accompanied by a strong infiltration into the tumor of $CD8^+$ T cells and ultimately tumor rejection (90). Interestingly, although primary colorectal tumors contain a significant memory and plasma B cell infiltrate suggestive of an active antitumor response, $CD24^{hi}CD38^{hi}$ Bregs were significantly increased in metastatic tissue indicating a shift toward active immunosuppression in the local microenvironment (91). Thus the current literature suggests that B cell infiltration and accumulation in the tumor site and draining lymph nodes establishes an immunosuppressive microenvironment that leads to inhibition of antitumor immune responses and expansion of the critical lymphatic network, thereby facilitating tumor growth and metastasis.

Investigations into the specific role of Bregs in cancer have recently increased following the discovery of a unique B cell subset termed tumor-evoked Bregs (tBregs) in Balb/c mice harboring 4T1 carcinoma cells (8). In this model it was shown that cancer-eradicating B cells were 'hijacked' by resident cancer cells within the tumor microenvironment and converted into immunosuppressive Bregs. These tBregs do not conform to any of the established Breg phenotypes; instead they resemble activated mature B2 cells ($CD19^+CD25^{hi}CD69^{hi}$ and B7-$H1^{hi}CD81^{hi}CD86^{hi}CD62L^{lo}IgM^{int}$). Incubation of non-regulatory $CD4^+$ T cells with tBregs bearing high expression levels of CD40, CD80, CD86, MHC class I and II molecules, and TGF-β production capacity led to the significant generation of $CD4^+CD25^+FOXP3^+$ Tregs which in turn inhibited $CD8^+$ T cell proliferation—thereby facilitating breast cancer escape and metastasis (8). More recently, it has been demonstrated that B cells infiltrating the mammary tumor bed are 'educated' through cognate interactions with resident tumor cells. These tumor-infiltrating B cells acquired PD-L1 expression and TGF-β competency which contributed to an immunosuppressive phenotype characterized by an enhanced inhibitory capacity against $CD4^+CD25^-$ T cells, $CD8^+$ T cells, and $CD49b^+$NK cells, as well as promotion of Treg expansion (92). Therefore, infiltrating B cells subverted into Bregs by the tumor microenvironment facilitates the necessary critical changes in the local immune profile to support a pro-tumorigenic outcome.

In humans, studies reveal that a higher frequency of Bregs is indicative of enhanced tumor aggressiveness and poorer prognosis. For instance, hepatocellular carcinoma patients harbor significantly more peripheral Bregs compared to healthy controls (93); malignancy in non-small cell lung cancer is associated with an increased frequency of IL-10-producing Bregs, $CD4^+CD25^{+/high}CD127^{low/-}$ Tregs, and MDSCs (94); patients with tongue squamous cell carcinoma, gastric cancer, and colorectal cancer also exhibit a higher frequency of Bregs within the tumor itself compared to unaffected neighboring tissues, with numbers positively correlated with an increased frequency of Tregs (91, 95, 96). Moreover, *in vitro* studies of lung cancer cells demonstrated their direct capacity to upregulate Treg and Breg phenotypes in lymphocyte cocultures (94). As for the mechanism, IL-10 and TGF-β competency appear to be the primary facilitators of suppression. For example, peripheral blood $CD19^+CD24^{hi}CD38^{hi}$ Bregs isolated from gastric cancer patients converted $CD4^+CD25^-$ effector T cells into $CD4^+FOXP3^+$ Tregs *via* expression of TGF-β (95). Moreover, IL-10 facilitates cross talk between $CD19^+IL\text{-}10^+$ Bregs and $CD4^+CD25^-$ T cells in tongue squamous cell carcinoma patients, resulting in the conversion of these resting T cells into Tregs (96). Likewise, IL-10 has been shown to induce the expansion of Tregs in peripheral lymphoid organs in gastric cancer patients (95).

Bregs AND FEMALE SEX HORMONES

The two major sex hormones involved in pregnancy are estrogen and progesterone, with each hormone playing a central role at different time points during gravidity. Progesterone promotes endometrial decidualization for the implantation of the embryo and maintains uterine relaxation throughout pregnancy (97). Estrogen on the other hand, plays a central role in angiogenesis which is needed for placentation and sustenance of the fetus (98). High estrogen levels and the withdrawal of progesterone promote the onset of labor, and ultimately, human parturition. Female sex hormones were found to have a significant influence on inflammatory responses when women with autoimmune conditions were reported to experience a change in their disease activity upon pregnancy. For instance, women that acquired SLE during pregnancy generally experienced a more severe affliction compared to non-pregnant patients (99, 100). Existing RA is often ameliorated by the surge of immunoinhibitory estrogen during pregnancy, which relapses postpartum upon an increase in the immunostimulatory hormone prolactin (101, 102). There is also evidence that female sex hormones are capable of modifying decidual immune cells, such as DCs and uterine natural killer cells, from being actively pro-inflammatory to being tolerogenic (103). Considering that pregnancy outcomes essentially rely on the effective control of the inflammatory response, essential pregnancy hormones should be investigated in any study of immune cell changes during pregnancy.

Estrogen regulation of immune cells, whether innate or adaptive, has been established in recent years. Its presence can increase splenic neutrophil numbers, alter the phagocytic capacity of macrophages, and enhance the maturation of DCs (2). It has also been shown to regulate the activities of $CD4^+$ and $CD8^+$ T cells and promote the expansion and activity of Tregs by increasing FOXP3, PD-1, and cytotoxic T-lymphocyte-associated protein 4 expression (34, 104, 105). RA and MS can both be ameliorated by estrogen-induced Treg expansion and activation which leads to immune suppression (104, 106). B cells are likewise affected by

the presence of estrogen. During lymphopoiesis, a rise in estrogen levels leads to a reduction of B cell precursors and an expansion of mature B cells. Estrogen activation therefore affects B cell maturation, and ensures that the immune system is equipped to defend the body against pathogens if required (5).

Two receptors are responsible for estrogen-mediated signaling—estrogen receptor α (ERα) and estrogen receptor β and upon activation act as transcription factors for a range of estrogen-sensitive genes, with ERα more predominant on B cells (107). Studies of EAE demonstrate that the protective effect of 17β-estradiol (E2) comes from its interaction with ERα on immune cells, including B cells (106). While many studies have explored the protective effect of estrogen in autoimmune diseases, primarily by focusing on effects on Tregs (34, 108), others have highlighted the role of B cells by showing that E2 conferred protection against the induction of EAE in mice even after ablation of Treg; a result unsuccessful in B cell-deficient mice (6, 109). Specifically, E2 augmented the frequency of splenic IL-10$^+$ Bregs *via* the PD-1/PD-L1 mechanism (110, 111) which led to a decline in the frequency of CD11b$^+$CD45^{hi}-activated macrophages, DCs, and infiltrating CD4$^+$ cells in the spinal cord (112). Together, these results suggest that Bregs may have carried out this protective effect *via* their responsiveness to estrogen. This was confirmed by studies which demonstrated that estrogen-receptor positive B cells upregulate Treg function in EAE (34).

The effect of progesterone on innate immune cells, mainly monocytes, DCs, and natural killer cells, has also been elucidated (103), and in a manner similar to estrogen, depends on the presence of progesterone receptors. These receptors also act as nuclear transcription factors upon activation and may either be progesterone receptor A (PR-A) or progesterone receptor B (PR-B) (113). The specific actions of progesterone relevant to myometrial function are mediated by the combined and distinct genomic actions of PR-A and PR-B. Myometrial cells express PR-B for most of the pregnancy, facilitating progesterone-mediated Th2-like maternal immune responses, resulting in reduced production of pro-inflammatory cytokines, and increased secretion of IL-10 (97, 114). At the latter part of pregnancy nearing the onset of parturition, PR-A gene expression becomes more predominant, blocking the transcriptional anti-inflammatory activity of PR-B and consequently increasing pro-inflammatory gene expression and promoting labor (97). Progesterone function in cancer and autoimmune diseases occurs comparably to that in pregnancy, in that progesterone inhibits T cell proliferation and pro-inflammatory cytokines IL-2, IL-17, and IFNγ expression while enhancing IL-4, IL-5, IL-6, and IL-10 cytokine production (98, 115). Moreover, there is a concurrent expansion of B cells associated with the increase in IL-10, and a lack of effect of progesterone on Tregs (116). Collectively, this suggests a possible signaling pathway wherein progesterone elicits IL-10 augmentation by activating Bregs.

The molecular mechanisms by which estrogen and progesterone operate appear to be distinct from one another. Whereas estrogen elicits a strong effect on Tregs, progesterone does not. However, both have been documented to affect the B cell immune profile. The current literature gives insights as to how the female sex hormones shape the immune response in various clinical conditions, and overall indicate that estrogen and progesterone foster a tolerant environment within the pregnancy milieu by expanding all regulatory cells. Changes in the circulating amount and distribution of these cells during pregnancy indicates that adaptations are in place to ensure that the mother's body is well equipped to conceive and grow a semi-allogeneic fetus.

Bregs IN PREGNANCY

Previous studies of Bregs in the context of autoimmune disease, graft transplantation, and cancer all indicate one thing—Bregs dampen the inflammatory response and foster a stable tolerant immune profile within the local microenvironment. Based on the current literature, it is therefore reasonable to speculate that Bregs may be a key player in pregnancy. Their anti-inflammatory role in autoimmunity and graft tolerance suggest relevance to the induction of maternal immunological tolerance, while their pro-tumorigenic role in cancer may be applicable in pregnancy, i.e., maternal–fetal cognate interactions may hijack naïve B cells and educate them to become Bregs thus ensuring the fetus' unperturbed growth. The pregnancy hormones estrogen and progesterone are likewise critically involved in the establishment, maintenance, and termination of pregnancy.

During pregnancy, as the mother's womb houses and protects the semi-allogeneic fetus, the response of the maternal immune system is the key determinant to pregnancy success, with failure to induce selective immune tolerance toward the fetus resultant in pregnancy loss. Modification of the maternal immune response is orchestrated by multiple cytokines that influence the nature and abundance of leukocyte subsets in the uterus and placenta and includes the reduction of antigen-presenting function of monocytes, macrophages, and DCs; inhibition of natural killer cells, T cells, and B cells; proliferation of uterine killer cells; maintenance of tolerogenic DCs; and the induction of Tregs (117). These immunological adaptations during early pregnancy pave the way for two main objectives—to protect the fetus from immune rejection and to facilitate the tissue remodeling processes needed for placental development (118).

Primarily, Bregs are considered highly relevant in the pregnancy milieu as they are a major cellular source of the potent anti-inflammatory cytokine IL-10. This cytokine is vital for optimal pregnancy outcomes, with IL-10 deficiency associated with fetal resorption, growth restriction, and even death (118–120). IL-10 is found in abundant amounts in the uterus and placenta during pregnancy and has been identified as a critical player for counteracting the pro-inflammatory cytokine response. Even at the initial conception stage, inflammation resulting from the recognition of paternal antigens is thwarted by the presence of IL-10 (121). Furthermore, administration of a sub-clinical dose of LPS to pregnant IL-10$^{-/-}$ mice resulted in a 10-fold reduction in viability of semi-allogeneic fetuses compared to unaffected controls. Analysis of maternal serum and uterine tissue in these LPS challenged IL-10$^{-/-}$ mice demonstrated high levels of the pro-inflammatory cytokines TNFα, IL-6, IL-1A, and IL-12p40, a result which could be abrogated by administration of exogenous IL-10 (118, 122, 123). Therefore, IL-10 confers pregnancy

protection by multiple mechanisms including inhibition of pro-inflammatory cells such as macrophages and monocytes, and suppression of pro-inflammatory cytokine and chemokine production such as TNFα, IL-6, IL-1a, IL-8, and IL-12 (118, 124, 125). These findings demonstrate the capacity of IL-10 to regulate the inflammatory response in the uterus and placenta during gestation.

Evidence of a direct role for Bregs was indicated in comparative mouse studies of normal and abortion-prone pregnancies which showed a diminished frequency of B10 cells in the maternal spleen of abortion-prone mice, whereas normal pregnancies harbored an increased number of B10 cells. Furthermore, adoptive transfer of B10 cells rescued pregnancies in abortion-prone mice, by inhibition of DC maturation and expansion of the Treg population (126). Another Breg phenotype reported as critical to pregnancy success are the MZ B cells that are found expanded in the spleen of normal pregnant mice, but lacking in mice undergoing pregnancy complications. Additionally, successful pregnancy was correlated with the capacity of MZ B cells to produce enhanced levels of immunoglobulin (IgM and IgA) which likely shifts the immune response from a Th1-like to a Th2-like profile (127). Although also IL-10 competent (22), there is no evidence yet linking MZ B cell cytokine production to their role in pregnancy well-being. In humans, IL-10-producing $CD19^{+}CD24^{hi}CD27^{+}$ B cells in the peripheral blood have been shown to be significantly higher in women undergoing normal pregnancies as opposed to non-pregnant women, or women who had suffered spontaneous abortions (128). Bregs isolated from peripheral blood of pregnant women taken during the first trimester successfully inhibited TNFα secretion by activated T effector cells *ex vivo* (128). Interestingly, $CD24^{hi}CD38^{hi}$ Bregs were found to be significantly lower during the third trimester of pregnancy and on delivery day compared to non-pregnant and postpartum controls, possibly as a result of a drop in female sex hormones that influence B cell activation (129). Although limited in number, these studies on Breg function in pregnancy, and the underpinning Breg-mediated immunosuppression, parallels that observed in autoimmune disease, graft tolerance, and cancer. In all conditions, Bregs impose a suppressive environment *via* induction and maintenance of Tregs, modification of the Th response, and inhibition of effector cell responses including cytolytic T cells, NK cells, and DCs.

As Breg function in pregnancy appears to be interlinked with that of Treg and DCs, defining the working mechanisms is

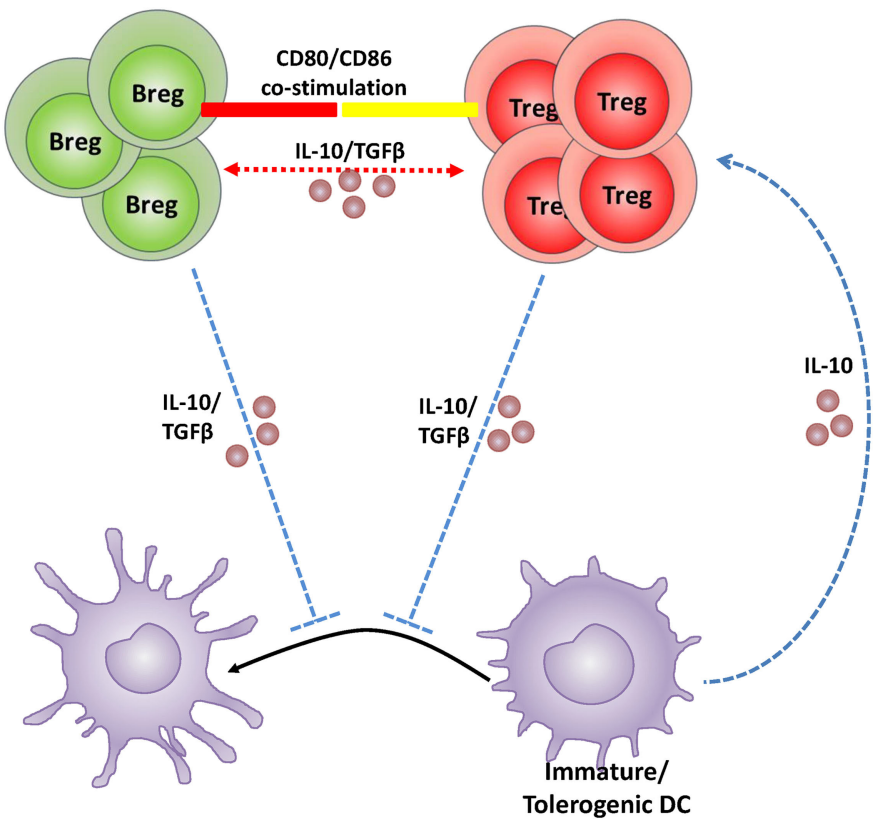


FIGURE 1 | Schematic illustration of the hypothetical feedback loop between regulatory B cell (Breg), regulatory T cell (Treg), and dendritic cells (DCs) during pregnancy. Bregs create a tolerant pregnancy milieu either by direct induction and expansion of the Treg population *via* CD80/CD86 costimulation and/or IL-10 and TGFβ production (depicted in red); or by inhibition of DC maturation causing retention of a tolerogenic phenotype and in turn expansion of the Treg population *via* IL-10 (depicted in blue). Tregs and tolerogenic DCs produce IL-10 and may maintain the feedback loop responsible for negative regulation within the pregnancy milieu. The exact mechanism by which Bregs recruit Tregs and inhibit DC maturation in the pregnancy context is, however, yet to be established.

requisite. Both Tregs and DCs are critical players in determining pregnancy outcomes. In normal pregnancy, the majority of decidual DCs remain immature which contributes to the maintenance of a fetal-tolerant local environment. These immature DCs are thus referred to as tolerogenic DCs (130). Tregs are likewise crucial contributors in achieving and maintaining maternal–fetal immune tolerance. In mice, a natural periodic accumulation of Tregs during the estrus stage is seen, purportedly in preparation for the implantation of a semi-allogeneic fetus (131). At the onset of embryo implantation, Tregs are recruited to the para-aortic lymph nodes draining the uterus (132). Expansion of the Treg population is critical in improving the rates of successful pregnancy as deficiency or harboring insufficient numbers of Tregs in the vicinity of the uterus at any time during pregnancy leads to abortion or miscarriage (4, 133). In the context of pregnancy, tolerogenic DCs and Tregs foster a fetal-tolerant environment *via* production of anti-inflammatory IL-10 and TGF-β (134).

During pregnancy, if an anti-inflammatory signal such as IL-10 or TGF-β is lacking, the DC phenotype remains such that it prevents the activation of T cells that protect the semi-allogeneic fetus (135). It is thus pertinent that DCs retain their immature state to acquire the tolerogenic phenotype. IL-10 and or TGF-β must then be initially present in the pregnancy milieu to either set off the induction of tolerogenic DCs followed by the induction of Tregs, or directly drive the induction of Tregs. However, given that Bregs are (1) established sources of both IL-10 and TGF-β, (2) significantly increased in numbers during normal pregnancy, and (3) are responsive to endocrine modulation, it is plausible that Bregs also play a critical role in pregnancy, especially in the initial induction of maternal immunological tolerance. In support, the findings of Jensen et al. (126) suggest that IL-10 production from B10 cells likely influences the non-maturation of IL-10-receptor rich DCs which then expand the Treg population in pregnant mice. Based on the current literature indicating similar immunosuppressive roles and direct interface among these three types of immune cells, a Breg–Treg–tolerogenic DC feedback loop is proposed for the maintenance of a tolerant pregnancy milieu (illustrated in **Figure 1**).

CONCLUSION

The function of Bregs in autoimmune diseases, graft tolerance, and cancer sheds light as to their emerging role in pregnancy. Studies of autoimmune disease and graft tolerance indicate that the immune response attempts to regain homeostasis by Breg-mediated negative regulation. In cancer, tumor-educated Bregs represent an adaptation that encourages a mutated self to survive and proliferate. Integrating these fields, it is reasonable to suggest that pregnancy represents a predicament common to both—the need for constant immune tolerance for the duration of the pregnancy, and the permission to allow a semi-allogeneic fetus to grow within. However to date, the direct role that Bregs play in maternal tolerance in pregnancy has not been fully investigated, and is the subject of ongoing investigations in our laboratory. Supporting studies suggest that the pregnancy sex hormones, estrogen and progesterone, may also be critical prerequisites for Breg activation and function, with a Breg–Treg–tolerogenic DC feedback loop potentially underpinning the induction and maintenance of a tolerant environment necessary for a successful pregnancy. Bregs therefore, are potential guardians of pregnancy well-being, and require further exploration as to their role in this context.

AUTHOR CONTRIBUTIONS

RG-G researched and wrote the manuscript. KD conceptualized the investigation and edited the manuscript.

FUNDING

This work was supported by NHMRC Project (APP1020984) and Fellowship (APP1012386) grants to KD; and University of South Australia President's Scholarship to RG-G.

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Conflict of Interest Statement: The authors declare that review of the literature was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Regulatory B Cells

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Advanced article

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Online posting date: 22nd January 2018

B cells are best known for their ability to produce antibodies and instigate immune memory necessary for the protection of the host against foreign stimuli. However, certain subsets of B cells are increasingly shown to be capable of negative immune regulation by counteracting inflammation and suppressing the immune response. These cells are aptly termed 'regulatory B cells' or 'Bregs' and carry out their function by multiple mechanisms that include production of anti-inflammatory cytokines and cognate interaction with other immune cells. Bregs are able to restore an immune response to homeostatic levels and as such play crucial roles in health conditions requiring suppression of inflammation and/or maintenance of tolerance such as in autoimmunity, graft tolerance, allergy, infection, cancer and pregnancy. Understanding how these potent and dynamic cells can be controlled in various immune situations offers a promising avenue for the development of novel prophylactic and immune therapy treatments.

Introduction

B lymphocytes (or B cells) are best known for their ability to produce antibodies following exposure to disease or vaccination. B cells also present antigens via MHC (major histocompatibility complex) class II to T cells to ensure optimal T-cell activation and effector responses. Recognition of antigens occurs by interaction with a specific B-cell receptor, which drives activation, clonal expansion and differentiation into antibody-producing plasma cells. These effector cells secrete antibodies with the same specificity as the B-cell receptor and are therefore specific for the original activating antigen. Some activated B cells will become memory cells that provide long-lasting immunity. Thus, B cells can positively regulate immune responses and inflammation via antibody production and antigen presentation for optimal T-cell activation. However, B-cell subsets with negative regulatory properties have also been reported. Initially called 'suppressor B cells' owing to their ability to limit proliferation and activation of lymphoid cells, they were first observed as immune cells that could inhibit delayed-type hypersensitivity skin reactions in guinea pigs (Katz *et al*., 1974). These suppressor cells were further defined in B-cell-deficient murine models of the autoimmune disease multiple sclerosis (MS) (known as experimental autoimmune encephalomyelitis or EAE). Here, the absence of B cells did not affect the incidence rate of EAE, but rather led to exacerbated forms of disease and the inability to recover, which clearly suggested a role for B cells in the immune regulation of EAE (Wolf *et al*., 1996). The formal description of this new B-cell subset came shortly thereafter in a murine study of chronic colitis, where the B-cell subset capable of suppressing inflammation *in vivo* were designated as 'regulatory B cells' or 'Bregs' (Mizoguchi *et al*., 2000). In the ensuing years, a plethora of studies have shown a role for Bregs in various autoimmune diseases and disorders, transplantation cases, allergy, infections, cancer and pregnancy. Bregs are found to have a profound effect on the immune response, despite making up less than 10% of B cells in circulation, and are capable of maintaining homeostasis by down-regulating active immune responses. **See also**: **B Lymphocytes**; **B Lymphocytes: Receptors**

eLS subject area: Immunology

How to cite:
Guzman-Genuino, Ruth Marian and Diener, Kerrilyn R (January 2018) Regulatory B Cells. In: eLS. John Wiley & Sons, Ltd: Chichester.
DOI: 10.1002/9780470015902.a0026235

Breg Identification

Bregs are currently defined as any B-cell population that exhibits an immunosuppressive quality. Unlike most regulatory T cells (Tregs) that can be identified by the expression of the key transcription factor Foxp3, Bregs are not yet known to possess

Table 1 Murine regulatory B-cell subsets

Subsets	Phenotype	Mechanism
B10	$CD5^{+}CD1d^{hi}$	Produce IL-10; suppress effector $CD4^{+}$ T cell and monocytes
MZ	$CD19^{+}CD21^{hi}CD23^{-}CD24^{hi}IgM^{hi}IgD^{hi}IgD^{lo}CD1d^{hi}$	Produce IL-10; induce Treg cells and suppress effector $CD8^{+}$ and $CD4^{+}$ T cells
T2-MZP	$CD19^{+}CD21^{hi}CD23^{hi}$	Produce IL-10; induce Treg cells and suppress effector $CD8^{+}$ and $CD4^{+}$ T cells
B-1a cells	$CD19^{+}CD5^{+}$	Produce IL-10; suppress effector $CD4^{+}$ T cells
TIM-1^{+} B cells	TIM-$1^{+}CD19^{+}$	Produce IL-10; suppress effector $CD4^{+}$ T cells
Plasma cells	$CD138^{+}IgM^{+}CD1d^{hi}CXCR4^{+}$MHC-$11^{lo}$	Produce IL-10 and IL-35; suppress NK cells, neutrophils and effector $CD4^{+}$ T cells
Plasmablasts	$CD138^{+}CD44^{hi}$	Produce IL-10; suppress DCs and effector $CD4^{+}$ T cells
GIFT-15 B cells	$B220^{+}CD21^{+}CD22^{+}CD23^{+}CD24^{+}CD1d^{+}CD138^{+}IgD^{+}IgM^{+}$	Produce IL-10; suppress IFN-γ response
PD-L1hi B cells	PD-$L1^{hi}CD19^{+}B220^{+}$	Regulate humoural responses through cognate interactions with PD-L1
Killer B cells	$CD19^{+}CD5^{+}CD178^{+}$	Induce cell death through FasL

Table 2 Human regulatory B-cell subsets

Subsets	Phenotype	Mechanism
B10	$CD24^{hi}CD27^{+}$	Produce IL-10; suppress effector $CD4^{+}$ T cells and monocytes
Transitional B cells	$CD19^{+}CD24^{hi}CD38^{hi}$	Produce IL-10; induce Tregs, suppress Th1 and Th17 cells
Memory B cells	$CD19^{+}CD27^{+}IgM^{+}$	Produce IL-10; suppress $CD4^{+}$ T-cell proliferation and effector function
Plasmablast	$CD19^{+}CD38^{hi}CD24^{hi}CD27^{int}$	Produce IL-10; suppress DCs and effector $CD4^{+}$ T cells
GrB+ B cells	$CD19^{+}CD38^{+}CD1d^{hi}IgM^{+}CD147^{+}GrB^{+}$	Express granzyme B; suppress T-cell proliferation
Br1 cells	$CD19^{+}CD25^{hi}CD71^{hi}CD73^{lo}$	Produce IL-10 and IgG4
TIM-1+ B cells	$CD19^{+}$TIM-1^{+}	Produce IL-10; suppress T-cell function

an equivalent unique unifying characteristic. Production of the anti-inflammatory cytokine interleukin-10 (IL-10) is most often used as a definitive Breg marker; however certain Breg subsets can induce negative regulation via IL-10-independent pathways. Of note, a Foxp3-negative CD4-positive type-1 regulatory T (Tr1)-cell subset has been described. These cells express high levels of IL-10 in an antigen-specific manner and are generated in the periphery after chronic activation of $CD4^{+}$ T cells in the presence of IL-10. Like Bregs, no unique marker has been found to identify Tr1 cell populations to date (Zeng *et al*., 2015). **See also**: **Regulatory T Cells**

In mice, the following B-cell subsets have been shown to suppress inflammation: IL-10-producing B cells (B10 cells), marginal-zone (MZ) B cells, transitional 2 marginal-zone precursor (T2-MZP) cells, follicular (FO) B cells, B-1a cells, killer B cells, GIFT-15 B cells, plasma cells, plasmablasts, TIM-1^{+} B cells and PD-$L1^{hi}$ B cells (**Table 1**). Bregs in humans include immature or transitional B cells, B10 cells, memory B cells, plasmablasts, GrB^{+} B cells, Br1 cells and TIM-1^{+} B cells (**Table 2**). The heterogeneity of these phenotypes suggests that Bregs are not derived from one lineage, but may acquire their suppressive ability through exposure to appropriate environmental cues. Breg activation is dependent on the engagement of activating molecules such as toll-like receptors (TLRs), CD40, B-cell receptor, CD80, CD86 and cytokine receptors (Baba *et al*., 2015). Whilst the search for a global identifier of Bregs remains ongoing, T-cell immunoglobulin and mucin domain 1 (TIM-1) has been proposed as an inclusive marker for murine Bregs, as it identifies about 70% of all IL-10^{+} B cells in all subsets mentioned (Ding *et al*., 2011).

Mechanisms of Suppression

Bregs can modulate many different types of immune cells, with their effect on the function and plasticity of T cells best studied. Research across various fields have established that Bregs can inhibit cytotoxic $CD8^{+}$ T cells; hinder activation of naïve $CD4^{+}$ T cells; promote the generation of $Foxp3^{+}$ Tregs and Tr1 cells; suppress helper T (Th) cell differentiation; inhibit the activation of monocytes and plasmacytoid dendritic cells (pDC); maintain the immature state of dendritic cells and stimulate natural killer T-cell activity (**Figure 1**). In most cases, Breg function is attributed to two or more mechanisms, indicating that Bregs employ a multimechanistic and likely cooperative approach for regulating immune responses. **See also**: **T Lymphocytes: Helpers**

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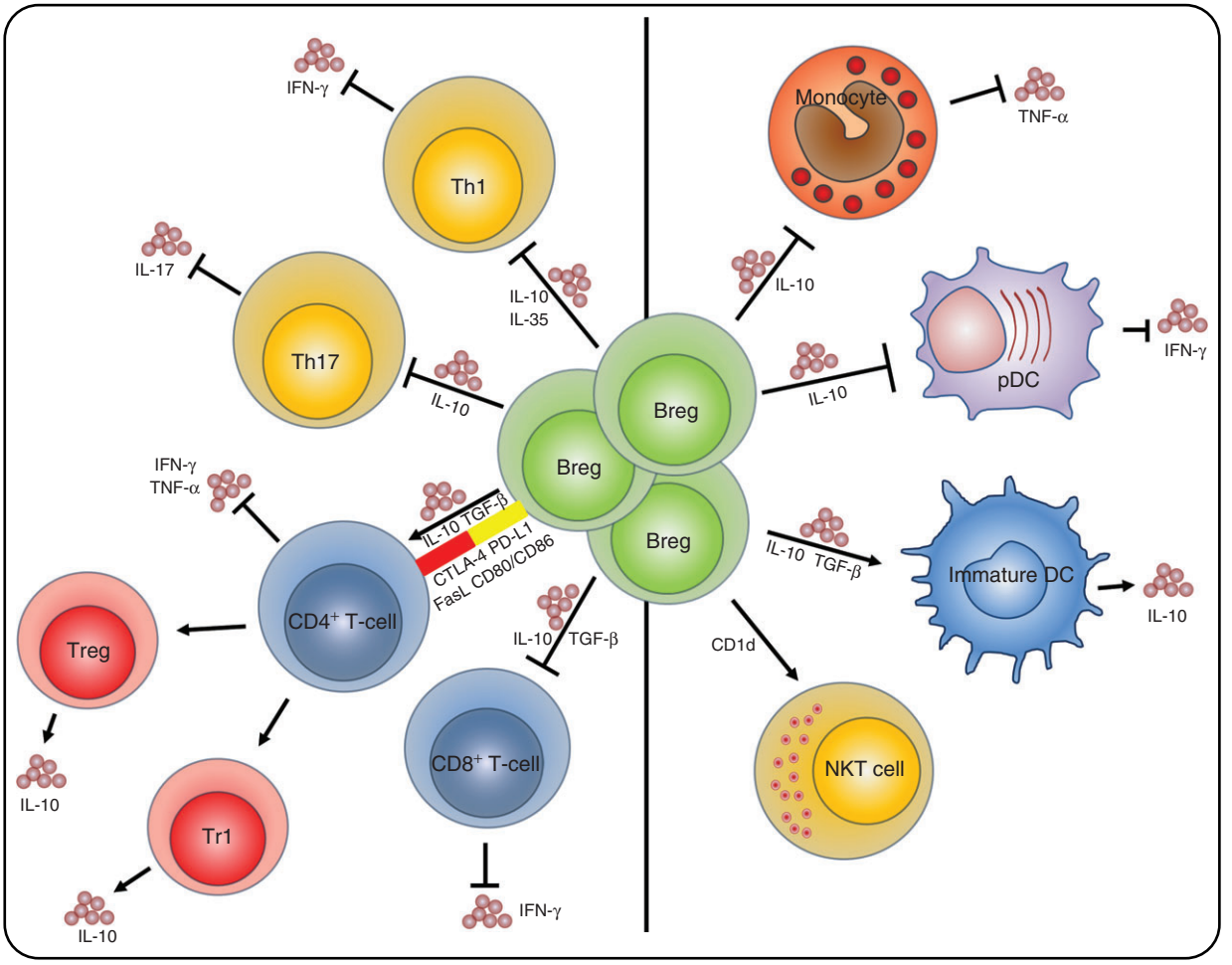


Figure 1 Mechanisms of Breg suppression. Bregs inhibit Th1 and Th17 responses, inducing a Th2-like bias to the T-cell response; Bregs also inhibit cytotoxic $CD8^+$ T cells, curtail effector $CD4^+$ T-cell proliferation, and abrogate the proinflammatory cytokine response, pushing instead the differentiation of $CD4^+$ T cells into Tregs and Tr1s (left panel). Bregs likewise affect other immune cells such as monocytes, dendritic cells and natural killer cells (right panel).

Cytokine production

Most studies confirm that production of IL-10 by Bregs is necessary for immune suppression. B10 cells constrain inflammation and maintain homeostasis by influencing T-cell processes, mainly through suppressing T-cell proliferation and inhibiting production of the proinflammatory cytokines interferon-gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α). B10 cells also inhibit $CD8^+$ effector T-cell responses and convert $CD4^+$ T cells into Tregs and Tr1 cells. For instance, in murine studies of chronic hepatitis B virus (HBV) infection, B10 cells are shown to inhibit the IFN-γ production by virus-specific $CD8^+$ T cells. Further, immune tolerance to chronic HBV infection in humans is associated with Breg-mediated suppression of $CD4^+$ Th cells and downregulation of the Th17 response, concurrent with enhanced recruitment and function of Treg and Tr1 cells (Das *et al*., 2012; Liu *et al*., 2016). Murine studies have also shown that B10 cells can affect innate immune cells by limiting monocyte and pDC activation and function and downmodulating the antigen-presenting capacity of dendritic cells (Matsushita *et al*., 2010; Menon *et al*., 2016).

While IL-10 production remains the hallmark of Bregs' negative regulatory function, other cytokines produced by Bregs have also been shown to perform a suppressive role. These include transforming growth factor beta (TGF-β), IL-35 and IL-17. TGF-β production by B cells leads to the promotion of Treg development in diabetes, transplant tolerance, allergic diseases and colitis (Ray *et al*., 2015). B10 cells in mice that also express IL-35 are notable in EAE and *Salmonella* infection, as IL-35 plays a key role in the recovery phase of the former and in improved resistance to the latter (Shen *et al*., 2014). IL-17 secretion by activated B cells is a crucial countermeasure for *Trypanosoma* infection. Trans-sialidase, the parasite cell surface enzyme necessary for *Trypanosoma* invasion, has been shown to trigger the secretion of IL-17A and IL-17F by B cells, an immunosuppressive mechanism requisite for pathogen control and importantly, resolution of infection (Bermejo *et al*., 2013).

Cell–cell contact

Crosstalk between Bregs and T cells mediated by costimulatory molecules CD80 and CD86 can inhibit Th1 and Th17 proinflammatory responses and thus facilitate immunosuppression. Moreover, direct Breg-T-cell contact via CD80 and CD86 can promote tolerance by regulating T-cell activation and expanding Treg populations. Other surface molecules on B cells considered instigators of negative regulation include programmed death receptor ligand 1 (PD-L1), granzyme B and Fas ligand (FasL). PD-L1-expressing B cells can transduce an inhibitory signal to $CD8^+$ T cells via interaction with the programmed death 1 (PD-1) ligand expressed on the cell surface of T cells. In EAE, adoptive transfer of PD-$L1^+$ B cells limited the Th1 response and induced Treg differentiation, thus mitigating the severity of disease (Bodhankar *et al*., 2013). B cells expressing FasL are shown to play a major role in inhibiting graft rejection and ameliorating autoimmune diseases by promoting apoptosis of autoreactive T cells and expanding Tregs (Lundy and Fox, 2009). Lastly, IL-21 induced granzyme B expression in B cells is demonstrated to be pivotal in inhibiting effector $CD4^+$ T-cell function in the tumour microenvironment (Lindner *et al*., 2013).

Bregs in Autoimmunity

The importance of Bregs was first realised in the studies of autoimmune pathologies where deficiencies in the frequency and function of these cells were linked to aggravation of the disease state. For instance, some autoimmune disease patients undergoing B-cell depletion therapy with rituximab (anti-CD20 antibody) have shown exacerbation of disease or the onset of a new immune-related pathology, a result attributed to severe suppression of IL-10 production (El Fassi *et al*., 2008; Goetz *et al*., 2007). These studies reinforce the notion that certain B-cell subsets play a major role in managing autoimmune diseases. **See also**: **Autoimmune Disease: Mechanisms**

Multiple sclerosis

Clinical studies show that the frequency and dysregulation of Breg function contribute to the disease activity of MS. MS patients possess B cells with a diminished capacity for IL-10 production on activation, compared to normal healthy individuals (Duddy *et al*., 2007). Furthermore, patients experiencing relapses demonstrate a decreased frequency of Bregs compared to patients in remission and healthy controls (Knippenberg *et al*., 2011). Studies of murine EAE have shown that Bregs can regulate disease severity through IL-10 production and expression of costimulatory molecules CD40, CD80 and CD86. These mechanisms limit the type-1 cytokine response resulting in decreased IFN-γ and TNF-α production and facilitate the recruitment of $Foxp3^+$ Tregs into the central nervous system (Wolf *et al*., 1996; Mann *et al*., 2007). Further, engagement of TLR-2 and TLR-4 triggers the regulatory function in B cells, which results in the suppression of Th1 and Th17 inflammatory responses and promotion of recovery from EAE (Lampropoulou *et al*., 2008). Preclinical murine studies demonstrate that IFN-β therapy is effective in ameliorating EAE in normal mice as IFN-β expands regulatory B-cell populations and boosts IL-10 secretion, a result which cannot be recapitulated in B-cell-deficient mice, thus reiterating the Breg-dependent pathway for recovery (Schubert *et al*., 2015). **See also**: **Multiple Sclerosis**

Systemic lupus erythematosus

Whilst patients with systemic lupus erythematosus (SLE) have similar levels of circulating immature $CD24^{hi}CD38^{hi}$ B cells compared with normal healthy controls, the IL-10-producing subset of these B cells is significantly reduced. B cells isolated from these patients are unresponsive to CD40 ligation and stimulation, which inhibited their ability to differentiate into Bregs, and resulted in a significant impairment in the capacity for immune regulation (Gao *et al*., 2014; Blair *et al*., 2010). Further, the regulatory feedback loop between Breg-inducing pDCs and B cells is disrupted in SLE such that pDCs are unable to drive the differentiation of immature B cells into IL-10-producing $CD24^+CD38^{hi}$ Breg cells (Menon *et al*., 2016). These recent developments make Bregs an integral component in the pathogenesis of SLE. **See also**: **Systemic Lupus Erythematosus**

Rheumatoid arthritis

Rheumatoid arthritis (RA) studies in mice expressing a B-cell-restricted IL-10 deficiency ($IL\text{-}10^{-/-}$ B cells) indicated that IL-10 production by B cells is critical in the primary induction and recruitment of Tregs and suppression of Th1 and Th17 differentiation, thereby promoting the amelioration of disease (Carter *et al*., 2012). In RA patients, IL-10-producing $CD19^+CD24^{hi}CD38^{hi}$ B cells restrict the differentiation of naïve T cells into Th1 cells and suppress Th1 responses, but are unable to suppress Th17 differentiation or convert naïve T cells into Tregs. Moreover, RA patients with active disease have profoundly lower Breg frequencies compared with patients in remission and healthy individuals (Flores-Borja *et al*., 2013). The reduced numbers and functional impairment of Bregs significantly impact the activity and regulation of RA. **See also**: **Rheumatoid Arthritis**

Bregs in Transplantation Tolerance

Renewed interest in the role of Bregs in transplant tolerance has been encouraged by cellular and gene expression profiling of operationally tolerant kidney transplant patients who, despite stopping all immunosuppression medication, do not reject their donated kidney. Immune profiles from these patients show significantly higher frequencies of naive and transitional $CD24^{hi}CD38^{hi}$ B cells coupled with reduced numbers of memory B cells compared with nontolerant patients (Newell *et al*., 2015). Recently, these operationally tolerant patients have been shown to also feature higher percentages of B-cell-producing IL-10, contributing to long-term allograft acceptance (Nova-Lamperti *et al*., 2017). Mouse transplantation studies have shown that Bregs effectively inhibit T-cell responses and actively expand Tregs through multiple mechanisms. For instance, IL-10- and IL-4-expressing

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TIM-1$^+$ Bregs are found to circulate within islet allografts in chemically induced diabetic recipient mice. Adoptive transfer of these TIM-1$^+$ B cells into diabetic B-cell-deficient allograft recipients prolonged graft survival primarily via IL-10-mediated activation of Tregs (Ding *et al*., 2011). Adoptive transfer of TGF-β-producing Bregs also protracted islet allograft survival by inducing Foxp3 expression in CD4$^+$CD25$^-$ T cells (Lee *et al*., 2014). In a murine MHC class I mismatched skin transplantation model, tolerised B cells exhibited regulatory properties and prolonged skin allograft survival via upregulated levels of TIM-1, downregulated costimulatory CD86 expression and cooperative crosstalk with graft-specific Tregs (Moreau *et al*., 2015). **See also**: **Graft Rejection: Immunological Suppression**

Bregs in Allergy

Bregs are strongly implicated in the development of allergen tolerance, as patients with allergic asthma and allergic rhinitis exhibit reduced frequencies of IL-10-producing CD24^{hi}CD27$^+$ Bregs, as well as Bregs with impaired capacity for IL-10 production (Kamekura *et al*., 2015; van der Vlugt *et al*., 2014a). Moreover, functional B10 cells produce about 15-fold more of allergen-specific IgG4 (antibodies that suppress allergy-induced symptoms) than non-IL-10-producing B cells. Patients allergic to phospholipase A2 (PLA), the major bee venom allergen, who successfully completed allergen-specific desensitisation therapy displayed up to a fivefold increase in PLA-specific B cells characterised by high production of IL-10 and mature IgG4 mRNA (messenger ribonucleic acid). These resultant levels were comparable to that of healthy beekeepers (van de Veen *et al*., 2013). Further, in murine asthma models where initial allergic airway disease can be resolved by ongoing chronic antigen exposure, local inhalation tolerance can be transferred to allergic recipients by adoptive transfer of TGF-β-producing CD5$^+$CD1d^{hi} B cells. Tolerance could only be transferred by Breg cells obtained from lung lymph nodes, suggesting that tolerance induction was locally induced and maintained after chronic antigen exposure. These Bregs were able to induce Foxp3 expression in CD4$^+$ T cells *in vitro*, and were found to be co-localised with Tregs *in vivo* in local lung lymph nodes (Natarajan *et al*., 2012). In a similar manner, TGF-β produced by CD5$^+$CX3CR1$^+$ Bregs is also pertinent in promoting the differentiation of Tregs and suppressing T-cell proliferation in food allergy-related intestinal inflammation (Liu *et al*., 2013a).

Bregs in Infections

Bregs have been implicated in the pathogenesis of bacterial, viral and parasitic infections. In general, Breg numbers are positively correlated with bacterial or viral load, as their suppressive mechanisms can alter the immune response in favour of pathogen survival, thus resulting in a worse prognosis.

In viral infections, studies on human immunodeficiency virus (HIV) and HBV have shown that Bregs can regulate disease-specific CD8$^+$ T cells, induce and expand Treg populations and impair helper T-cell function through IL-10 production and PD-L1 cognate interactions (Das *et al*., 2012; Siewe *et al*., 2013). This deleterious effect of Bregs was recently highlighted in studies of HIV-infected individuals, whose increased circulating Breg and Treg frequencies were associated with poor immune responses to influenza vaccination (Weinberg *et al*., 2013).

In bacterial infections, lipopolysaccharide and lipoteichoic acid found in the outer membranes of Gram-negative and Gram-positive bacteria, respectively, have the capacity to engage B cells via TLR (TLR 2, 4, 5, 7/8 and 9) expression. Following TLR activation, populations of B cells such as CD5$^+$, CD138$^+$ and MZ B cells can produce IL-10 and interact with T cells and macrophages in a negative regulatory manner. Bregs can thus facilitate pathogen survival and increase disease severity. For instance, *Salmonella typhimurium* infection induces IL-10 production in CD138$^+$ B cells which suppresses effector cells such as natural killer cells, neutrophils and effector T cells (Neves *et al*., 2010). Susceptibility to *Listeria* is also supported by the high production of IL-10 by marginal zone B cells encountered by the bacteria in the spleen (Lee and Kung, 2012).

Lastly, the persistence of helminth and protozoan infections is largely owing to the reduced or lack of inflammatory responses necessary for eradication. This is owing in part to the capacity of regulatory cells to polarise the Th1/Th2 cytokine balance towards a more Th2-like phenotype. Exposure of B cells to protozoan and helminth infections leads to induction of regulatory properties and consequent suppression of Th1 function, as seen in cases of *Schistosoma* and *Leishmania* infections. *Schistosoma*-induced Bregs secrete IL-10 and increase Th2 responses while inhibiting the production of proinflammatory cytokines IFN-γ, IL-4 and IL-17 (van der Vlugt *et al*., 2014b). Protozoan parasite *Plasmodium berghei*, the causative agent of malaria, has also been shown to expand B10 cells that in turn reduce populations of natural killer cells and cytotoxic CD8$^+$ T cells to aid survival (Liu *et al*., 2013b).

Bregs in Cancer

Cancer escape occurs when the immune system fails to eradicate individual mutated cells. In the local microenvironment, the presence of regulatory cells such as Bregs can aid tumour growth by the suppression of effector immune cells. The presence of Bregs can therefore be indicative of enhanced tumour aggressiveness and poorer prognosis. As such, interest in the role of Bregs in cancer has continued to grow, and has led to the identification of a B-cell subset termed tumour-evoked Bregs (tBregs). These cells were initially found in mice harbouring 4T1 carcinoma cells (Olkhanud *et al*., 2011). These circulating cancer-specific B cells were shown to be recruited and 'hijacked' by resident cancer cells and turned into immunosuppressive Bregs. These tBregs are not of any established Breg phenotype, however they do resemble activated mature B2 cells (CD19$^+$CD25^{hi}CD69^{hi} and B7-H1^{hi}CD81^{hi}CD86^{hi}CD62L^{lo}IgM^{int}). tBregs are characterised by the high expression of surface markers CD40, CD80, CD86 and MHC class I and II molecules. TGF-β production by tBregs and their costimulating cognate interactions with CD4$^+$ T cells can generate CD4$^+$CD25$^+$Foxp3$^+$ Tregs within the tumour microenvironment, which in turn inhibits CD8$^+$ T-cell

proliferation. Similarly, B cells infiltrating mammary tumours were shown to be 'educated' to exhibit a suppressive phenotype following cognate interactions with resident tumour cells. These B cells express both PD-L1 and TGF-β which support their immunosuppressive functions (Zhang *et al*., 2016). In humans, patients with hepatocellular carcinoma, tongue squamous cell carcinoma, gastric cancer, nonsmall cell lung cancer and colorectal cancer possess more peripheral Bregs compared with healthy controls. Bregs also preferentially accumulate at the tumour site rather than within unaffected neighbouring tissues (Sarvaria *et al*., 2017). Clinically, both IL-10 and TGF-β play key roles: $CD19^{+}CD24^{hi}CD38^{hi}$ Bregs isolated from gastric cancer patients were found to induce and expand $CD4^{+}Foxp3^{+}$ Tregs via TGF-β (Wang *et al*., 2015); while IL-10-mediated crosstalk between B10 and $CD4^{+}CD25^{-}$ T cells in tongue squamous cell carcinoma patients led to the differentiation of resting T cells into Tregs (Zhou *et al*., 2016). **See also**: **Tumour Immunology**

Bregs in Pregnancy

Bregs are increasingly implicated as having a role to play in the maintenance of pregnancy; primarily via production of IL-10, a cytokine essential in preventing foetal loss and growth restriction. In spontaneous abortion-prone murine models, a reduced frequency of B10 cells is found within the maternal spleen compared to normal pregnancies, which suggests that Bregs play a critical role in supporting maternal tolerance and therefore pregnancy success. Further, adoptive transfer of syngeneic B10 cells into abortion-prone mice rescued pregnancies by inhibiting dendritic cell maturation and expanding Tregs (Jensen *et al*., 2013). Marginal zone B-cell numbers are also increased in the spleens of normal pregnant mice, but are lacking in mice exhibiting pregnancy pathologies. Moreover, the success of the pregnancy correlated with the capacity of MZ B cells to produce enhanced levels of IgM and IgA, immunoglobulins essential in shifting the immune response from a Th1-like to a Th2-like profile (Muzzio *et al*., 2016).

Similar patterns of Breg expression are observed in humans wherein numbers of $CD24^{hi}CD27^{+}$ B cells in the peripheral blood of normal pregnant women in their first trimester are significantly higher compared to nonpregnant women or women who experienced spontaneous abortions (Rolle *et al*., 2013). In contrast, $CD24^{hi}CD38^{hi}$ Bregs are significantly lower during the third trimester of pregnancy and on delivery day compared to nonpregnant and postpartum controls, suggesting a diminished role as the body induces the inflammatory state necessary for parturition (Lima *et al*., 2016). **See also**: **Fetal-Maternal Immunological Relationships**; **Reproductive Immunology**

Use of Bregs in Immune Therapy

As negative regulators of immune responses and promoters of immunological tolerance, the use of Bregs to prevent or treat various immune conditions clinically is theoretically a viable and conceivable initiative. In circumstances where inflammation is excessive such as in autoimmune diseases, transplantation and allergy, induction and expansion of these immunosuppressive cells may restore homeostasis. In cancer and infections, however, Breg depletion, rather than expansion may be the required approach. Therefore, the use of Bregs for immune therapy requires careful calibration in order to achieve the desired outcome.

Currently, many immune therapies employed in treating diseases are nonselective. In autoimmunity, rituximab-mediated B-cell depletion is used as a successful method of treating disease, however as it targets all B cells, beneficial Bregs are also depleted which can result in the generation of new inflammatory conditions (El Fassi *et al*., 2008; Goetz *et al*., 2007). Natalizumab, a humanised monoclonal antibody against the integrin α_4 (Itga4) currently used as an effective MS-modifying therapy, has been shown to neutralise B cells, including Bregs, in peripheral lymphoid organs leading to the development of secondary progressive multifocal leukoencephalopathy (Glatigny *et al*., 2016). In cancer, Bregs have been found to accumulate specifically in the tumour-draining lymph nodes, but in some cases, they neither produce IL-10 nor recruit Tregs, instead they inhibit T-cell function via PD-L1 and CD39 cognate interactions which facilitate tumour progression (Ganti *et al*., 2015). Nonselective depletion will obliterate the Bregs, however it will also deplete effector B cells that eradicate tumours. Targeted cell therapy where specific B-cell subsets are selectively depleted will address these issues; however the lack of specific cell markers for efficient identification and isolation of all subsets presents a challenge to therapy development. In addition, the diversity of Breg subsets across different disease conditions suggests that Breg differentiation and function are context-dependent, thus any immune therapy needs to be tailored to the specific disease of interest.

Currently, amassed knowledge of Breg induction and function has led to many prospective observational studies exploring the relevance of Bregs in diseases such as SLE and thymoma. Results are eagerly awaited as these endeavours and future research insights will herald further understanding of context-dependent Breg physiology, information requisite in designing novel disease-specific therapeutic approaches.

Glossary

Adoptive transfer The method of transferring donor immune cells of interest into a recipient patient or specimen.

Allograft Transplant of cells, tissue, or organ from one individual to another of the same species.

Autologous Cells or tissues that are obtained from the same individual.

Leukoencephalopathy A progressive neurological disorder describing the deterioration of the brain and spinal cord's white matter.

Marginal zone Region at the interface between the non-lymphoid red pulp and the lymphoid white-pulp of the spleen.

Operational tolerance Ability of recipients to maintain graft function without the need for immunosuppressive medication.

Semi-allogeneic Partial sharing of genes, as in the case of an offspring borne through sexual reproduction that carries genes from both the mother and the father.

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frontiers in Immunology

ORIGINAL RESEARCH
published: 11 December 2019
doi: 10.3389/fimmu.2019.02899

Uterine B Cells Exhibit Regulatory Properties During the Peri-Implantation Stage of Murine Pregnancy

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OPEN ACCESS

Edited by:
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Reviewed by:
Damián Muzzio, University of Greifswald, Germany
Anne Schumacher, Otto von Guericke University Magdeburg, Germany

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Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *10 September 2019*
Accepted: *26 November 2019*
Published: *11 December 2019*

Citation:
Guzman-Genuino RM, Eldi P, Garcia-Valtanen P, Hayball JD and Diener KR (2019) Uterine B Cells Exhibit Regulatory Properties During the Peri-Implantation Stage of Murine Pregnancy. Front. Immunol. 10:2899. doi: 10.3389/fimmu.2019.02899

A successful outcome to pregnancy is dependent on the ability of the maternal uterine microenvironment to regulate inflammation processes and establish maternal tolerance. Recently, B cells have been shown to influence pregnancy outcomes as aberrations in their numbers and functions are associated with obstetric complications. In this study, we aimed to comprehensively examine the population frequency and phenotypic profile of B cells over the course of murine pregnancy. Our results demonstrated a significant expansion in B cells within the uterus during the peri-implantation period, accompanied by alterations in B cell phenotype. Functional evaluation of uterine B cells purified from pregnant mice at day 5.5 post-coitus established their regulatory capacity as evidenced by effective suppression of proliferation and activation of syngeneic $CD4^+$ T cells. Flow cytometric analysis revealed that the uterine B cell population has an expanded pool of IL-10-producing B cells bearing upregulated expression of co-stimulatory molecules CD80 and CD86 and activation marker CD27. Our investigations herein demonstrate that during the critical stages surrounding implantation, uterine B cells are amplified and phenotypically modified to act in a regulatory manner that potentially contributes toward the establishment of maternal immunological tolerance in early pregnancy.

Keywords: uterine B cells, regulatory B cells, implantation, early pregnancy, suppression

INTRODUCTION

Pregnancy is a model of immune tolerance wherein the mother carries a semi-allogeneic fetus that in principle triggers a maternal immune response due to the presence of paternally derived fetal alloantigens; however the inflammation is kept at a tolerable level and the pregnancy progresses. In early pregnancy, it is well-accepted that for implantation to occur, a regulated pro-inflammatory microenvironment that permits tissue remodeling and angiogenesis at the maternal-fetal interface is required. This is then followed by a shift to an immune tolerogenic profile that allows for fetal growth and development (1, 2). The necessity of a controlled inflammatory environment during this time implies a dynamic feto-maternal crosstalk of immune, endometrial, and fetal cells. A multitude of preclinical and clinical pregnancy studies show immune cells such as uterine dendritic

cells, decidual macrophages, decidual natural killer cells, and regulatory T cells undergo changes during pregnancy for the purposes of calibrating inflammation and mediating maternal immune tolerance (3). For B cells, their role in the induction of maternal immune tolerance in pregnancy to date remains poorly understood.

Although studies on B cells in pregnancy are limited, there is an emerging view of their involvement in the physiology of pregnancy. Studies on murine models have demonstrated that percentages of B cell subsets are altered throughout gravidity. In the bone marrow and the spleen, murine B cells undergo lymphopenia mid-pregnancy as numbers of bone marrow pre/pro and immature B cells as well as splenic follicular B cells drop substantially at day 14 post-coitus (4). However, splenic marginal zone B cells and mature B cells in the para-aortic lymph nodes that drain the uterus were also reported to expand (4, 5). A normal pregnancy was also associated with an enhanced production of immunoglobulins by marginal zone B cells, speculated to reduce the occurrence of autoreactive B cells that may cause pregnancy complications (5). Similarly, changes in the proportions of B cell subsets during pregnancy have been documented in the clinical setting. Analysis of peripheral blood showed a significant decrease in the percentages of $CD19^{+}CD24^{bright}CD38^{bright}$ transitional B cells during normal pregnancy and relatively higher percentages of non-switched memory B cells were associated with recurrent miscarriages (6, 7).

Recently, there has been interest in B cells taking on a regulatory role during pregnancy as a specific subset of B cells called "regulatory B cells" or Bregs have been implicated in the network of immune cells crucial for maintaining pregnancy. Interestingly, Bregs do not have a specific phenotypic marker, rather they are defined by their functional capacity for suppressing other immune cells (8). Among the known Breg subtypes, IL-10-producing B cells or B10 cells are the most commonly studied. Adoptive transfer of B10 cells induced from splenic B cells was previously shown to restore tolerance and rescue pregnancies in abortion-prone mice (9). Peritoneal $CD19^{+}IL\text{-}10^{+}$ cells were also shown to increase throughout normal pregnancy whereas a significant reduction was seen in abortion-prone pregnant females (10). Additionally, clinical studies correlate inadequate number of circulating IL-10-producing $CD24hiCD27^{+}$ B cells with higher risk of spontaneous abortion (11). These studies however have been restricted to B cells in the spleen, peritoneum, and in the blood while studies of B cell function within the pregnancy microenvironment is noticeably lacking. The main hindrance to studying the B cells in the uterine mucosa and the decidua stem from the paucity of B cells within these tissues. Uterine cell profiling of the murine estrous cycle showed that B cells constituted 1.5% of viable cells at estrus (12). In humans, B cells are considered rare lymphocyte subpopulations in the uterus with a median of 2% in pre-implantation uterine tissue and 5% in the decidua (13, 14). Moreover, the functional promiscuity of B cells also poses an added layer of complexity in describing the specific role of B cells in pregnancy.

In the present work, we used a murine C57Bl/6J x BALB/c semi-allogeneic pregnancy model to describe the phenotypes and functions of B cells within the uterine microenvironment throughout gestation. Our results indicate that these uterine B cells undergo changes in frequency and phenotype, particularly during the peri-implantation phase of pregnancy between days 2.5 and 8.5 post-coitus (pc). B cells were shown to be significantly expanded as well as express markers and cytokines indicative of regulatory qualities. Furthermore, we show in *ex vivo* experiments that uterine B cells collected from pregnant females at day 5.5 pc significantly suppressed proliferation and activation of syngeneic $CD4^{+}$ T cells via cell-cell interactions. We thus posit that uterine B cells at peri-implantation exhibit immunosuppressive characteristics and likely take part in fostering the generation of maternal immune tolerance during early pregnancy.

MATERIALS AND METHODS

Animals

All mice were housed in a specific-pathogen free (SPF) animal facility with optimal lighting and food and water *ad libitum*. Female C57Bl/6J mice (6–9 weeks of age) were checked for estrous cycle stage by vaginal flushing with 20 μL sterile PBS and assessing the types and ratios of cells present by bright field microscopy. Females in proestrus or early estrus stages were placed with a BALB/c stud for mating overnight. The following day, female mice were visually inspected for the presence of a vaginal copulation plug indicative of a successful mating event. This was designated day 0.5 post-coitus (d0.5 pc). Virgin age-matched females at estrus were used as experimental controls.

Tissue Dissociation and Cell Purification

At time points indicated for each experiment, the following organs were collected: spleen, the uterus draining para-aortic lymph nodes (PALNs), and uterus.

Single cell preparations from the spleen and lymph nodes were isolated by standard methods. Briefly, using the plunger of a small syringe, spleens, or lymph nodes were crushed through a 70 μm cell strainer. The resultant single cell suspension was spun (300 × g, 5 min), and resultant pellet resuspended in 1 mL of ammonium-chloride-potassium (ACK) lysis buffer for 5 min to eliminate red blood cells. RPMI culture medium supplemented with 2% fetal bovine serum (FBS) was then added to neutralize the lysis buffer. Splenocytes were washed twice with PBS, and resuspended at 10^{7} cells/mL.

Cells were extracted from uterine tissue using the gentleMACS^{TM} Octo Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Uterine tissue was dissected into small pieces then transferred into gentleMACS^{TM} C tubes suspended in RPMI culture medium supplemented with Collagenase A (10 mg/mL, Roche, Basel, Switzerland) and DNAse I (280 U per sample, Sigma-Aldrich, MO, USA) for efficient connective tissue digestion. Heating adaptors were used for optimal temperature control of tissue dissociation. The

resultant homogenized tissue suspension was passed through a 70 μm cell strainer, resuspended in culture medium and washed twice with PBS. Collected cells were resuspended at 10^7 cells/mL.

In experiments requiring B or T cells, the harvested mixed cells were further purified by magnetic isolation. B or T cells were negatively selected for using the MojoSort™ Mouse Pan B Cell Isolation Kit (480052; Biolegend, San Diego, CA, USA) or MojoSort™ Mouse CD4 T Cell Isolation Kit (480033; BioLegend) according to the manufacturer's instructions. Isolated B cells or $CD4^+$ T cells were routinely achieved >90% purity as assessed by flow cytometry (**Supplementary Figure 1**).

For uterine tissue, the resultant cell suspension was briefly incubated with anti-mouse CD16/32 (FC block; eBioscience, San Diego, CA, USA) for 15 min at 4°C to block Fc receptors, washed, then stained with conjugated monoclonal antibody anti-mouse CD19-APC-Cy7 (BD Biosciences, NJ, USA) for 40 min in the dark on ice. $CD19^+$ B cells were subjected to more restrictive FSC-W × FSC-H and SSC-W × SSC-H assessment to ensure single cells were purified by sorting on a FACS Aria II (BD Biosciences). A 100 μm nozzle at a flow rate of 2,000 events/s generated a sorting efficacy between 95 and 100%. Sorted uterine $CD19^+$ B cells were used in the suppression assay described below.

Phenotypic Analysis by Flow Cytometry

Multi-parameter flow cytometry was used to identify the expression of B cell phenotype markers in cells isolated from the organs of interest. Harvested cells were collected, washed, and then incubated with anti-mouse CD16/32 for 15 min on ice. After washing once, cells were stained with a cocktail of monoclonal antibodies diluted in buffer for 40 min. Rat/Armenian hamster anti-mouse monoclonal antibodies used are as follows: CD19-APC-Cy7 (1D3; BD Biosciences), B220-APC (RA3-6B2; BioLegend), CD1d-FITC (1B1; BD Biosciences), CD5-BV510 (53-7.3; BD Biosciences), CD38-BV421 (90/CD38; BD Biosciences), CD138-BV510 (281-2; BD Biosciences), TIM-1-BV421 (RMT 1-4; BD Biosciences), PD-L1-PE-Cy7 (MIH5; eBioscience), CD80-PE-Cy7 (16-10A1; BioLegend), CD86-AF488 (GL-1; BioLegend), CD21-BV421 (7E9; BioLegend), CD24-BV421 (M1/69; BioLegend), CD27-PE-Cy7 (LG.3A10; BioLegend), I-A/I-E-AF488 (M5/114.15.2; BioLegend), IgM-AF488 (RMM-1; BioLegend), and IgD-PE-Cy7 (11-26c.2a; BioLegend). Stained cells were washed twice with PBS then further stained with a fixable viability dye (eFluor™ 660; eBioscience) for 20 min and washed with PBS prior to flow cytometric analysis (Cytoflex; Beckman Coulter, Brea, CA, USA). Post-acquisition analysis was carried out using Kaluza analysis software (Beckman Coulter). Unstained and single color controls were used to set gates and identify the positive population (**Supplementary Figure 2**).

Phenotypes of B cell subsets were identified as follows: $CD19^+B220^+CD80^+CD86^+$ activated B cells, $CD19^+B220^+CD21^+CD27^+$ memory B cells, $CD19^+B220^+CD27^{hi}CD38^+CD138^+$ plasmablasts, and $CD19^+B220^+CD24^+IgM^{hi}IgD^{lo}$ transitional B cells.

In vitro Suppression Assay

To examine the suppressive activity of B cells collected from pregnant or virgin mice on $CD4^+$ T cells, B cells from the spleen and PALNs and T cells from a syngeneic spleen were purified by magnetic isolation while B cells from the uterus were purified by FACS sorting. Purified $CD4^+$ splenic T cells were labeled with Cell Proliferation Dye e450 (eBioscience) for 10 min at 37°C in the dark, then washed with 10% cold RPMI culture medium twice before resuspending in pre-warmed 10% RPMI culture medium supplemented with IL-2 (10 ng/ml, Peprotech, NJ, USA) and Dynabeads™ mouse T-activator CD3/CD28 (eBioscience) for T cell expansion and activation. Cells were then dispensed into a 96-well round-bottom plate at 1×10^5 cells/well, with purified B cells subsequently added to a final ratio of 0.5:1, 1:1, and 2:1 relative to T cell numbers, with unstimulated T cells and T cells with Dynabeads alone as controls. After 3 days in culture, cells were analyzed using flow cytometry to determine T cell proliferation as indicated by sequential dye dilution.

T cell proliferation was expressed as the proliferative index (PI) (15). The PI denotes the total number of divisions divided by the number of cells that went into division, and is calculated using the following formula:

$$\mathrm{PI} = \frac{(n_{pp} + \mathrm{nG1} + \mathrm{nG2} + \mathrm{nG3} + ...\mathrm{nGn})}{[(\frac{\mathrm{n}_{G1}}{2^1}) + (\frac{\mathrm{n}_{G2}}{2^2}) + (\frac{\mathrm{n}_{G3}}{2^3}) + ...(\frac{\mathrm{n}_{Gn}}{2^x})]}$$

where n = number of cells in each fluorescent peak, with the peaks identified as follows: "pp" refers to the parental undivided peak of T cells, "G1" is the first T-cell division peak, "G2" the second, "G3" the third etc. until peak differentiation is not discernible from the background fluorescence. The PI for each sample was calculated using the fitting and modeling processes of the software FCS Express on cell division profiles (DeNovo Software, California, USA).

Activation of proliferating $CD4^+$ cells was assessed by staining with CD4-FITC (RM4-5; eBioscience) and CD25-APC (PC61.5; eBioscience) antibodies for 40 min in the dark on ice and assessing expression by flow cytometric methods. Unstained, single-color controls, and FMOs were used as gating controls.

Intracellular Staining of IL-10^+ B Cells

Single cell suspensions were incubated for 5 h in a stimulation cocktail [50 ng/mL phorbol-myristate-acetate (PMA); 500 ng/mL ionomycin; 5 μg/mL lipopolysaccharide (LPS 0111:B4, Sigma)] and 1 μg/mL Brefeldin A, to induce cytokine production and inhibit Golgi transport enabling accumulation of cytokines within the cell. Cells were then washed twice and incubated with anti-mouse CD16/32 (eBioscience). Cells were washed once and stained with anti-mouse B220/CD45R-BV650 (RA3-6B2; BD Biosciences) for 40 min in the dark on ice. Post-staining, cells were fixed and permeabilized using the BD Fix/Perm Kit (BD Biosciences) as per manufacturer's instructions. Permeabilized cells were incubated with PE anti-mouse IL-10 (JES5-16E3;

BioLegend) or isotype control PE Rat IgG2b κ (RTK4530; BioLegend) for 40 min at room temperature. Lastly, cells were washed twice with Perm Buffer, resuspended in FACS buffer, and analyzed for B220$^+$IL-10$^+$ cells via flow cytometry. Matched isotype controls were used to ensure correct gating for IL-10$^+$ cells.

Generation of Induced IL-10$^+$ B Cells

Splenic B cells were magnetically isolated as above and cultured with purified anti-mouse CD40 (HM40-3; BD Biosciences) for 48 h as previously described (16). The cell culture supernatant was also collected and used for the measurement of secreted IL-10 levels by ELISA.

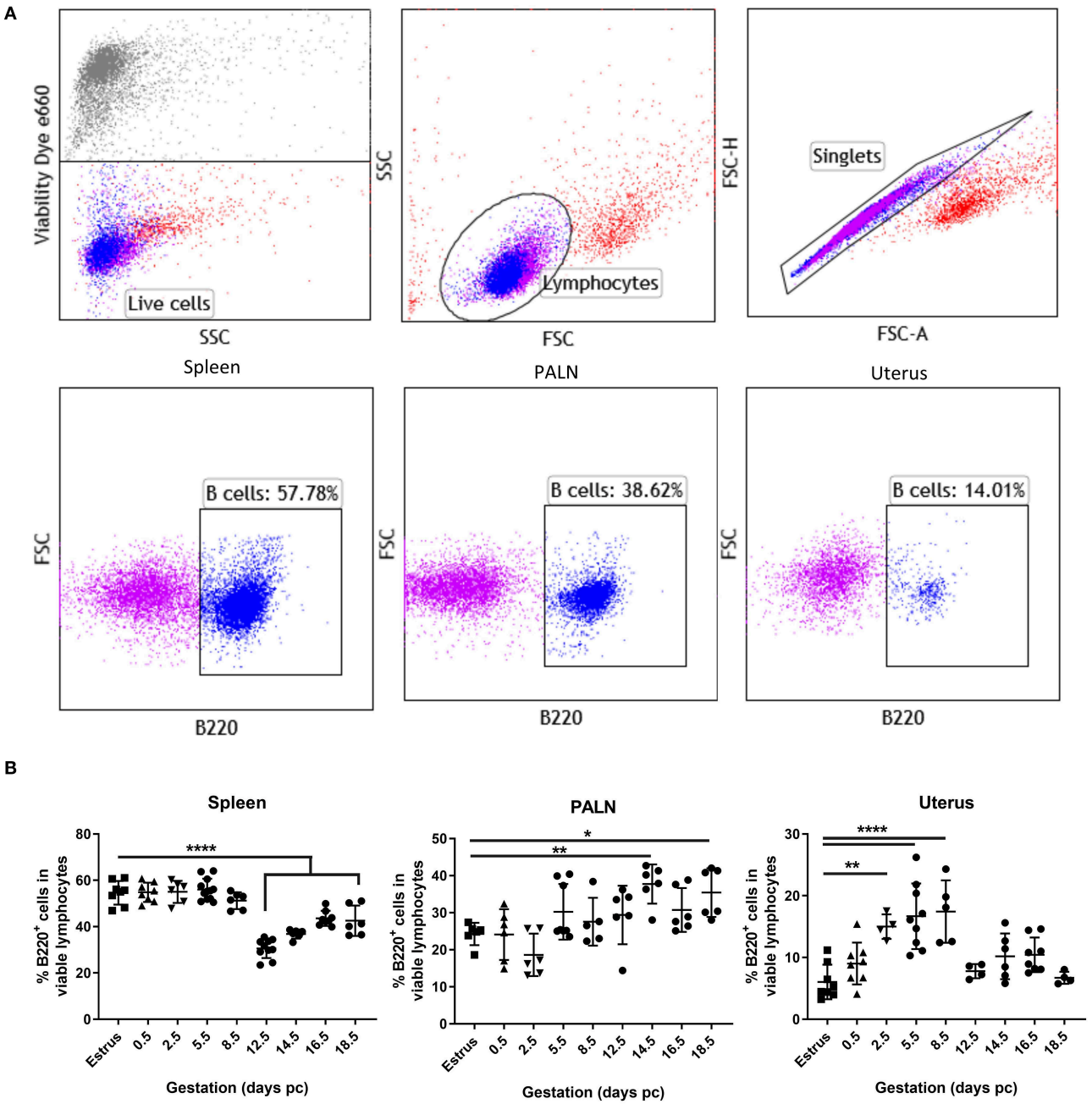


FIGURE 1 | Proportions of uterine B cells expand significantly at peri-implantation. **(A)** Flow cytometric gating strategy used to identify B cells in the isolated cell suspensions from spleen, para-aortic lymph nodes, and the uterus taken at day 5.5 pc. Single viable lymphocyte cells were gated as per exclusion of the viability dye, FSCvSSC plots showing lymphocyte population, doublet discrimination, and single color controls. **(B)** Graphical summary of the percentages B220$^+$ B cells in the viable lymphocyte cell populations obtained from estrus and mated mice at various days pc (n = 4–9 per time point). Data is shown as mean ± SEM and compared by one-way ANOVA, followed by Dunnett's *post-hoc* multiple comparisons test. *$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.0001$.

IL-10 Detection by ELISA

IL-10 quantities in supernatants were measured using the ELISA Max^{TM} Standard Set Mouse IL-10 Kit (Biolegend) following the manufacturer's instructions. Briefly, high-binding 96-well plates were coated with 100 μL of the capture antibody and kept at 4°C overnight. After washing, the wells were blocked with 200 μL of 1% BSA-PBS for an hour. Samples and standards were added and incubated for 3 h, washed, and 100 μL of biotin-conjugated anti-IL-10 antibodies added. After washing, avidin-horseradish peroxidase (HRP) was added for 30 min. Wells were washed and 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution added for 30 min for colorometric detection of IL-10. The reaction was stopped with 100 μL of 1M HCl, and plates read at 450 nm with a differential filter set at 570 nm using the MultiSkanTM FC (Thermo Fisher Scientific, USA) plate reader.

Statistical Analysis

All experiments were performed at least in triplicate. The results were analyzed for significant differences using the Student's *t*-test or the Mann-Whitney test for parametric and non-parametric samples, respectively. For multiple comparisons, one-way ANOVA with Dunnett's *post-hoc* test was used. Differences between groups were considered statistically significant at $p < 0.05$ as analyzed using GraphPad Prism 7.03 software (GraphPad, San Diego, CA, USA).

RESULTS

B Cell Proportions Are Increased in the Uterine Tissue at Peri-Implantation

To investigate the impact of pregnancy on B cell populations throughout gestation, we used a murine model of allogeneic mating where C57Bl/6J females were mated with BALB/c male studs. The percentages of B220$^+$ B cells in the spleen, para-aortic (uterine draining) lymph nodes, and the uterus itself were determined at succeeding time points and compared to basal B cell numbers found in comparable organs of virgin female controls at estrus.

Representative flow cytometric panels indicating the gating strategy and cell surface staining of viable B220$^+$ B cells found within the spleen, para-aortic lymph nodes, and uterus organs at day 5.5 pc are shown in **Figure 1A**. Kinetic assessment of organ B cells clearly showed the induction of lymphopenia in the spleen commenced mid-pregnancy, around day 12.5 pc (**Figure 1B**, left panel), with a contrasting significant increase in B cells in the uterine draining lymph nodes occurring around day 14.5 pc (**Figure 1B**, middle panel), results that concurred with data shown in previous studies (4). In the uterus however, a statistically significant increase in B cell proportions commenced pre-implantation, at day 2.5 pc, and continued post-implantation to day 8.5 pc (**Figure 1B**, right panel). This increase in uterine B220$^+$ B cells was limited to this peri-implantation phase as

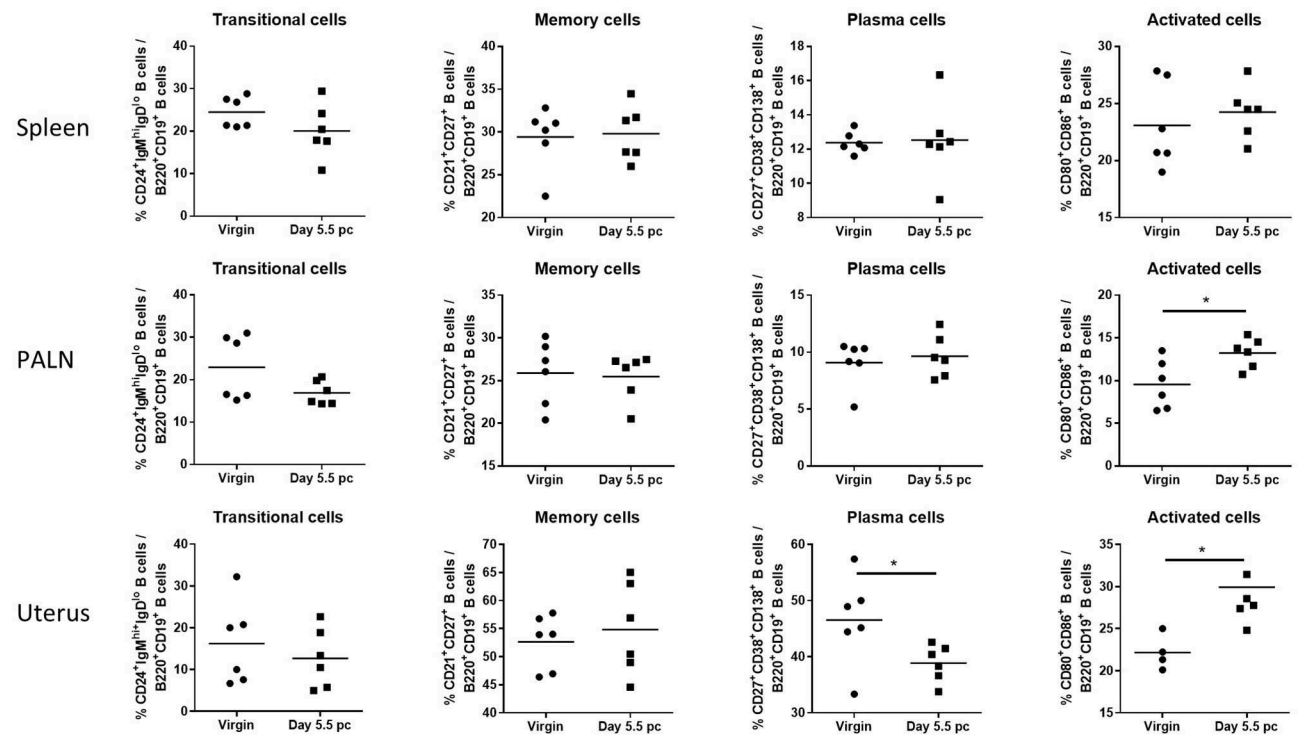


FIGURE 2 | B cell subsets in the para-aortic lymph nodes and uterus are altered post-implantation. Percentages of transitional or mature naive B cells (CD24$^+$IgM^{hi}IgD^{lo}), memory B cells (CD21$^+$CD27$^+$), plasma B cells (CD27$^+$CD38$^+$CD138$^+$), and activated B cells expressing co-stimulatory molecules (CD80$^+$CD86$^+$) were investigated in the spleen, para-aortic (uterus draining) lymph nodes, and uterus of virgin and pregnant mice at day 5.5 pc ($n = 6$, p-values calculated by unpaired *t*-test). *$p \leq 0.05$.

assessment of later time points revealed similar B cell frequencies to that of virgin mice in estrus. Based on these results, we focused on the day 5.5 pc time point, i.e., just after implantation, for all subsequent analyses.

Alterations in B Cell Subsets During the Peri-Implantation Stage of Pregnancy

Assessment of B cell subsets from spleen, uterus draining lymph nodes, and the uterus itself at day 5.5 pc indicated that proportions of $CD24^{+}IgM^{hi}IgD^{lo}$ transitional B cells and $CD21^{+}CD27^{+}$ memory B cells remained the same as those obtained from virgin female controls (**Figure 2**). In contrast, levels of $CD27^{+}CD38^{+}CD138^{+}$ plasma B cells decreased only in the uterine tissue, with no observed changes in the spleen and the para-aortic lymph nodes. However, $CD80^{+}CD86^{+}$ activated B cells were found to be significantly higher in both the uterus and the para-aortic lymph nodes at day 5.5 pc. Together this suggests that expanded numbers of B cells at the site of implantation, and associated draining lymph nodes, are activated and upregulate co-stimulatory markers indicative of an increased capacity to interact and engage with other immune cells.

Uterine B Cells From Pregnant Mice Suppress $CD4^{+}$ T Cell Proliferation and Activation

Given the observed changes in B cell percentages and subset composition, we hypothesized that the upregulated expression of co-stimulatory molecules CD80 and CD86 in uterine B cells prompted a directed engagement with T cells. Potentially, co-stimulation of T cells by B cells is a pathway for T cell activation that directly affects their rate of proliferation. Thus, we sought to examine whether uterine B cells influenced $CD4^{+}$ T cell activation and proliferation.

Uteri from day 5.5 pc pregnant and virgin non-pregnant control mice were collected and processed for single cell suspensions. Viable $CD19^{+}$ B cells sorted from the uterine cell

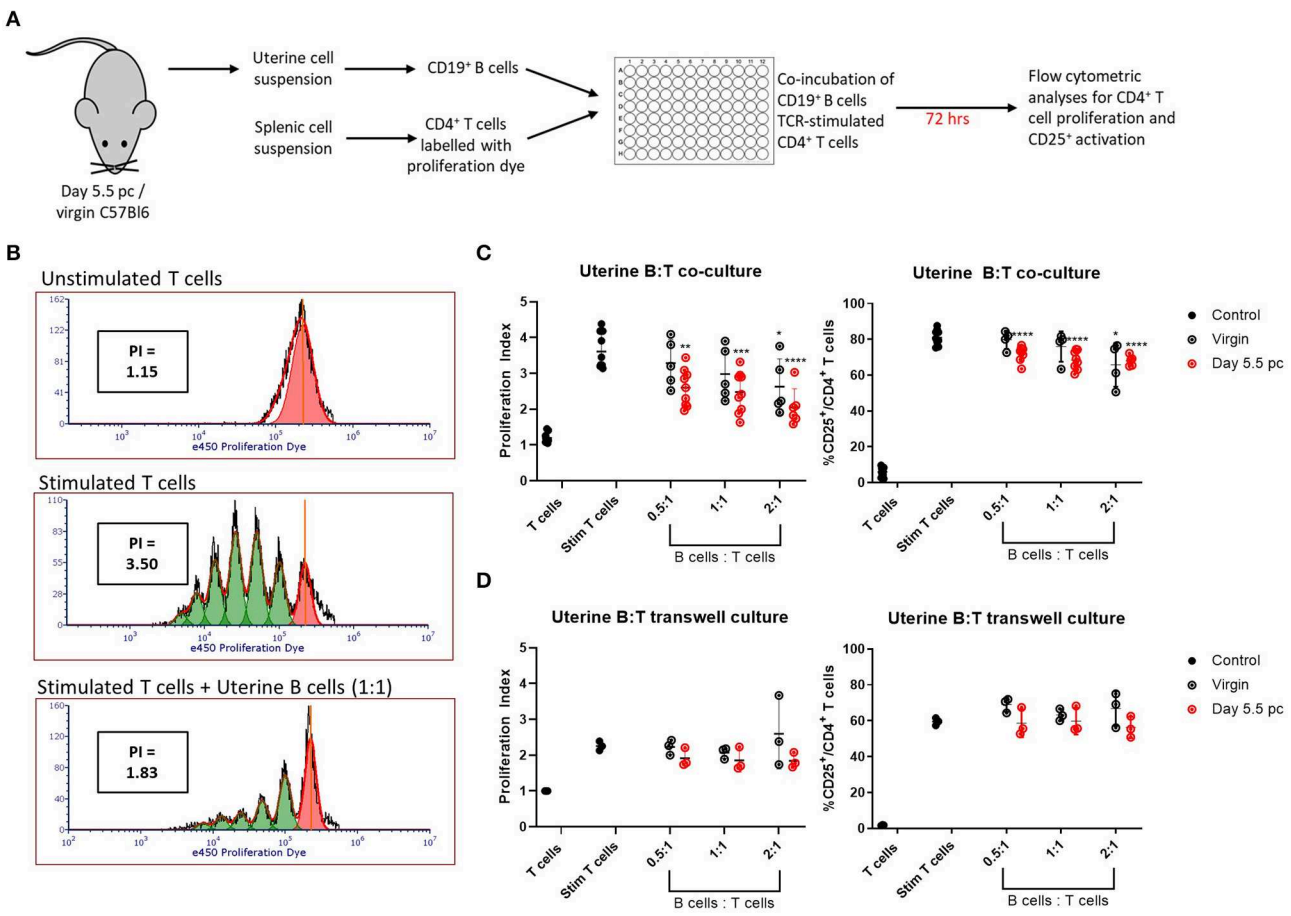


FIGURE 3 | Uterine B cells from pregnant mice post-implantation suppress $CD4^{+}$ T cell proliferation and activation. **(A)** Schematic diagram of the suppression assay used to assess the effect of B cells on $CD4^{+}$ T cell proliferation and activation. **(B)** Representative flow cytometric panels illustrating the inhibitory effect of B cells on T cell proliferation quantified via calculation of individual proliferation index (denoted as PI). **(C)** Graphical summaries of biologically independent experiments assessing amount of $CD4^{+}$ T cell proliferation and CD25 expression with and without co-culture of increasing ratio of uterine B cells. **(D)** The same experiments as in **(C)** except T and B cells were kept physically apart by a 0.4 μm transwell membrane ($n = 3$–8). Data is presented as mean ± SEM and compared by one-way ANOVA followed by Dunnett's *post-hoc* test. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. Data were combined from five independent experiments.

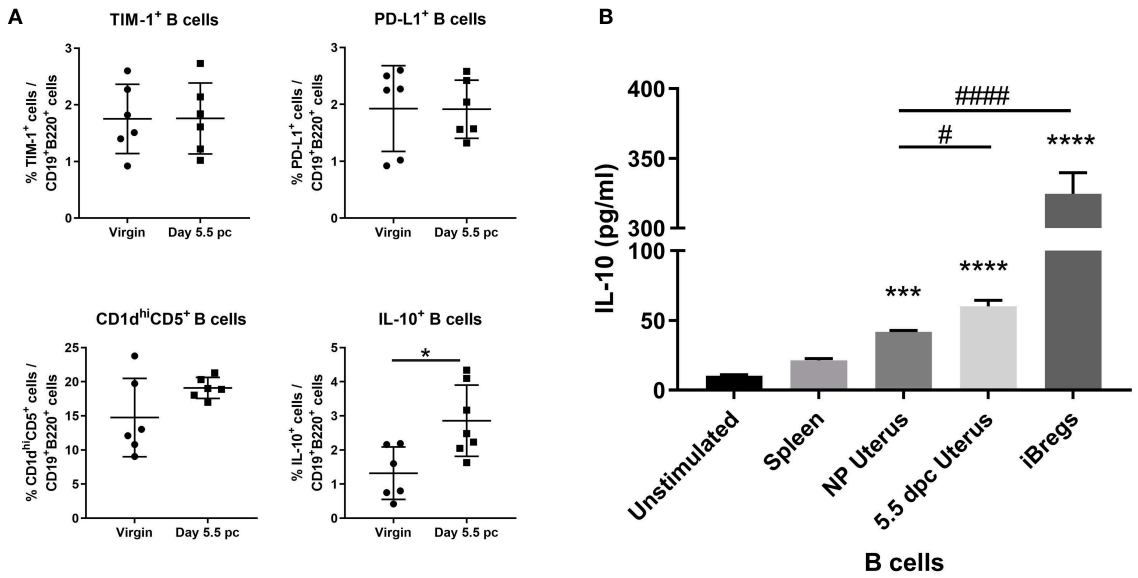


FIGURE 4 | An expanded pool of IL-10$^+$ B cells are present in the uterus after implantation. **(A)** Known regulatory B cell subsets were analyzed from B cells collected from non-pregnant and pregnant uteri ($n = 6$–7, unpaired *t*-test). **(B)** IL-10 production by stimulated populations of purified B cells obtained from the spleen, virgin uterus, day 5.5 pc uterus, or *in vitro* induced Bregs was assessed by ELISA. Data represent pooled supernatants of duplicate wells from three independent experiments. Data is presented as mean ± SEM and compared by one-way ANOVA, followed by Dunnett's *post-hoc* multiple comparisons test against unstimulated control. $^*p \leq 0.05$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$. Additional Dunnett's analysis assessed against non-pregnant virgin uterus reveal a significant increase in IL-10 produced by day 5.5 pc uterine B cells and iBregs ($^{\#}p \leq 0.05$, $^{\#\#\#\#}p \leq 0.0001$).

suspensions were co-cultured with syngeneic TCR-stimulated CD4$^+$ splenic T cells for 72 h, after which the T cells were assessed for cell proliferation and activation by upregulation of CD25 expression (**Figure 3A**). Representative histograms of T cell proliferation from unstimulated and stimulated controls, and T and B cell co-cultures are shown with corresponding proliferation index (**Figure 3B**). Combined results clearly indicated the ability of uterine B cells from pregnant mice to significantly inhibit the proliferation of syngeneic CD4$^+$ T cells in a dose-dependent manner, with the proliferation indices significantly decreased in response to an increase in ratio of uterine B cells to T cells (**Figure 3C**, left panel). Similarly, inhibition of proliferation was also associated with decreased numbers of CD4$^+$ T cells expressing the activation marker CD25 (**Figure 3C**, right panel). Furthermore, this inhibitory ability of B cells from pregnant mice was tissue-specific, as no inhibitory capacity was observed by B cells obtained from the spleen or uterus-draining lymph nodes in the same pregnant mice (**Supplementary Figure 3A**). B cells obtained from virgin control mice could only inhibit T cell proliferation and activation at the highest ratio of B:T cells (2:1) indicating that pregnancy amplified this basal uterine B cell response.

To determine whether the inhibitory effect was evoked by B-T cell cognate interactions or via soluble molecules produced by B cells, we conducted the same experiment but with the B cells and T cells physically separated by a 0.4 μm transwell membrane. No significant increase in inhibition of proliferation or CD25 activation was observed (**Figure 3D**; **Supplementary Figure 3B**), which suggested that the suppressive effect of uterine B cells on T cells is mediated by cognate cell-to-cell interactions rather than through soluble mediators.

Uterine B Cells at Peri-Implantation Stage Exhibit an Enhanced Regulatory Phenotype

The previous results indicated that uterine B cells exhibited a suppressive phenotype at day 5.5 pc, post-implantation. We next sought to investigate whether these uterine B cells possessed surface and functional markers typically seen in Bregs such as the expression of TIM-1, PD-L1, CD1d, CD5, as well as the anti-inflammatory cytokine IL-10 (8). We focused particularly on investigating if there were differences in the frequencies of these known Breg phenotypes between uterine B cells from virgin mice and uterine B cells from pregnant mice that could account for their differing effects on CD4$^+$ T cells.

Assessment of surface markers indicated statistically similar proportions of TIM-1$^+$ B cells, PD-L1$^+$ B cells, and CD1d^{hi}CD5$^+$ B cells within the uterus of virgin and pregnant mice. In contrast, IL-10 cytokine expression in uterine B cells was found to be significantly increased after implantation (**Figure 4A**). Furthermore, *ex vivo* stimulated uterine B cells at day 5.5 pc produced levels of IL-10 that were significantly higher than unstimulated controls, stimulated splenic B cells, and stimulated uterine B cells from virgin mice, although levels are 4–5-fold less than the amount of IL-10 produced by the positive control, *in vitro* generated splenic IL-10$^+$ B cells (**Figure 4B**). These results indicated that the capacity of uterine B cells from pregnant mice to regulate T cell proliferation and activation may be due to a significant increase in the proportion of IL-10-producing B cells within the uterine B cell population.

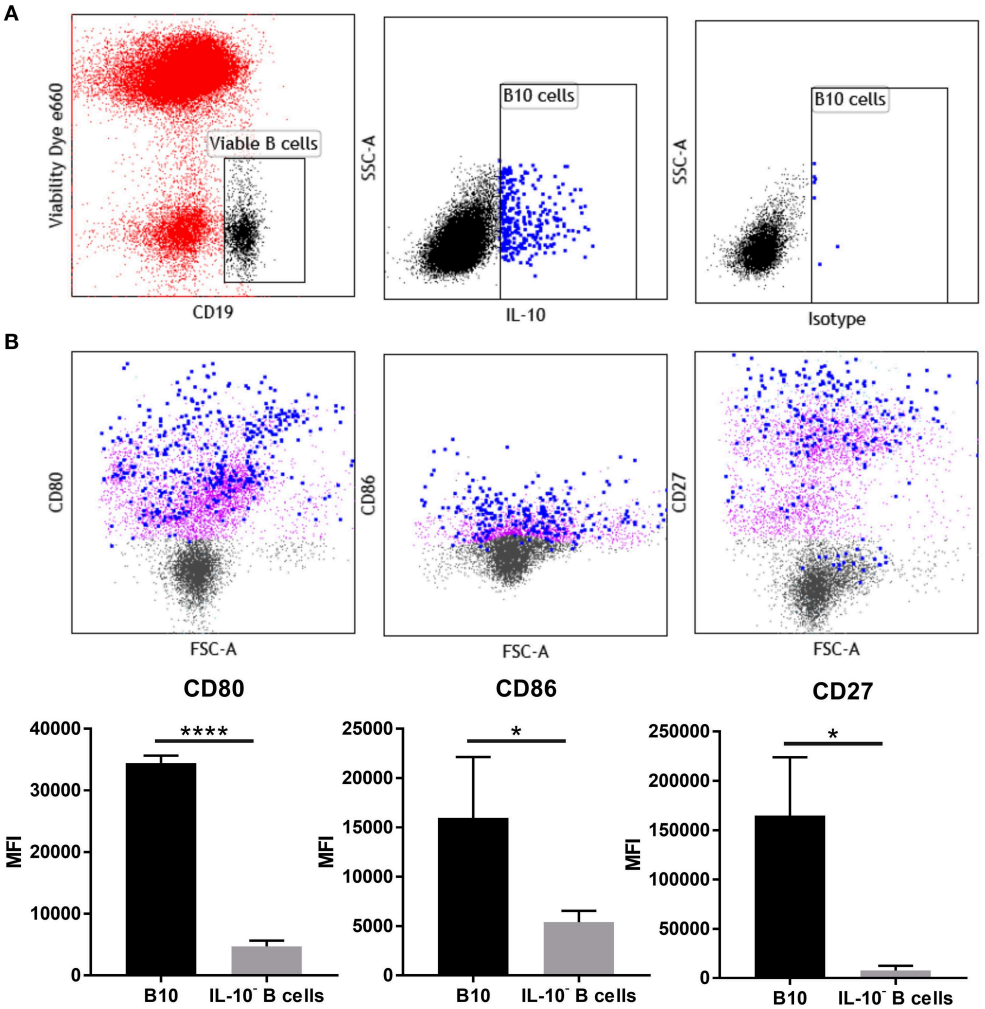


FIGURE 5 | IL-10$^+$ B cells at day 5.5 pc have upregulated expression of CD27, CD80, and CD86. **(A)** Gating strategy for IL-10$^+$ cells within the viable CD19$^+$ cell population. **(B)** Representative flow cytometric profiles of B10 cells (blue) and IL-10$^-$ B cells (purple) and their upregulated expression of activation marker CD27 and co-stimulatory molecules CD80 and CD86, and the corresponding graphical presentation of the mean fluorescence intensity (MFI) of B10 cells compared to IL-10$^-$ B cells by unpaired *t*-test. *$p \leq 0.05$, ****$p \leq 0.0001$. Data shown are from three independent experiments.

Phenotype Profile of Uterine IL-10$^+$ B Cells at Peri-Implantation Show Increased Activation and Co-stimulatory Markers

Phenotypic analysis of uterine B cells, as identified by their expression of CD19 and B220, showed that at day 5.5 pc they co-expressed two B cell receptor isotypes IgM and IgD, as well as major histocompatibility complex class II (MHC class II), signifying their antigen-driven development and maturity, as well as their capacity to act as professional antigen-presenting cells. These cells also expressed CD24, CD21, and CD38, surface markers associated with B cell differentiation and activation (**Supplementary Figure 4**). However, multi-parameter flow cytometric analysis of uterine IL-10$^+$ B cells (**Figure 5A**), indicated that these cells expressed higher levels of co-stimulatory molecules CD80 and CD86 compared to non-IL-10 expressing B cells. Notably, expression of activation marker CD27 was also significantly increased in uterine IL-10$^+$ B cell cells (**Figure 5B**). The differential expression of these markers suggests the involvement of uterine IL-10$^+$ B cells in regulation of the maternal immune response at implantation, particularly through their increased capacity to engage and interact with T cells. Together with the previous findings, we posit that the enhanced expression of these co-stimulatory and activation markers accompanied with the increased production of anti-inflammatory IL-10, enable uterine B cells to regulate the proliferation and activation of CD4$^+$ T cells.

DISCUSSION

B cells are a major component of the lymphoid immune system. Their functional promiscuity is due to the variety of B cell subsets that can carry out one or more functions such as antibody

production, antigen presentation, or secretion of modulatory cytokines. Historically, B cells in pregnancy and early life have been poorly studied due to their scarcity in the pregnancy microenvironment. However, studies over the past decade have only strengthened the need to explore the role of B cells in gestation as links between B cell abnormalities and obstetric complications have been repeatedly shown (17–19).

Variations in frequency and modification in phenotype and function have been demonstrated in maternal B cells from the bone marrow and spleen during murine pregnancy, but examination of the B cell compartment within the uterine microenvironment remains limited. As the molecular architecture and function of B cells are heavily influenced by the extracellular environment and their exposure to foreign antigens (20), we hypothesized that B cells within uterine tissue in close proximity to the implanting semi-allogeneic embryo undergo changes in phenotype and function to support the development of maternal-fetal tolerance. In this study, we investigated the modifications in B cells, particularly within the uterine mucosa, in a mouse allogenic pregnancy model to provide a clearer picture of the role they play during early pregnancy.

Assessment of B cell proportions in the spleen over the gestational period recapitulate the B cell lymphopenia previously reported at mid-gestation (4). However, we also show a significant expansion in $B220^+$ cells in the uterus over the peri-implantation period (days 2.5–8.5 pc; **Figure 1**). This expansion at the time points approaching, during, and post-implantation, suggests their involvement in the control of the inflammation required for successful decidualization and implantation. At the peri-implantation period, the morphologic and functional changes in the endometrium are accompanied by major changes in the proportion and number of endometrial immune cells (21, 22). Uterine dendritic cells, decidual macrophages, and regulatory T cells are some of the immune cells recruited into the endometrium to accumulate around the embryo at implantation (23–25). Alteration in the number and function of these cells in the local pregnancy milieu has been demonstrated to be instrumental in the recognition of fetal alloantigens, decidual differentiation, and the establishment of maternal immune tolerance (21, 22). Recently, we have shown *in vitro* that human trophoblasts can educate B cells to acquire regulatory properties that protect against T cell-mediated inflammation that may otherwise be detrimental to implantation (26). Our results herein showing the expansion of B cells in the murine pregnancy microenvironment further suggest an intimate involvement of B cells in facilitating induction of immune tolerance during the peri-implantation phase of pregnancy.

Focusing on the peri-implantation time point at day 5.5 pc (**Figure 2**), we showed the pregnant uterus harbors a significantly lower population of $CD138^+$ plasma cells compared to unmated controls. The transmembrane marker CD138 is a syndecan specifically found in plasma cells that has long been correlated with the occurrence of chronic endometritis, an inflammatory condition of the uterine lining often associated with negative reproductive outcomes such as recurrent pregnancy loss, implantation failure, and infertility (27, 28). The decrease in the plasma cell population in the uterus, but not in the spleen or lymph nodes, gives credence to clinical evidence that plasma cell numbers should be at a tolerable level in the pregnancy microenvironment (16). Thus, the lower plasma cell count is likely suggestive of a normal implantation event.

The increased population of uterine B cells bearing $CD80^+$ and $CD86^+$ at day 5.5 pc is particularly interesting, as it implies increased co-stimulatory function that may direct the T cell-mediated immune response (29). Upregulation of CD80 and CD86 expression on B cells is obtained by triggering and activating the BCR through a variety of stimuli such as ligands and cytokines (30, 31). Subsequently, the crosstalk facilitated by the enhanced expression of these two co-stimulatory molecules on activated B cells is capable of skewing T cell activity (29). In pregnancy, it has been demonstrated that increased CD86 expression in murine peritoneal cavity B1-a B cells is associated with perturbations to pregnancy in a CBA/J mice model, an effect attributed to the increased capacity for Th1 and Th17 differentiation (32). It may however be a different case within the pregnancy microenvironment as investigated here, where the presence of the blastocyst and associated alterations in cytokine production within the microenvironment may serve as triggers for BCR activation and lead to enhanced expression of both CD86 and CD80 on uterine B cells. Moreover, previous studies demonstrated the upregulated expression of CD80 and CD86 on murine splenic antigen presenting cells such as dendritic cells and macrophages at the pre-implantation period (day 3.5 pc). This was shown to be crucial in modulating T regulatory cell (Treg) abundance, cytokine production, and ultimately pregnancy outcome (33, 34). A follow up study examining splenic B cells showed similar results, with CD86 protein expression significantly increased in normal pregnant mice compared to abortion-prone models at pre-implantation (35). Thus, we postulate that the differential expression of CD80 and CD86 in uterine B cells may be predisposing them to act in a similar manner, that is elevated expression of these molecules on uterine B cells may contribute to establishment of maternal tolerance and therefore appropriate implantation. These findings are certainly intriguing and warrant further investigation that was unfortunately, out of scope of this current study.

Amongst the B cell subsets, regulatory B cells or Bregs have drawn attention as potentially critical players in pregnancy well-being. Previous murine studies have shown that splenic Bregs can rescue pregnancies in abortion-prone models (9), and a clinical study has revealed that deficiency in circulating IL-10-producing Bregs is correlated with higher risk of spontaneous abortions (11). Furthermore, it was recently shown that the lack of $IL\text{-}10^+$ B cells in murine pregnancies led to diminished embryo sizes and stagnated Treg pools, as well as increased susceptibility to LPS which caused intrauterine fetal death (36). With the expansion of uterine B cells at the peri-implantation phase, we speculated whether these cells possessed regulatory properties, and indeed demonstrated that uterine B cells from day 5.5 pc pregnant mice effectively suppressed syngeneic $CD4^+$ T cell proliferation and activation as seen by the significant reduction in proliferation and diminished expression of the activation marker CD25 in a cell contact dependent manner (**Figure 3**).

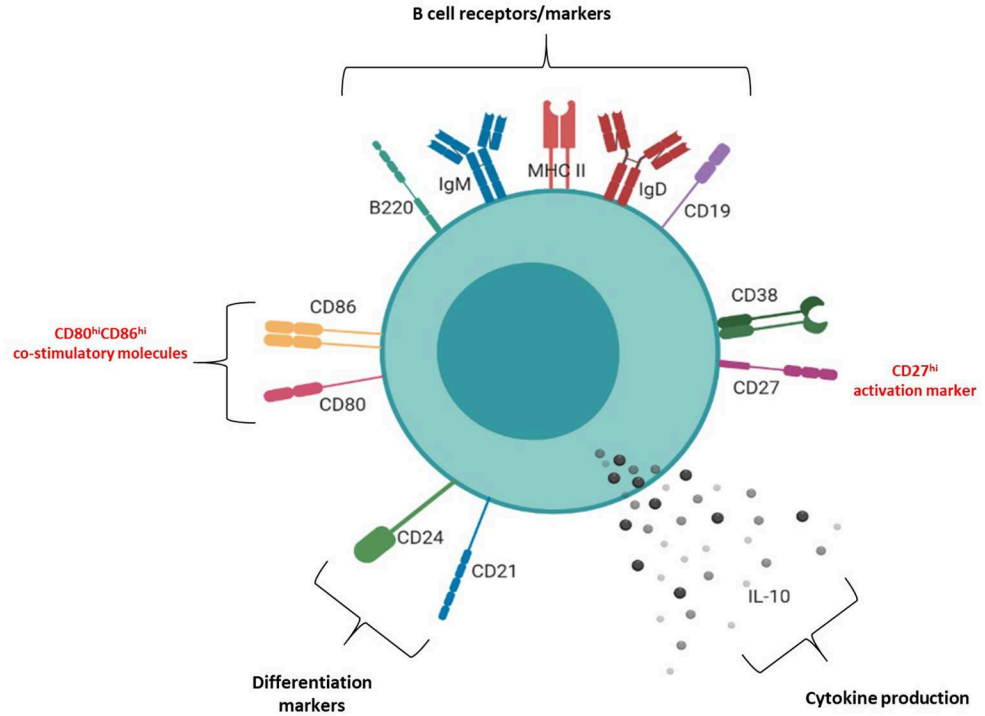


FIGURE 6 | Phenotypic structure of an IL-10$^+$ uterine B cell at peri-implantation. Schematic diagram of the phenotypic markers expressed by uterine IL-10$^+$ B cells at peri-implantation, including B cell receptors and markers (CD19, B220, IgM, IgD, MHC II), activation markers (CD27, CD38), differentiation markers (CD21, CD24), co-stimulatory molecules (CD80, CD86), and IL-10 cytokine production.

Bregs are defined as any B cell carrying out negative regulation of an immune response and as such, a plethora of studies have attributed this ability to various subsets of B cells including TIM-1$^+$ B cells, PD-L1$^+$ B cells, CD5$^+$CD1d^{hi} B cells, and IL-10$^+$ B cells (8). Among these subsets, only the IL-10$^+$ B cells were significantly expanded in the uterus at the peri-implantation stage (day 5.5 pc) of pregnancy, with more IL-10 produced by B cells compared to non-pregnant controls and splenic B cells (**Figure 4**). In pregnancy, IL-10 is found in high amounts in placental and decidual tissues at early gestation in pre-clinical and clinical studies and is well-established as a vital cytokine for optimal pregnancy outcomes (37). Mainly thought to be produced by trophoblasts, uterine natural killer cells, monocytes, dendritic cells, and Tregs, IL-10 has been shown to be a key cytokine for the balance of pro- and anti-inflammatory signals that establish maternal immune tolerance during the implantation process (38–40). While it was desirable to assess the suppressive capacity of these uterine IL-10$^+$ B cells on CD4$^+$ T cells specifically, the low percentage of IL-10$^+$ B cells (2–5%) within an already scarce population of uterine B cells from an individual pregnant mouse proved to be a major limitation to conducting these experiments.

The molecular surface architecture of uterine IL-10$^+$ B cells is summarized in **Figure 6** and illustrates how these cells may facilitate their suppressive function. We determined that the majority of uterine B cells isolated from pregnant mice at the peri-implantation time point take on a phenotype descriptive of mature and antigen-driven differentiated B cells: they bear both IgM and IgD, antigen presenting cell marker MHC class II, differentiation markers CD24 and CD21, and activation marker CD38 (**Supplementary Figure 4**). The dual expression of IgM and IgD is particularly interesting, as IgD co-expression is suggested to indicate a "tolerogenic" role via regulating autoreactivity brought about by chronic exposure to antigen (6). Likewise, the retention of differentiation markers CD24 and CD21 as well as expression of activation marker CD38 suggested that uterine B cells are mature, but yet to have differentiated into antibody-producing plasma cells (41, 42). Furthermore, ∼20 and 50% of IL-10 negative uterine B cells express the ligands CD80 and CD86, respectively (**Figure 5B**) which suggests that a significant proportion of the uterine B cells have the capacity for co-stimulating T cell activation, potentially leading to negative regulation by way of Treg expansion and modification of cytokine production (33, 34). Interestingly, the small proportion observed as IL-10$^+$ B cells within the uterine B cell population exhibited higher expression of co-stimulatory molecules CD80 and CD86 as well as CD27, an activation marker of memory and plasmablast lineages in murine B cells. In this study, these cell surface molecules, together with the cells' capacity for producing anti-inflammatory IL-10, is likely to have contributed to the regulatory phenotype that uterine B cells from pregnant mice exhibit. The differential expression of these surface markers indicates that IL-10$^+$ B cells may be involved in facilitating changes in the maternal immune network as it adjusts to the presence of

fetal allo-antigens in early pregnancy. The increased capacity and engagement of these activated IL-10^+ B cells for T cell co-stimulation potentially contributes to the range of the T cell repertoire generated in early pregnancy, a process critical for setting the conditions for implantation.

CONCLUSION

To our knowledge, this is the first comprehensive study on the phenotype and functional characteristics of uterine B cells in early murine allogeneic pregnancy. In this study, we demonstrate an expanded pool of uterine B cells at the time points associated with embryo implantation, which harbor expanded subpopulations of $CD80^+CD86^+$ and IL-10^+ B cells, which collectively suppresses $CD4^+$ T cell proliferation and activation. Thus, we propose that at this crucial time point, B cells within the uterine microenvironment undergo changes in both population frequency and phenotype that supports a regulatory role and contributes to the immune changes necessary for the progression of a successful pregnancy.

DATA AVAILABILITY STATEMENT

The raw data that support the findings of this study are available from the corresponding authors upon reasonable request.

ETHICS STATEMENT

The animal study was reviewed and approved by University of South Australia Animal Ethics Committee. All animal work was conducted in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition (2013) as defined by the National Health and Medical Research Council of Australia. All experiments were conducted with approval from The University of Adelaide and University of South Australia Animal Ethics Committees.

AUTHOR CONTRIBUTIONS

KD and RG-G conceived and designed the study. RG-G performed the experiments, analyzed the data, and wrote the manuscript. PE, PG-V, and JH assisted in experimental design and provided technical expertise. KD evaluated the results and edited the manuscript. KD, JH, PE, and PG-V supervised the study.

FUNDING

This work was supported by NHMRC Project (APP1020984) and Fellowship (APP1012386) grants to KD, Robinson Research Institute grant to KD, and Research Training Program Domestic Scholarship to RG-G.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02899/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Received: 25 June 2019 | Revised: 25 August 2019 | Accepted: 29 August 2019

DOI: 10.1111/aji.13187

Check for updates

AJRI American Journal of Reproductive Immunology | WILEY

ORIGINAL ARTICLE

Trophoblasts promote induction of a regulatory phenotype in B cells that can protect against detrimental T cell–mediated inflammation

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Funding information
National Health and Medical Research Council, Grant/Award Number: APP1012386 and APP1020984; Research Training Program Domestic (RTPd) Scholarship; Department of Obstetrics and Gynecology, Yale School of Medicine

Abstract

Problem: A successful outcome to pregnancy is critically dependent on the initiation of maternal immune tolerance before embryo implantation. Cells of embryonic origin that come in contact with the uterine microenvironment can exert influence over the phenotype and function of immune cells to facilitate robust implantation; however, what influence they may have on B cells remains unknown. In this study, we investigate the effect of human trophoblast cells on B-cell phenotype and the subsequent effect on peri-implantation events.

Method of study: We cultured purified human B cells with the first-trimester human trophoblast cell line Swan 71 to investigate trophoblast-B-cell interactions and utilized trophoblast spheroids in an in vitro implantation model of migration and invasion.

Results: Trophoblast-educated B cells or TE-B cells were found to consist of B cells in committed lineages such as plasmablasts and memory B cells, as well as increased proportions in subsets of $CD24^{hi}CD27^{+}$ regulatory B cells and $CD19^{+}IL\text{-}10^{+}$ B cells. Conditioned media from the TE-B cells showed reduced production of pro-inflammatory cytokines that influenced the T-cell proliferation and cytokine production. Using trophoblast spheroids, we assessed the role of TE-B cells in trophoblast invasion and migration. Our results demonstrate a protective effect of TE-B-conditioned media against deleterious inflammation as evidenced by survival of the trophoblast spheroid in the presence of an immune assault and promotion of a migratory phenotype.

Conclusion: We posit that trophoblast-mediated education of B cells leads to their acquisition of properties capable of modulating inflammation in the uterine environment during the peri-implantation period.

KEYWORDS

B cells, inflammation, regulatory phenotype, spheroid, trophoblast

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1 | INTRODUCTION

In pregnancy, maternal immune cells in the local uterine microenvironment are critical players in the physiological processes that lead to successful conception, implantation, gestation, and ultimately, parturition.[1] At the outset of pregnancy, the foremost objective is to build and foster a coordinated interplay of immune cells, hormones, and cytokines that is oriented toward securing blastocyst attachment and implantation into the uterine decidua. At periimplantation, innate and adaptive immune cells including macrophages, natural killer cells, dendritic cells, and T cells accumulate in the endometrium. Interestingly, the phenotypes of endometrial and decidual immune cells in early pregnancy are altered compared with the non-pregnant state; that is, uterine dendritic cells (uDC) retain an immature tolerogenic state,[2] uterine natural killer cells (uNK) exhibit reduced cytotoxic capability,[3] and $CD8^+$ T cells in the decidua have reduced expression of perforin and granzyme B.[4] These modified functional roles are recognized as beneficial and contributory to a receptive uterus and vital for the development of the placenta.[5]

The first point of contact between embryonic cells and the maternal uterine microenvironment is via the invading trophoblast cells of the blastocyst. Trophoblast cells present an interesting immunologic occurrence in that they are semi-allogenic and should, in principle, trigger an adverse inflammatory response. Instead, the trophoblast thrives in the tolerant immunological milieu at the implantation site. Previous studies have shown that trophoblast cells can regulate immune cell function through various pathways. First-trimester trophoblast cells secrete high levels of TGF-β and thymic stromal lymphopoietin (TSLP), and have been observed to actively recruit and differentiate regulatory T cells (Tregs).[2,6] Exosomes produced by trophoblast cells can act as recruiting agents for monocytes and downregulate NKG2D receptors on NK, $CD8^+$, and γδ T cells to reduce their cytotoxic capacity.[7,8] Trophoblast cells have also been shown to induce a predominantly tolerogenic profile in dendritic cells in vitro.[9] Trophoblast cells, therefore, are suggested to be paramount in shaping the manner in which maternal uterine immune cells respond to invasion of the blastocyst.

So far, studies on lymphocytes within the uterine microenvironment have been largely limited to T cells, which contribute immensely to the controlled inflammatory environment requisite for uterine receptivity leading to implantation and placentation.[10] However, there are now a number of studies outlining an emerging role for B cells as being active participants in pregnancy. Recent studies have shown a protective effect of IL-10-producing B cells (B10) in restoring tolerance and rescuing pregnancy. Mouse splenic B10 cells are expanded during normal pregnancy, and their adoptive transfer to abortion-prone mice rescued pregnancies.[11] Similarly in humans, IL-10-producing $CD19^+CD24^{hi}CD27^+$ regulatory B cells (Bregs) are significantly higher in women undergoing normal pregnancies in their first trimester as opposed to non-pregnant women, or women who suffered spontaneous abortions.[12] These studies suggest that B cells play a role in early pregnancy; thus, there is an impetus to investigate events critical at this stage.

In this study, we aimed to investigate the effect of first-trimester trophoblast cells on the human B-cell phenotype and function. Here, we provide in vitro evidence that trophoblast cells can promote the differentiation of regulatory B cells, transitional B cells, IgM-memory B cells, and plasmablasts, which subsequently produced a dampened inflammatory cytokine profile. We also show that these trophoblast-educated B (TE-B) cells can regulate T-cell proliferation and cytokine production. Lastly, using trophoblast spheroids as an in vitro implantation model, we demonstrate that the addition of conditioned media from TE-B cells protect the spheroid from damage and aid trophoblast migration, despite the presence of deleterious cytokines from highly activated T cells. Thus, we propose that first-trimester trophoblast cells can educate B cells to acquire phenotypes and functional properties that contribute in calibrating inflammation that otherwise could be detrimental to a successful implantation event.

2 | METHODS

2.1 | Cell lines and culture conditions

The human first-trimester trophoblast cell line, SWAN 71 (Sw.71), was derived from a 7-week placenta and immortalized by ectopic expression of the catalytic subunit of the human telomerase (hTERT)[13] and used for all experiments performed. Cells were maintained in DMEM/F12 (Gibco Invitrogen) and passaged when approximately 85% confluent. For some experiments, hTERT-immortalized human endometrial stromal cells (HESC)[14] were used and maintained in DMEM (Gibco Invitrogen) and passaged every 3 days. All culture media were supplemented with 2 mmol/L L-glutamine, 10% fetal bovine serum (FBS), 1 mmol/L sodium pyruvate, 0.1 mmol/L non-essential amino acids, 1 mmol/L sodium pyruvate, 100 units/mL penicillin-streptomycin, and kept at 37°C and 5% CO_2.

2.2 | Peripheral blood mononuclear cells (PBMC) and B-cell isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood obtained from consenting healthy non-pregnant female donors as approved by the Human Investigation Committee of the Yale University Institutional Review Board (IRB). Briefly, whole blood collected in EDTA-coated vacutainers was diluted 1:2 with PBS and carefully overlaid into a tube containing equal volume of Lymphoprep™ (StemCell Tech). Cells were spun at 750*g* without brake and acceleration for 25 minutes at room temperature with resultant mononuclear cells harvested from the interface of plasma and Lymphoprep™ and washed twice with large volumes of PBS. B cells were then purified by negative selection from the PBMCs using the MojoSort™ Human Pan B Cell Isolation Kit (BioLegend) following

the manufacturer's instructions, resulting in highly purified population of $CD19^+$ Cells (>90% purity).

2.3 | Collection of conditioned media

To obtain conditioned media from trophoblast cells, Sw.71 cells were plated at 25% confluency into T-175 flasks. Cells were incubated for 48 hours after which the cell supernatant was collected and centrifuged to remove all cellular debris. Cell-free supernatant was then aliquoted and stored at −80°C until further use.

Highly purified $CD19^+$ B cells were resuspended in RPMI-1640 media (Gibco Invitrogen) and seeded at 1×10^6 cells/well in a 24-well flat-bottom plate (Sigma-Aldrich). Cells were incubated either unstimulated for the non-treated control or stimulated with Sw.71 conditioned media diluted 50% in RPMI medium for 48 hours at 37°C and 5% CO_2. Cell supernatant was collected and spun down to exclude cellular debris in the collection of cell-free supernatant. Aliquots were prepared and stored at −80°C until further use.

2.4 | Preparation of differentiated and activated T cells

T cells were purified from isolated PBMCs by negative selection using the EasySep™ Human T cell Isolation Kit (StemCell Tech) following the manufacturer's instructions resulting in purity of >95% $CD3^+$ cells. For non-specific T-cell receptor (TCR) stimulation, 1×10^5 cells T cells were seeded in 96-well flat-bottom plate pre-coated with anti-CD3 (10 ug/mL, BD Biosciences) and anti-CD28 (1 ug/mL, BD Biosciences) antibodies in RPMI medium supplemented with 10% FBS and IL-2 (2 ng/mL, PeproTech). The cells were left to incubate at 37°C and 5% CO_2 for 3 days after which the stimulated cells were transferred to a new plate in normal 10% RPMI medium and rested for 2 more days. In some experiments, these pre-primed T cells were activated with Cell Stimulation Cocktail (eBioscience) containing phorbol 12-myristate 13-acetate (PMA) and ionomycin for 5 hours, to induce differentiation into activated T cells.

2.5 | Flow cytometry

Multi-parameter flow cytometry was used to assess B cell phenotypes using a panel of conjugated mouse antihuman monoclonal antibodies (mAb) specific for the following markers: CD19-FITC Clone HIB19 (BD Biosciences), CD24-PerCP Clone ML5 (BD Biosciences), CD27-APC Clone O323 (BioLegend), CD38-PE Clone HIT2 (BD Biosciences), IgM-PECy7 Clone G20-127 (BD Biosciences), and IL-10-FITC Clone JES3-9D7 (BioLegend). B cells were collected, washed twice, and then incubated with heat-inactivated human serum for 20 minutes on ice. Cells were washed and stained with the cocktail of mAb in FACS buffer for 30 minutes in the dark on ice. For IL-10 intracellular staining, cells were stimulated using a Cell Stimulation Cocktail (plus protein transport inhibitors; eBioscience) for 5 hours prior to fixing and permeabilizing using the BD Cytofix/Cytoperm Kit (BD Biosciences). The cells were then stained with IL-10-FITC for 30 minutes at room temperature. Cells were washed and transferred to FACS tubes for flow cytometry analysis. For T cells, the following markers were used: CD4-APC Clone OKT4 (SunGene Biotech), CD25-PE Clone HI25A (SunGene Biotech), and Foxp3-FITC Clone PCH101 (eBioscience).

Cell phenotype was determined by four-color flow cytometry analysis (FACSCalibur; BD Biosciences), and the data were analyzed using FlowJo software (Treestar). Unstained, single color, FMO, and isotype controls were used to set gates and identify the positive populations.

2.6 | Classification of B cell subsets

The following B-cell subsets were identified by previous studies as regulatory in function via their production of IL-10 and thus were used to classify the B cells acquired: regulatory B cells ($CD19^+CD24^+CD27^+$), transitional B cells ($CD19^+CD24^{hi}CD38^{hi}$), IgM-memory B cells ($CD19^+IgM^+CD27^+$), and plasmablasts ($CD19^+CD27^{hi}CD38^{hi}$).

2.7 | Cytokine/chemokine analysis

The effect of trophoblast supernatant on immune cell chemokine and cytokine production was determined using Bio-Plex Pro™ Human Cytokine 17-plex Assay (Bio-Rad) following the manufacturer's instructions. Briefly, wells of a 96-well filter plate were loaded with the prepared standard solution or cell-free supernatant and incubated with the 17-plex ProBead Mix at ±800 rpm for 30 minutes in the dark at room temperature. Wells were then vacuum-washed three times then incubated with 25 µl of biotinylated detection antibody at ±800 rpm for 30 minutes at room temperature in the dark. After three washes, streptavidin-phycoerythrin was added to each well and incubated for 10 minutes at ±800 rpm in the dark. After a final wash, the beads were resuspended in sheath buffer for measurement with the LUMINEX 200 (LUMINEX).

2.8 | RNA isolation, RT-PCR, and quantitative PCR analysis

RNA was extracted from purified B cells cultured in TB-CM, or normal medium as controls, using the RNeasy kit (Qiagen). RNA concentration and purity were quantified via spectrophotometric analyses of 260/280 ratios with a cut-off ratio of 1.8 or above for samples to be used for analysis. Pellets were resuspended in 20 µL of DNAse/RNAse-free water and quantitation using NanoDrop 2000 (Thermo Fisher). For quantitative analysis of messenger RNA (mRNA), 1 µg of RNA from each sample was reverse-transcribed using iSCript reverse transcription Supermix (Bio-Rad). The complementary DNA (cDNA) was diluted to 1:5 in nuclease-free water, and 5 µL was used in the polymerase chain reaction (PCR). iTaq Universal SYBR green Supermix (Bio-Rad) and gene-specific primers were added to the reverse transcriptase reactions and run on the CFX96, C1000 system

quantitative (qPCR) machine (Bio-Rad). The primer pair sequences are as follows: IL-10 forward 5′-GTGATGCCCCAAGCTGAGA-3′ and reverse 5′-CACGGCTTGCTCTTGTTTT-3′; internal normalizer gene GADPH forward 5′-TTAAAAGCAGCCCTGGTGAC-3′ and reverse 5′-CTCTGCTCCTCCTGTTCGAC-3′. Data were collected in the SYBR channel, and the final cycle was followed by a thermal melt curve. The relative expression to reference GAPDH levels were calculated using the $\Delta\Delta C_t$ method (ΔC_t = ΔC_t treated−ΔC_t control), and the results expressed as the fold change over control (Fold change = $2^{-\Delta\Delta C_t}$).

2.9 | Formation of trophoblast spheroids

For formation of trophoblast spheroids, 4 × 10^5 Sw.71-GFP cells were seeded into each well of a 96-well ultra-low attachment plate (Sigma-Aldrich) and centrifuged at 400*g* for 5 minutes to cluster the cells together. The plate was incubated for 48 hours to allow the clumped trophoblast to form into a spheroid. The spheroids were individually collected and transferred onto other plates for migration or invasion assays. This in vitro model of trophoblast implantation is described in full by You et al.[15]

2.10 | Trophoblast spheroid invasion assay

The matrigel invasion assay directly measures the invasive capability of the trophoblasts as conditioned by the environment. Briefly, a trophoblast spheroid was carefully plated into the center of a well layered with a matrigel (Corning Inc) solution comprised of matrigel and the treatment medium. Serum-free media supplemented with treatment medium was used to drive trophoblast invasion as measured by the presence of cellular protrusions within the matrigel layer. Decidualized stromal cell-conditioned medium and serum-free culture medium were used as positive and negative controls for invasion, respectively. The invasion of trophoblast cells into the matrigel matrix was monitored from 2 to 4 days and visualized under the Revolve Pro microscope (Echo Laboratories). To indicate the level of activity of individual spheroids across biologically independent experiments, a scoring system was devised based on calculated area and visual characteristics of 2D images. Differences in spheroid activity assessed at 48 and 96 hours of culture were scored as follows: 0 = cell death; 1 = viable spheroid without change in area; 2 = >10% increase in area; 3 = >10% increase in area with peripheral protrusions; 4 = >10% increase in area and prominent peripheral protrusions. Area ($A = \pi r^2$) was calculated from 2D images where radius (*r*) was set as half the average of two perpendicular diameter lengths of the horizontal and vertical axes.

2.11 | Trophoblast spheroid migration assay

For the migration assay, trophoblast spheroids were individually seeded in a 96-well plate and grown in serum-free culture media supplemented with experimental treatment medium. The spheroids were monitored for 48 hours with time-lapse documentation at 2-hour intervals using the IncuCyte ZOOM System (Essen BioScience). Conditioned medium from decidualized stromal cells and normal culture medium were used as positive and negative controls, respectively. To indicate the level of activity of individual spheroids across biologically independent experiments, a scoring system was devised based on visual characteristics of 2D images. Differences in spheroid activity assessed at 0 and 48 hours of culture were scored as follows: 0 = cell death; 1 = viable spheroid; 2 = viable spheroid with migration of trophoblast cells; 3 = viable spheroid with extensive migration of trophoblast cells.

2.12 | Statistical analysis

All experiments were performed in triplicate. The results were analyzed for significant differences using the Student's *t* test or the Mann-Whitney test for parametric and nonparametric samples, respectively. For multiple comparisons, one-way ANOVA with Dunnett's post hoc test was used. Data are presented as the mean ± standard error of the mean. Differences between groups were considered statistically significant at *P*-value <.05 as analyzed using GraphPad Prism 7.03 software (GraphPad).

3 | RESULTS

3.1 | Trophoblast cells modify the phenotype of B cells and reduce pro-inflammatory cytokine production

Our first objective was to determine whether trophoblast cells could influence B-cell differentiation. To test this, we cultured purified peripheral blood B cells with either immortalized first-trimester trophoblast cells, trophoblast-conditioned medium, or normal medium as controls for 48 hours. Cells were subsequently collected, and the B cell phenotype determined by flow cytometry. Figure 1A shows representative flow cytometry panels for the evaluated $CD19^+$ B cells subsets: $CD24^{hi}CD27^+$ regulatory B cells, $CD24^{hi}CD38^{hi}$ transitional B cells, $CD27^+IgM^+$ memory B cells, and $CD38^{hi}CD27^{hi}$ plasmablasts. Our results indicate that trophoblast cells can directly, or indirectly by secreted factors, induce B cells to undergo differentiation characterized by a significant increase in the proportion of B regulatory, transitional, IgM-memory, and plasmablast subpopulations (Figure 1B). The fact that culture with trophoblast-conditioned medium or trophoblasts directly yielded similar alterations in phenotypes suggested that the greater part of the observed phenotypic changes can be incurred through soluble factors present in the conditioned medium rather than cognate interactions with the trophoblast cells. For this reason, in all following experiments we focused on using B cells cultured with trophoblast-conditioned medium and refer to them henceforth as trophoblast-educated B cells or TE-B cells.

Next, we characterized the cytokine/chemokine profile of TE-B cells by multiplex assay analysis (Figure 2). Interestingly, B cells cultured in trophoblast-conditioned medium showed major differences

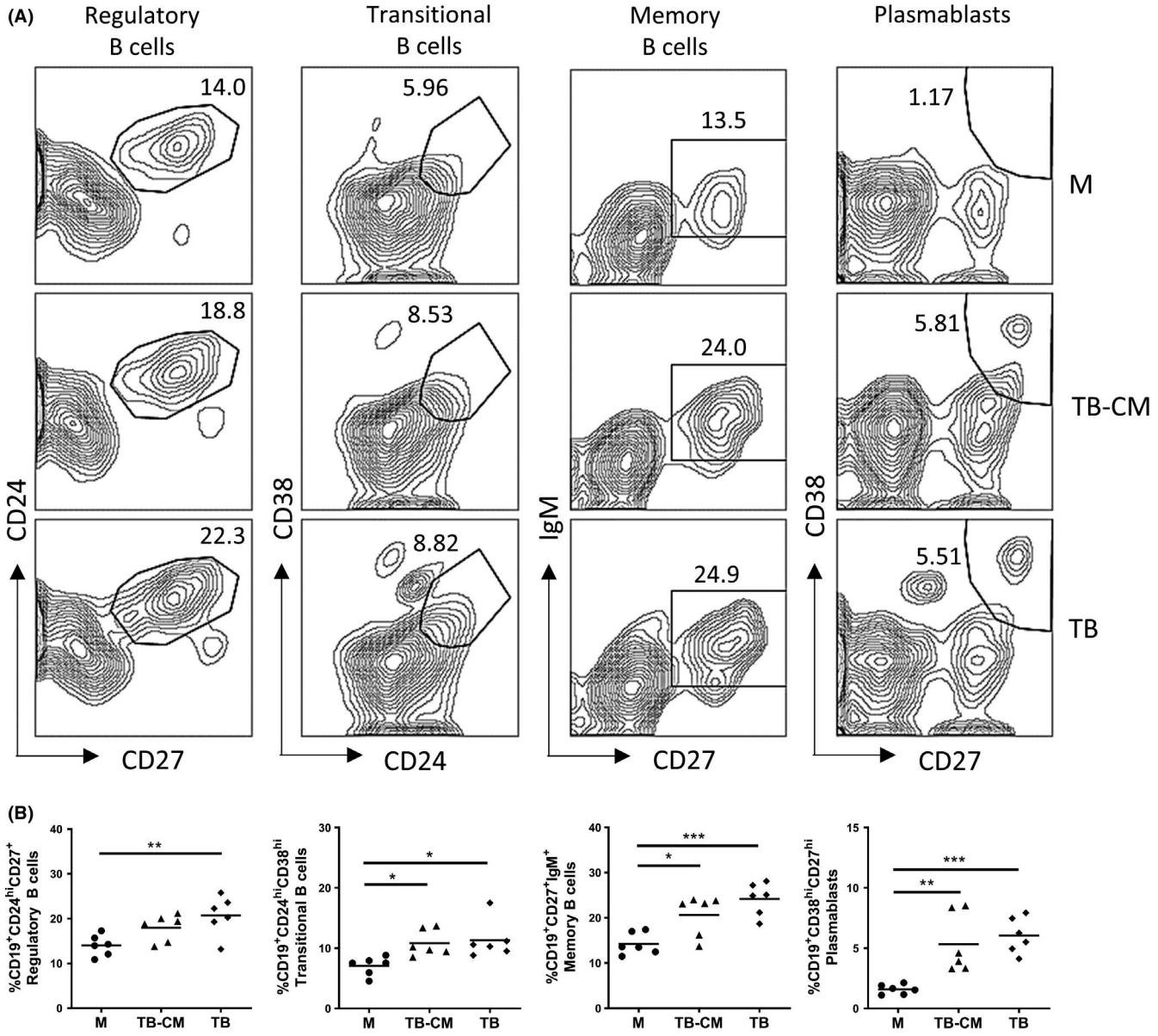


FIGURE 1 Trophoblast cells modify B-cell phenotype. Purified B cells (10^5 cells/100 μL medium) were cultured in normal medium (M), trophoblast-conditioned medium (TB-CM), or directly co-cultured with trophoblast (TB) cells. After 48 h, recovered B cells were stained with B cell lineage surface markers CD19, CD24, CD27, CD38, and IgM to discriminate between the Breg, transitional, IgM-memory, and plasmablast subpopulations. Representative flow cytometry panels indicating gating strategy shown in (A), with combined results from three independent experiments performed in duplicate shown in (B). $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$

in their secreted cytokine profile, with concentrations of the pro-inflammatory cytokines IL-17, IL-1β, IL-6, TNFα, and the chemokine RANTES, all significantly reduced compared to that of control medium. All other analytes were not significantly different between cultures. This indicated that secreted factors found in the conditioned medium from trophoblast cell cultures dampened the basal inflammatory cytokine profile in B cells.

The modification in phenotype and the general reduction in pro-inflammatory cytokines alluded to the induction of regulatory properties in these TE-B cells. To verify, we looked specifically at the capacity of these cells to generate the regulatory cytokine IL-10. Expression of IL-10 mRNA was significantly increased in B cells cultured in trophoblast-conditioned medium compared to control medium (Figure 3A). Similarly, assessment of intracellular IL-10 indicated that the percentage of IL-10-producing B cells increased with culture in trophoblast-conditioned medium. A further increase was shown in B cells cultured with trophoblast cells directly, which yielded similar percentages of IL-10^+ cells to that observed with CpG (TLR9 agonist), a known factor responsible for the expansion of IL-10^+ B cell frequencies in humans[16] (Figure 3B). Lastly, analysis of surface markers showed that IL-10^+ B cells have increased expression of CD24, CD38, and IgM, and

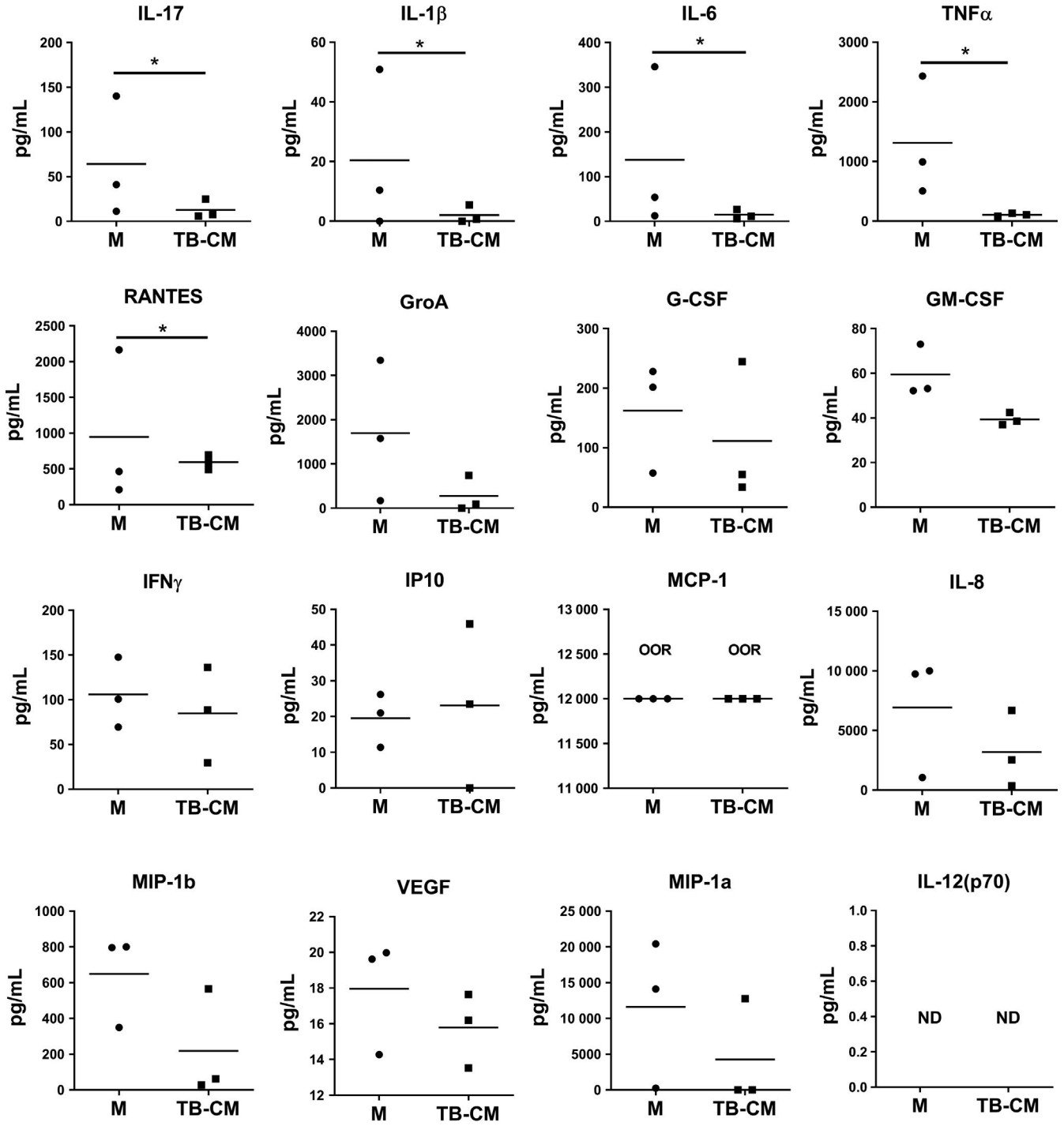


FIGURE 2 Trophoblast-educated B cells have diminished pro-inflammatory cytokine production. Cell-free supernatant from cultures of purified B cells incubated in trophoblast-conditioned medium (TB-CM) or control medium (M) for 48 h were quantified against TB-CM alone using Bio-Plex® multiplex assay. Results were expressed in pg/mL ± SEM with data presented as concentration minus concentrations found in TB-CM alone. IL-12 was not detected (ND) and MCP-1 readouts were out of range (OOR) and could not be extrapolated in the assay. Data represent pooled supernatants of duplicate wells from three independent experiments. *$P < .05$

intermediate expression of CD27, similar to that of CpG-generated IL-10$^+$ B cells. The surface marker expression profile indicates that IL-10$^+$ B cells are found in the suppressive B-cell subsets, namely within the groups of regulatory B cells (CD19$^+$CD24$^+$CD27$^+$), transitional B cells (CD19$^+$CD24^{hi}CD38^{hi}), and IgM-memory B cells (CD19$^+$IgM$^+$CD27$^+$; Figure 3C).

3.2 | TE-B cells regulate T-cell proliferation and cytokine production

B cells perform a range of immunological functions, some of which require close interactions with T cells. At the implantation stage, various T-cell subsets participate in the development of a receptive

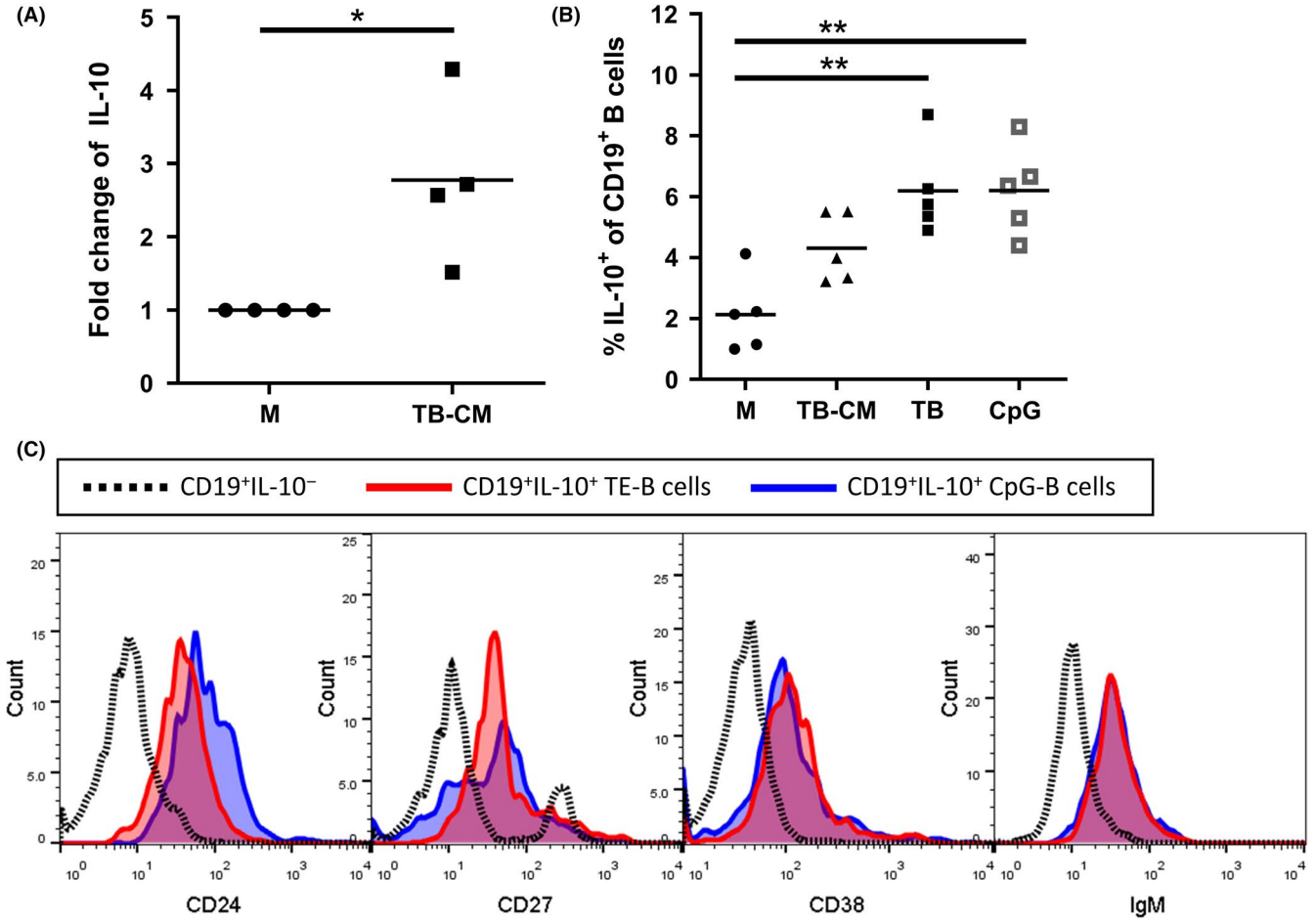
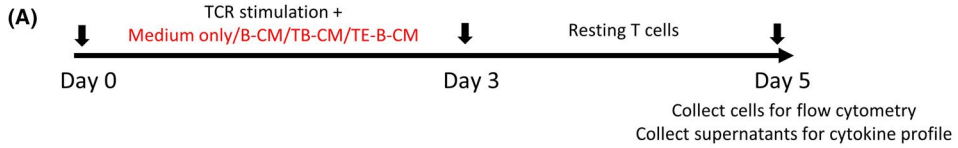


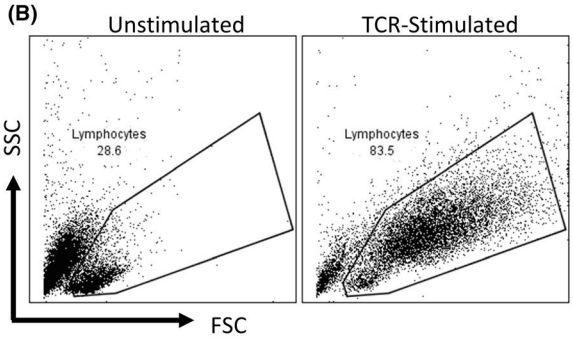
FIGURE 3 Trophoblast cells secrete factors that upregulate IL-10 expression in B cells. A, Purified B cells were cultured with trophoblast-conditioned medium (TB-CM) or normal medium (M) for 48 h after which B cells were processed for IL-10 mRNA expression by RT-qPCR analysis. Results are shown as fold increase over controls. B, Production of IL-10 by B cells cultured with M, TB-CM, or trophoblast cell (TB) cells directly was assessed by intracellular flow cytometric methods against positive control cultures stimulated with CpG. Results shown represent data collected from 4-5 independent experiments. C, Expression of surface markers CD38, CD24, CD27, and IgM on $CD19^+IL\text{-}10^+$ B cells after trophoblast education (red) and CpG treatment (blue) was compared to non-IL-10 producing B cells (dotted line). $^*P < .05$, $^{**}P < .01$

endometrium. Critical events such as generation of Th1/Th2/Th17 cells, development of memory T cells, and induction of Treg cells require an expanded $CD4^+$ T-cell subset to generate these lineages.[17-19] Post-implantation, decidual $CD4^+$ T cells are in much lower proportions due to the general predominance of $CD8^+$ T cells.[4] Here, we explored whether B cells differentiated under the influence of trophoblast secreted factors are functional and able to influence T-cell subset composition and T-cell cytokine production.

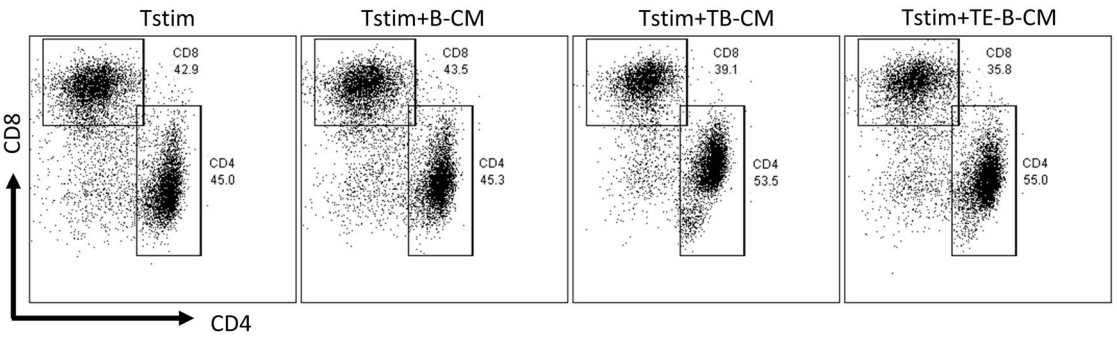
To test this hypothesis, we non-specifically stimulated purified T cells into proliferation with anti-CD3 and anti-CD28 antibodies alone, or in the presence of conditioned medium from cultures of normal B cells, trophoblast cells, and TE-B cells. Cells were then left to rest after which CD4 and CD8 T-cell subset composition and cytokine expression was analyzed (Figure 4A). Representative flow cytometric panels and gating strategy for the $CD4^+$ and $CD8^+$ T-cell subsets are shown in Figure 4B, as well as the tabulated $CD4^+/CD8^+$ ratios following differentiation. Our data indicated that exposure of proliferating T cells to trophoblast or TE-B-cell-conditioned media increased the proportion of $CD4^+$ T cells compared to proliferating T cells cultured in medium from B cells alone (Figure 4C). We next examined whether the expansion in $CD4^+$ T cells was a result of an increase in the Treg population. Whilst TGFβ, the positive control for $CD4^+Foxp3^+$ Treg expansion, generated the highest percentage of $CD4^+Foxp3^+$ Tregs, no such response was observed in our expanded populations suggesting that conditioned medium from TE-B cells are not responsible for Treg differentiation (Figure 4C).

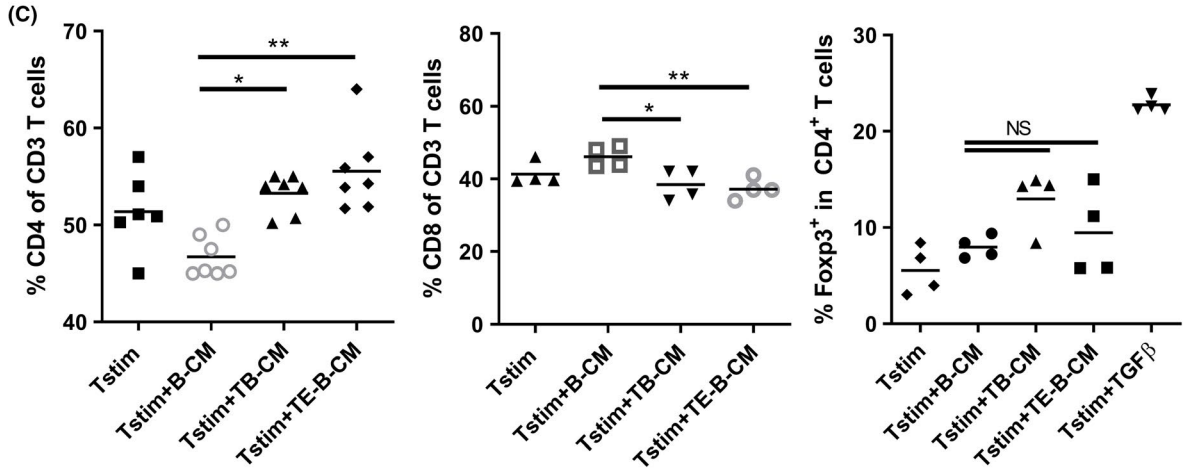
However, the presence of TE-B-cell-conditioned medium during TCR stimulation had an independent effect on the cytokine profile of proliferating T cells. We observed a significant increase in the expression of the pro-inflammatory cytokines IL-6 and IL-8 by T cells exposed to secreted factors from TE-B cell cultures compared to conditioned medium from cultures of trophoblasts and normal B cells (Figure 4D). Both IL-6 and IL-8 are cytokines that have been





Treatment	$CD4^+/CD8^+$ ratio
Tstim	1.24
Tstim+B-CM	1.01
Tstim+TB-CM	1.38
Tstim+TE-B-CM	1.49





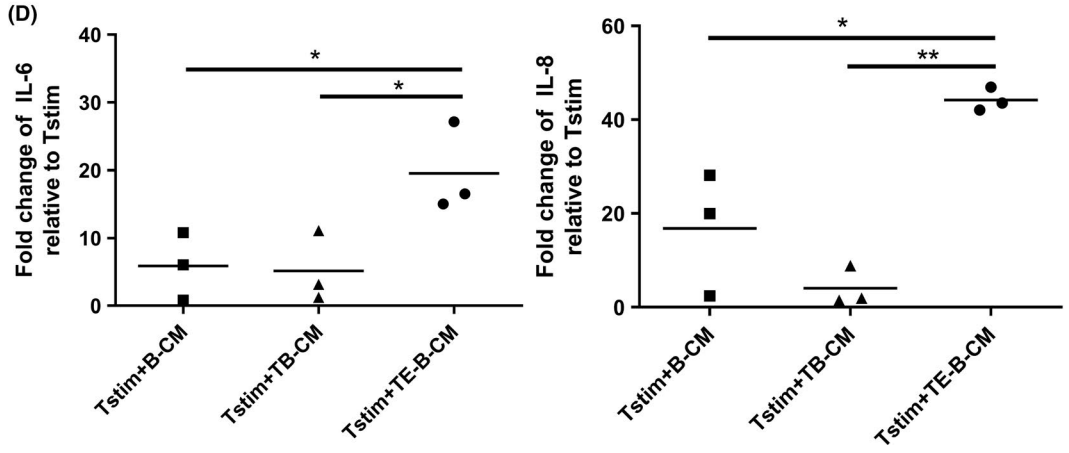


FIGURE 4 TE-B cells impact on T-cell proliferation and cytokine production. Purified T cells were (A) TCR-stimulated with anti-CD3 and anti-CD28 antibodies in normal medium alone (Tstim), or with addition of conditioned medium obtained from cultures of normal B cells (B-CM), trophoblast cells (TB-CM), or TE-B cells (TE-B-CM). B, Representative flow cytometry plots comparing side and forward scatter of unstimulated and TCR-stimulated T cells (upper panels), the gating strategy for $CD4^+$ and $CD8^+$ subsets (lower panels), and the summarized $CD4^+/CD8^+$ ratios. C, Graphical presentation of data combined from a minimum of four independent experiments showing $CD4^+$ and $CD8^+$ T cells as a percent of all $CD3^+$ T cells and expression of transcription factor Foxp3 in $CD4^+$ T cells. D, Fold change increase of IL-6 and IL-8 cytokine production by proliferating T cells cultured in indicated conditioned medium over normal medium controls. $^*P < .05$, $^{**}P < .01$

repeatedly shown as important factors in implantation as reduced levels have been correlated with a reduction in trophoblast invasion resulting in early miscarriages.[20,21] These findings demonstrate a network of cell communication where trophoblast cells promote B-cell differentiation into unique B-cell subtypes with the capacity to regulate T-cell proliferation and cytokine production.

3.3 | TE-B cells contribute to the control of inflammation at implantation

To investigate the potential functional role of TE-B cells at implantation, we utilized a novel 3D in vitro model to evaluate trophoblast migration and invasion.[15] Trophoblast cells were grown as blastocyst-like spheroids and transferred onto matrigel-coated wells and assessed for evidence of cellular protrusions indicative of invasion into the matrigel matrix. Whilst stromal cell-conditioned medium clearly induced prominent protrusions as expected, conditioned medium from TE-B cells or control B cells elicited effects similar to that of media controls (Figure 5A,B, left panel). Similarly, stromal cell-conditioned medium induced rapid attachment and migration of trophoblast cells (from 12 hours), an effect not replicated with conditioned medium from TE-B-cell cultures. Furthermore, TE-B cells did not impact on trophoblast attachment and migration induced by stromal cell-conditioned medium (Figure 5B,C, right panel) indicating that TE-B cells did not secrete factors that directly or indirectly affected trophoblast attachment, migration, or invasion.

We then examined if TE-B cells might have an indirect effect on trophoblast invasion and migration via modification of T-cell activity. We collected the cell-free supernatant from rested T cells non-specifically stimulated in the presence of normal medium or conditioned medium from TE-B or normal B-cell cultures (Figure 6A). These supernatants were combined with matrigel and plated into individual wells for trophoblast invasion assays. Conditioned media from stimulated T cells alone or in combination with TE-B cell or B cell–conditioned mediums were able to induce protrusions from trophoblast cells with varying consistency (Figure 6B and 6C, left panel). However, no conditioned media could induce migration of trophoblast cells (Figure 6D and 6C, right panel). Taken together, these results suggest that soluble factors produced by T cells in a non-inflammatory state have a limited effect on the functional activity of trophoblast cells.

Th_1 inflammation is essential for implantation; however, the level of inflammation should fall within a range that is not detrimental to the embryo. In these experiments, we investigated whether TE-B cells could modulate the level of inflammation in a novel trophoblast model of deleterious inflammation where conditioned medium from cultures of PMA/ionomycin activated pre-primed T cells are added to trophoblast spheroids. By combining TCR stimulation by anti-CD3/CD28 antibodies with PMA/ionomycin stimulation which activates the cells through the $Ca2^+$/calmodulin-dependent and PKC signaling pathways, we were able to induce heightened activation in T cells. The resultant conditioned medium derived from these supra-activated cells is predominantly comprised of pro-inflammatory cytokines. Incubation with the activated T cell–conditioned medium resulted in restricted spheroid growth and active disintegration of peripheral cells as visualized by a fuzzy outermost layer of dead trophoblast cells clearly identifiable by 96 hours in invasion assays and 48 hours in migration assays (Figure 7). This cell death was confirmed by CellTox green dye binding (Dimova T., Yuan Y., Aldo P., and Mor G., manuscript in preparation). To determine whether TE-B cells could dampen the deleterious inflammatory response, conditioned medium from TE-B cell or normal B cell cultures was added to rested TCR-stimulated T cells prior to PMA/ionomycin activation, with resultant supernatants added to wells containing trophoblast spheroids and observed for invasion and migration capacity (Figure 7A). Soluble factors from these activated T cells clearly inhibited generation of cellular protrusions; rather, death of the outer spheroid cells is evident (Figure 7B,C, left panel). However, activation of T cells in the presence of conditioned medium from TE-B cells could negate this effect, as indicated by increased survival of these trophoblast cells. This effect was also seen when conditioned medium from normal B cells was used. In a similar manner, medium from activated T cells inhibited spheroid growth, attachment, and migration; rather, death of peripheral trophoblast cells was observed (Figure 7C,D, right panel). This outcome could be inhibited by prior addition of conditioned medium from TE-B cells or normal B cell cultures, although conditioned medium from TE-B cells was also able to induce partial trophoblast migration. Together these results suggest that soluble factors produced by TE-B cells may play a role in the regulation of inflammation such that it can protect against embryo cell death and contribute to maintaining the cytokine environment necessary for progression of implantation.

4 | DISCUSSION

Pregnancy is a delicate condition requiring a complex and coordinated interplay of immune cells, endometrial cells, and endocrine factors. From the outset, the maternal immune system is tasked with protecting the mother against foreign assault as well as protecting the conceptus from damage. The conceptus on the other hand imposes an influence on the maternal immune system such that it is

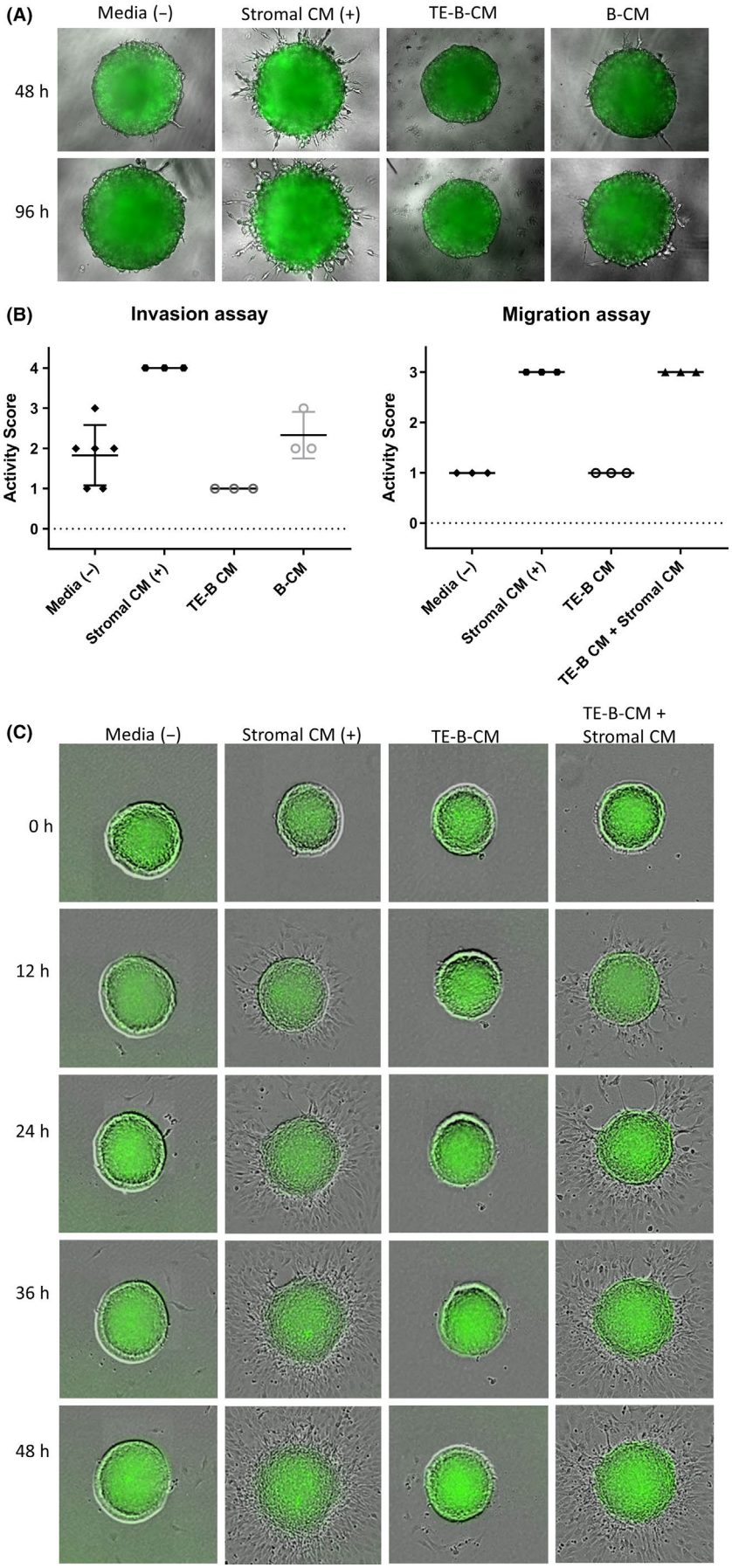


FIGURE 5 TE-B cells do not express soluble factors that affect the invasion and migration activity of trophoblast spheroids. A, For invasion assays, trophoblast spheroids were cultured on matrigel solution combined with normal medium (negative control) or conditioned medium from decidualized stromal cell cultures (stromal CM) as a positive control, or cultures of TE-B cells (TE-B-CM) or normal B cells (B-CM). The formation of trophoblast cell protrusions indicative of invasion into the matrigel matrix was monitored for up to 96 h, with protrusions clearly generated by stromal CM as expected, but not from any other conditioned or control medium. Representative photographs at 48- and 96-h timepoints of the trophoblast spheroids in the invasion assay are shown. B, Independent invasion (left panel) and migration (right panel) experiments were assessed and given an activity score. Combined data from at least three biologically independent experiments are shown. C, For migration assays, trophoblast spheroids were individually seeded into 96-well plate with attachment and migration of trophoblast cells monitored for 48 h. Stromal CM induced cell attachment and migration; however, no such effect was seen with control or TE-B-cell-conditioned media. TE-B-CM was also not capable of inhibiting migration induced by stromal CM. Representative photographs of trophoblast spheroids at 0-, 12-, 24-, 36-, and 48-h timepoints in the migration assay are shown. All photographs were taken using the Revolve Pro microscope in (A), or the IncuCyte ZOOM System in (B) at 200× magnification

accepted into the uterine microenvironment despite its semi-allogeneic nature. At implantation, feto-maternal immune cross-talk is initiated by the trophoblast cells. Trophoblast-immune interactions are categorized into three stages: (a) recruitment—where the trophoblast transmits signals to encourage influx of immune cells in the implantation site, (b) education—where the trophoblast influences the phenotype and function of immune cells, and (c) response—where the immune cells educated by the trophoblast cells carry out functions that support implantation and placental formation.[22] The trophoblast's role in the recruitment and phenotype modification of some immune cells has been studied over the years, including the trophoblasts impact on the form and functions of monocytes, NK cells, regulatory T cells, macrophages, and dendritic cells.[2,6,7,23,24] Interestingly, the B cells' role in implantation has not been extensively explored. In this study, we focused on the effects of trophoblast cells on B-cell phenotype and function, and the subsequent role of these trophoblast-educated B cells (TE-B cells) in the context of implantation.

B-cell lymphopoiesis generates a diverse repertoire of peripheral B cells, starting from B cells exiting the bone marrow and entering peripheral circulation as immature transitional B cells and naïve B cells. Upon acquiring cognate antigens in secondary lymphoid locations, B cells become activated and differentiate into memory B cells, plasmablasts, or fully differentiated plasma cells bearing combinations of surface markers including CD21, CD24, CD27, CD38, and IgM.[25] In recent years, B cells have been attributed a role in pregnancy due to their production of protective antibodies against paternal antigens as well as the production of damaging auto-antibodies that have been implicated in some pregnancy pathologies.[26] IL-10-producing regulatory B cells have also been identified as important cells in pregnancy well-being.[27] In our study, we show that trophoblast cells have a profound effect on the phenotype and cytokine production of B cells, mainly by inducing the differentiation of B cells into transitional B cells and committed B-cell lineage subsets such as regulatory B cells, IgM-only memory B cells, and plasmablasts. We hypothesize here that the Sw.71 trophoblast cells preferentially differentiate and expand B cells that can potentially function for immune regulation (regulatory B cells and IL-10$^+$ transitional B cells) as well as protection against foreign antigen assaults (memory B cells and plasmablasts).

It is tempting to speculate that the observed effect on B cell phenotype and cytokine profiles may stem from the polarizing effect of Sw.71 trophoblast-derived exosomes, a phenomenon that has been previously reported in monocytes wherein their recruitment and differentiation were modified to support trophoblast function.[7] In the same study, trophoblast-derived exosomes also decreased the production of pro-inflammatory cytokine IL-17 and chemokines CCL5 and macrophage migration inhibitory factor (MIF). Our results emulate their findings of cell-contact-independent modification of immune cells by Sw.71 cells, with B cells undertaking differentiation into specific B cell lineages and production of reduced levels of pro-inflammatory cytokines IL-17, IL-1β, IL-6, and TNF-α. Furthermore, Sw.71 cells are characterized as representing extravillous cytotrophoblasts that express fetal fibronectin secretin, cytokeratin 7, vimentin, and HLA-G, as well as low levels of human chorionic gonadotrophin (hCG).[13] It is possible that hCG from the Sw.71 trophoblast cells may have also influenced the observed phenotypic changes in B cells. There is growing evidence that hCG can mediate immune-modulatory effects that promote tolerance at the maternal-fetal interface specifically in early pregnancy, and recent studies have demonstrated the capacity of hCG to expand the population of CD24^{hi}CD27$^+$ regulatory B cells as well as boost IL-10 production in CD19$^+$ B cells.[12,28-30] Additionally, hCG has been shown to alter the secretory profile of dendritic cells and human endometrial epithelial cells to support endometrial receptivity and blastocyst implantation.[31,32]

Feto-maternal cross-talk in early pregnancy is essential for the establishment of maternal immune tolerance, regulation of trophoblast activity, and remodeling of the uterine spiral arteries.[33] Local immune cells in the endometrium are hypothesized to possess embryotrophic properties by providing a cytokine network that controls the immune and endocrine systems, as well as trophoblast function at implantation. The expression of cytokines mediates the embryo-maternal paracrine dialog necessary for the early stages of implantation and trophoblast invasion.[34,35] Our findings demonstrate a modified cytokine profile of TE-B cells, suggesting their involvement in the balance of cytokines in the maternal-fetal interface which may contribute to implantation success. The decreased production of pro-inflammatory cytokines IL-17, IL-1β, IL-6, and TNF-α of TE-B cells can potentially downregulate inflammatory reactions and provide cytokine regulation in a predominantly pro-inflammatory environment required during implantation. Moreover, the increased mRNA expression of regulatory cytokine IL-10 indicates that the TE-B cells may also have the capacity to counteract inflammation in the microenvironment when needed. Recently, IL-10 produced by B cells has been demonstrated to provide protection against fetal death in murine pregnancies jeopardized by the presence of lipopolysaccharide (LPS),[36] further highlighting the influence of B cells in the immune balance at the maternal-fetal interface. The findings on B cell phenotype demonstrate that trophoblast education of B cells helps set the stage for a balanced immune condition that is conducive for implantation.

Although considered as scarce populations in the uterine microenvironment, B cells are present and active in the endometrium and decidua.[17,37,38] In this study, we focused on the interaction of B cells and T cells, specifically the effect of TE-B cells on T-cell subset distribution and cytokine production. In most studies of pregnancy, the CD4$^+$/CD8$^+$ T-cell ratio has been reported to be approximately 1 or slightly decreased as the number of CD8$^+$ T cells has been found to be higher than CD4$^+$ T cells.[39] However, in pre-pregnancy endometrium, which is arguably the best representation of the endometrium at the onset of pregnancy, there is a predominance of CD4$^+$ T cells and an increased CD4$^+$/CD8$^+$ ratio compared to term decidua and peripheral blood[17,40] presumably in preparation for differentiation into effector T cells necessary for inflammation. Treatment with TE-B-conditioned media alongside TCR stimulation maintained the

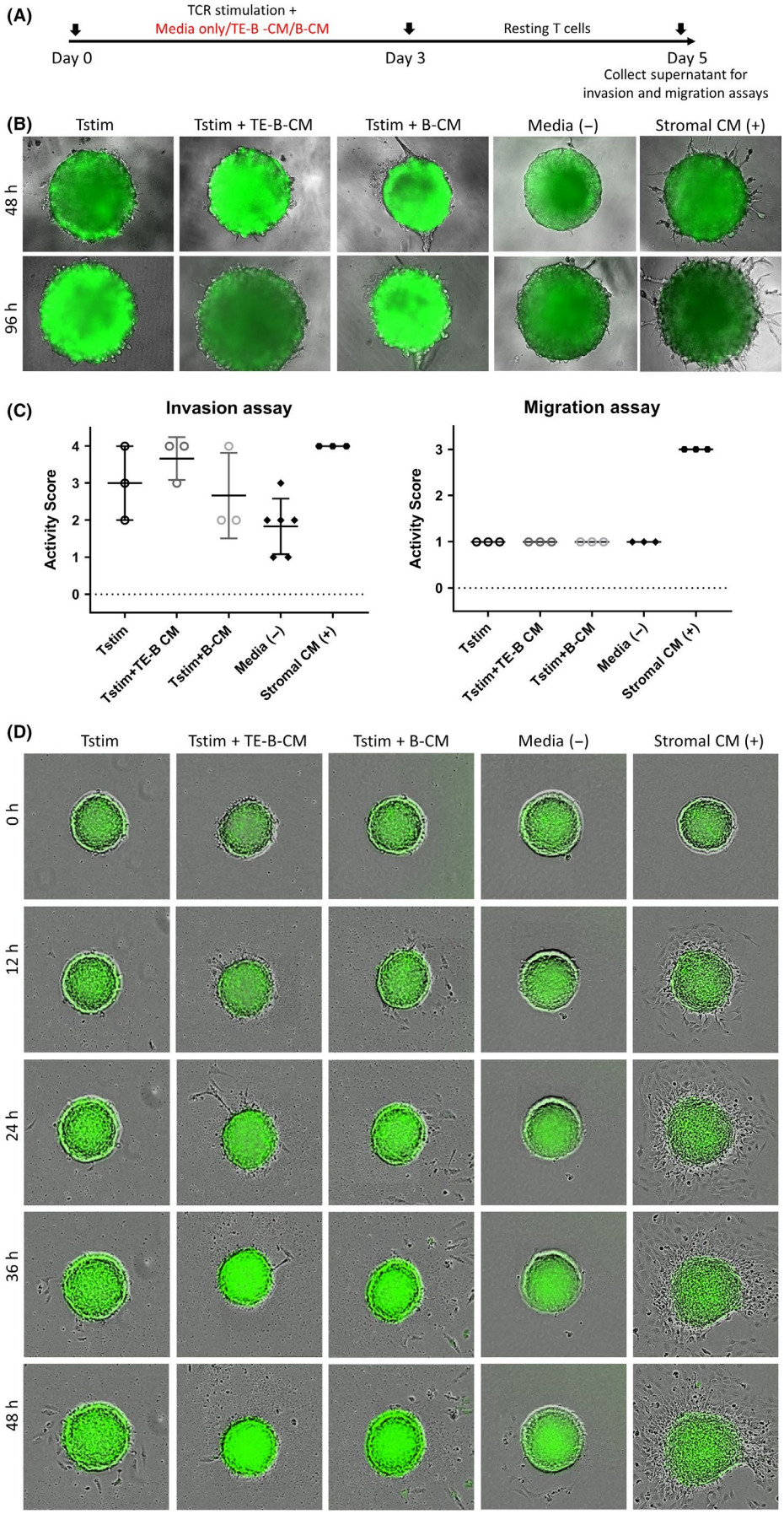

(A)
TCR stimulation +
Media only/TE-B -CM/B-CM
Resting T cells
Day 0
Day 3
Day 5
Collect supernatant for invasion and migration assays
(B)
Tstim
Tstim + TE-B-CM
Tstim + B-CM
Media (–)
Stromal CM (+)
48 h
96 h
(C)
Invasion assay
Migration assay
Activity Score
Tstim
Tstim+TE-B CM
Tstim+B-CM
Media (–)
Stromal CM (+)
(D)
Tstim
Tstim + TE-B-CM
Tstim + B-CM
Media (–)
Stromal CM (+)
0 h
12 h
24 h
36 h
48 h

FIGURE 6 TE-B cells do not promote trophoblast invasion or migration by indirect effect on T cells. A, Timeline of T-cell stimulation, resting period, and collection of media is shown. B, For invasion assays, trophoblast spheroids were incubated on matrigel combined with medium from TCR-stimulated T cells alone (Tstim) or in the presence of conditioned medium from TE-B cells (TE-B_CM) or normal B cells (B-CM). Medium alone or stromal cell medium was used as negative and positive controls, respectively. No effect on invasion by any of the conditioned media was observed. Representative photographs at 48- and 96-h timepoints of the trophoblast spheroids in the matrigel invasion assay are shown. C, Independent invasion (left panel) and migration (right panel) experiments were assessed and given an activity score. Combined data from at least three biologically independent experiments are shown. D, For migration assays, trophoblast spheroids were individually monitored for attachment and migration of trophoblast cells for 48 h. No migration was induced by stimulated T-cell culture media with or without conditioned media from TE-B or normal B cells. Representative photographs of trophoblast spheroids at 0-, 12-, 24-, 36-, and 48-h timepoints in the migration assay are shown. All photographs were taken using the Revolve Pro microscope or the IncuCyte ZOOM System at 200× magnification and are representative of three other independent experiments performed in duplicate

high $CD4^+/CD8^+$ T-cell ratio. However, comparison with other controls such as trophoblast-conditioned medium treatment showed that this is likely a consequence of trophoblast factors and TE-B-conditioned media only enhances that effect. The increase in IL-6 and IL-8 production by TE-B treated T cells is interesting as these cytokines play critical roles in implantation events, particularly in spiral artery remodeling. Inadequate levels of IL-6 and IL-8 have been associated with reduced trophoblast invasion translating to spontaneous abortions.[20,41] IL-8 in particular has been shown to stimulate extravillous trophoblast invasion and is involved in uterine natural killer cell-mediated stimulation of trophoblast invasion.[42,43] The amplified production as per TE-B treatment is indicative of cytokine production being orchestrated to accommodate the invading trophoblasts. Lastly, we determined if TE-B cells can contribute toward expansion of the $CD4^+Foxp3^+$ regulatory T cells via induction of Foxp3 expression in $CD4^+$ T cells. The results showed levels of Foxp3 expression similar to the treatment with just the trophoblast-conditioned medium, supporting the idea that Treg recruitment at implantation is achieved by fetal antigens,[6] and so at this point there is no indication that soluble factors produced by TE-B cells are involved in Treg induction.

Uterine receptivity pertinent for embryo implantation is brought about by a Th1 inflammation in the endometrium, with the process of trophoblast migration and invasion dependent on the chemotactic gradients and cytokines conceived in the implantation milieu by resident immune cells. This inflammatory activity conditions the uterine microenvironment to receive the invading trophoblast and permit cells of embryonic origin to migrate into maternal tissue.[44] The various regulatory mechanisms by both immune and decidualized endometrial cells to keep the pro-inflammatory cytokines and chemokines in check indicate that this inflammation is a tightly controlled process. Previous studies as well as the results thus far in this study provide evidence for a significant role of trophoblast cells in orchestrating control of inflammation. In this study, TE-B cells in a non-inflammatory, non-dangerous state by themselves do not have any significant effect on the activity of trophoblast spheroid cells (Figure 5), although combined with conditioned medium from TCR-stimulated T cells, TE-B cells may promote invasion of trophoblast cells (Figure 6). However, they do demonstrate functional activity within a system with prevailing inflammatory conditions (Figure 7). In our activated T cell–mediated inflammation model, we demonstrated a deleterious condition where the inflammation is elevated to toxic levels for the spheroid. This detrimental effect was negated upon the addition of TE-B-conditioned medium prior to PMA/ionomycin activation of the stimulated T cells. Furthermore, TE-B-conditioned medium treatment in the migration assay resulted in trophoblast cells migrating out of the spheroid in 2 out of 3 independent experiments, as opposed to the activated T-cell treatment alone or that with B cell–conditioned medium, suggesting that soluble factors from TE-B cells may have prevented and interfered with the normal expression and function of the activated T-cell cytokine profiles to a more tolerable level such that spheroid survival and subsequent activity was permitted. This may be due directly to increased levels of IL-6 and IL-8 cytokines found within the TE-B-conditioned medium, or indirectly by changes in the proportions of cytokines produced by activating T cells in the presence of TE-B-conditioned medium, thus creating a more favorable chemotactic gradient and prompting migration to occur. Whilst these effects demand further investigation in primary trophoblast cell lines and more physiological settings such as in vivo mouse models, the in vitro models used here imply that B cells may play a specific regulatory role in maintaining an environment conducive for trophoblast invasion and migration, a hallmark event in implantation.

The confirmation that TE-B cells can only partially restore normal migration activity is a testament that the process of implantation is regulated by multiple cellular players working together. The results gathered from the invasion and migration assays emphasize two things: (a) that controlled inflammation is necessary for implantation events to proceed and that (b) trophoblast-educated B cells contribute in calibrating the inflammation to a tolerable level.

5 | CONCLUSION

In toto, we demonstrate in this study that the interactions and interplay of Sw.71 trophoblast cells and B cells influence the generation of a tolerable inflammatory environment that is favorable for trophoblast health and activity. Successful implantation is dependent on a network of immune cells and endometrial cells working together for a balanced inflammatory response necessary in achieving a receptive uterine microenvironment. Cells of embryonic origin, primarily trophoblasts, have been previously reported as significant modifiers of immune function in order to facilitate acceptance of the invading embryonic cells as well as prime cells

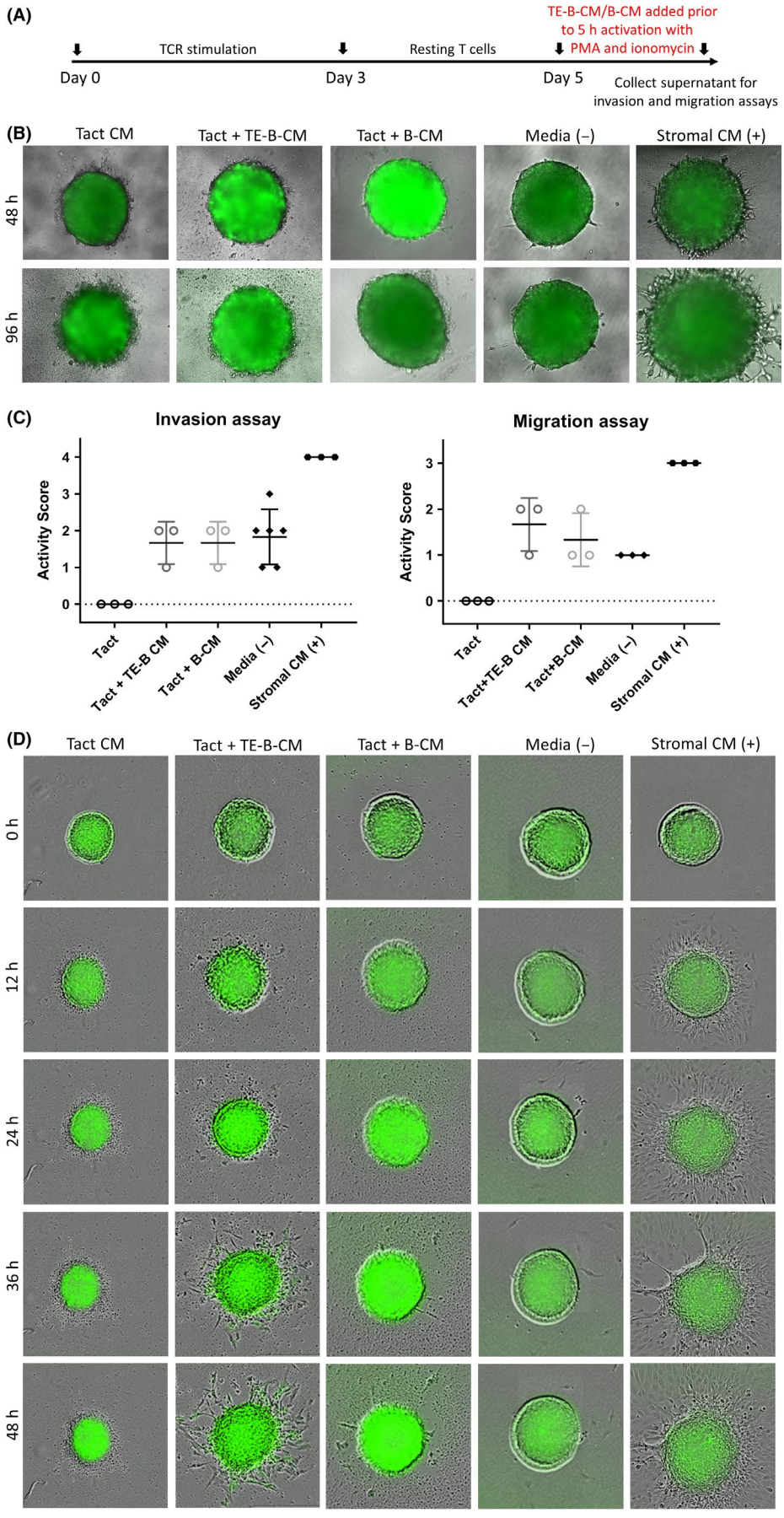

(A)
TE-B-CM/B-CM added prior to 5 h activation with PMA and ionomycin
TCR stimulation
Resting T cells
Day 0
Day 3
Day 5
Collect supernatant for invasion and migration assays
(B)
Tact CM
Tact + TE-B-CM
Tact + B-CM
Media (–)
Stromal CM (+)
48 h
96 h
(C)
Invasion assay
Migration assay
Activity Score
Tact
Tact + TE-B CM
Tact + B-CM
Media (–)
Stromal CM (+)
Tact+TE-B CM
Tact+B-CM
(D)
Tact CM
Tact + TE-B-CM
Tact + B-CM
Media (–)
Stromal CM (+)
0 h
12 h
24 h
36 h
48 h

FIGURE 7 Soluble factors from TE-B cells can protect the trophoblast spheroid from cell damage and aid migration in conditions of deleterious inflammation. A, Timeline of non-specific T-cell stimulation, resting period, and T-cell activation by PMA/ionomycin in normal or conditioned media from cultures of TE-B cells (TE-B-CM) or normal B cells (B-CM). B, Trophoblast spheroid invasion assays. Trophoblast spheroids were plated onto matrigel matrix solutions containing conditioned medium from activated T cells (Tact CM), T cells activated in the presence of conditioned medium from TE-B cells (Tact + TE-B-CM) or normal B cells (Tact + B-CM) and assessed at 48 and 96 h. Normal medium and stromal cell medium acted as negative and positive controls, respectively. C, Independent invasion (left panel) and migration (right panel) experiments were assessed and given an activity score. Combined data from at least three biologically independent experiments are shown. D, Trophoblast migration assays. Individual trophoblasts were plated in the presence of conditioned medium from activated T cells as above and assessed every 12 h for 2 d. Photographs are representative of three independent experiments. All photographs were taken using the Revolve Pro microscope or the IncuCyte ZOOM System at 200× magnification

to ensue immune reactions against foreign antigens should the need arise. In this study, we describe the effect of first-trimester trophoblast cells on B cells in vitro by showing the phenotypic and cytokine profile changes. TE-B cells are comprised of higher percentages of regulatory cells, IgM-memory cells, plasmablasts, and IL-10-producing immature/transitional cells that exhibit reduced cytokine/chemokine production. These TE-B cells were shown to help maintain an expanded $CD4^+$ T-cell subset and stimulate the production of vital cytokines IL-6 and IL-8. Lastly, we demonstrate the role of B cells in counterbalancing inflammation due to activated T cells, enabling our in vitro implantation model of trophoblast spheroids to survive and undertake implantation hallmarks. Collectively, our results imply that trophoblast education of B cells may be one of the regulatory mechanisms in place ensuring the success of implantation in pregnancy.

ACKNOWLEDGMENTS

This work was supported by NHMRC Project (APP1020984) and Fellowship (APP1012386) grants to KD; and Research Training Program Domestic (RTPd) Scholarship and UniSA Research Degree Students International Travel Grant to RG-G. Additional support was provided by the Department of Obstetrics and Gynecology, Yale School of Medicine.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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How to cite this article: Guzman-Genuino RM, Dimova T, You Y, et al. Trophoblasts promote induction of a regulatory phenotype in B cells that can protect against detrimental T cell–mediated inflammation. *Am J Reprod Immunol*. 2019;82:e13187. https://doi.org/10.1111/aji.13187