Ex vivo expansion of human IL-10^{+} B cells with regulatory function

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For Alexander, the most splendid of chaps.

ABSTRACT

Introduction: Solid organ transplantation is the gold standard therapy for many end-stage organ diseases. Although short-term graft survival is excellent due to the use of potent immunosuppressive regimens, incidence of chronic allograft dysfunction and graft failure remains unchanged. Global immunosuppressants are also associated with adverse side effects including severe infection, malignancy and target end-organ damage. Novel, targeted approaches are required to more effectively modulate the immune system and to improve long-term graft outcomes. One such strategy is that of cellular therapy. An enriched population of human IL-10^+ B cells with regulatory function (Breg) has been identified in tolerant, human kidney transplant recipients; mouse IL-10^+ Breg confer protection of allograft in mouse models of transplantation. However, human Breg are poorly characterised, are rare in peripheral blood and there is currently no method by which to obtain such cells in large numbers; thus limiting the potential for human Breg as a clinical therapy. Here we describe, for the first time, *ex vivo* generation and characterisation of human IL-10^+ B cells with both *in vitro* and *in vivo* regulatory function (EXPB10 cells).

Methods: Human $CD19^+$ B cells from healthy individuals were negatively selected and stimulated for 7 days with $CD154^+$ CHO cells and the cytokine combination IL-2, IL-4 and IL-10 to generate IL-10^+ B cells. Phenotype and suppressive function of EXPB10 cells were examined *in vitro.* The ability of EXPB10 cells to regulate *in vivo* immune responses was tested using a clinically relevant humanised mouse model of skin transplantation. Human $CD19^+$ B cells in peripheral blood from both healthy human subjects and patients suffering from squamous cell carcinoma of the skin were examined for human Breg subsets by flow cytometry.

Results: EXPB10 cells produced IL-10, up-regulated expression of the putative Breg marker TIM-1, and suppressed $CD4^+$ T cell proliferation and Th1 cell differentiation *in vitro*. Suppression was partially dependent on IL-10 and CD154. EXPB10 cells were highly activated, $CD19^+$TIM-$1^+CD25^+CD71^+CD73^-$ B cells which demonstrated

dependency on autocrine IL-10. TIM-1 may have modulated intracellular pathways downstream of IL-10, thereby driving EXPB10-mediated suppression of $CD4^+$ T cell responses. A second, CD154-independent suppressive mechanism was identified which may rely on purinergic signaling. EXPB10 cells significantly prolonged human graft survival in a humanised mouse model of skin transplantation and were able to generate and expand functionally suppressive human T regulatory cells (Treg). Examination of $CD19^+$ B cells in human peripheral blood identified an endogenous $CD19^+CD25^+CD71^+CD73^-$ B cell subset which was enriched for TIM-1, similar to EXPB10 cells. This endogenous B cell subset was also significantly enriched in patients with squamous cell carcinoma of the skin compared to a control cohort of healthy human subjects.

Discussion: Human $CD19^+IL\text{-}10^+$ B cells with regulatory function can be generated *ex vivo* and can prolong human allograft survival in a complex, biological environment. Mechanistic data demonstrated that this human Breg subset was dependent on the cognate interaction between CD154 and CD40, as well as the autocrine action of IL-10. TIM-1 may act as a gatekeeper of regulatory function by modulating the EXPB10 cell's response to IL-10. Such subtleties in cellular behaviour offer the potential for further manipulation to better control an immune response. The identification of $CD19^+$ B cells in human peripheral blood with a similar phenotype as EXPB10 cells, and moreover the finding that this subset was enriched in patients diagnosed with cancer, suggests that endogenous Breg may exist with similar cellular properties to that of EXPB10 cells. This potent suppressor population may represent a novel cellular therapy which could be used alone or as an adjunct to other immunosuppressive regimens in the context of transplantation, autoimmunity and inflammation.

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ABBREVIATIONS

7-AAD	7-Amino-Actinomycin D
ACR	Acute Cellular Rejection
ADO	Adenosine
AICD	Activation-Induced Cell Death
APC	Antigen Presenting Cell
APRIL	A Proliferation Inducing Ligand
ATP	Adenosine Triphosphate
B10	IL-10^{+} B Cell
B10pro	Progenitor B Cell
BAFF	B Cell Activating Factor
BANK1	B-Cell Scaffold Protein With Ankyrin Repeats 1
BCMA	B Cell Maturation Antigen
BCR	B Cell Receptor
BLyS	B Lymphocyte Stimulator
Breg	Regulatory B Cell
c.RPMI	Complete media
CAD	Chronic Allograft Dysfunction
cAMP	Cyclic Adenosine Monophosphate
CBA	Cytokine Bead Array
CD	Cluster of Differentiation
CFA	Complete Freund's Adjuvant
CFSE	Carboxyfluorescein Succinimidyl Ester
CHO	Chinese Hamster Ovary
CIA	Collagen Induced Arthritis
CNS	Central Nervous System
CpG-ODN	CpG-oligodeoxynucleotides
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
DC	Dendritic Cell
Dil	1,1'-Dioctadecyl
DSA	Donor-Specific Antibody
DTH	Delayed-Type Hypersensitivity
EAE	Experimental Autoimmune Encephalitis
ELISA	Enzyme-Linked Immunosorbent Assay
EXPB10	Expanded Human IL-10^{+} B Cell
FACS	Fluorescence-Activated Cell Sorting
FCS	Foetal Calf Serum
FoxP3	Forkhead Box P3
GVHD	Graft vs. Host Disease
Hglc	Hyperglycaemic
HIV-1	Human Immunodeficiency Virus-1
HLA	Human Leucocyte Antigen
HSC	Haematopoietic Stem Cell
Hu	Human
IBD	Inflammatory Bowel Disease

ICAM-1	Intercellular Adhesion Molecule 1
IDO	Indoleamine 2,3-Dioxygenase
IFNγ	Interferon Gamma
Ig	Immunoglobulin
IL	Interleukin
iNKT	Invariant Natural Killer T Cell
iNOS	Inducible Nitric Oxide Synthase
IP	Intraperitoneal
iTreg	Induced Regulatory T Cell
IV	Intravenous
JAK	Janus Kinase
KTR	Kidney Transplant Recipient
LAP	Latency-Associated Peptide
Lnglc	Long-Term Normoglycaemic
LPS	Lipopolysaccharide
LTKTR	Long-Term Kidney Transplant Recipient
mAb	Monoclonal Antibody
MDCs	Myeloid Dendritic Cells
MDDCs	Monocyte-Derived Dendritic Cells
mf-1	Mean Fluorescence Minus 1
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MLR	Mixed Leucocyte Reaction
MOG	Myelin Oligodendrocyte Glycoprotein
mRNA	Messenger Ribonucleic Acid
MST	Median Graft Survival
MyD88	Myeloid differentiation primary response gene 88
MZ	Marginal Zone
ND_{50}	50% Neutralisation Dose
NHSBT	National Health Service Blood and Transplant
NO	Nitric Oxide
NON-EXPB10	Non-Expanded Human $CD19^+$ B Cell
nTreg	Natural Regulatory T Cell
PBMC	Peripheral Blood Mononuclear Cells
PD-L1	Programmed Death Ligand 1
PD-L2	Programmed Death Ligand 2
PI3K	Phosphoinositide 3-Kinase
PLA_2	Phospholipase A_2
PMA	Phorbol Myristate Acetate
PRE-INC	48 hour Pre-Incubation
RA	Rheumatoid Arthritis
rATG	Rabbit Anti-Thymocyte Globulin
ROS	Reactive Oxygen Species
RT-PCR	Real-Time Polymerase Chain Reaction
SAC	*S aureus* Cowan I antigen
SCC	Squamous Cell Carcinoma
SCID	Severe Combined Immunodeficiency
SLE	Systemic Lupus Erythematosus
STAT3	Signal Transducer and Activator of Transcription 3
STIM	Stromal Interaction Molecule

T2	Transitional-2
T2-MZP	Transitional 2 Marginal Zone Precursor
TACI	Transmembrane Activator and Calcium Modulator and Cyclophilin Ligand Interactor
TAMs	Tumour Associated Macrophages
TCR	T Cell Receptor
Teff	T Effector Cells
TGFβ	Transforming Growth Factor β
Th1	Type 1 T Helper Cell
Th17	Type 17 T Helper Cell
Th2	Type 2 T Helper Cell
TIM-1	T-cell Immunoglobulin and Mucin domain 1
TLR	Toll-Like Receptor
TNFR	Tumour Necrosis Factor Receptor
TNFα	Tumour Necrosis Factor α
Tr1	Type 1 Regulatory Cell
TRAF	TNFR-Associated Factor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
Treg	Regulatory T cell
TSDR	Treg-Specific Demethylated Region
VPD	Violet Proliferation Dye
WT	Wild Type

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1.0 INTRODUCTION

Solid organ transplantation has become the therapy of choice for many end-stage organ diseases. While short-term graft survival is excellent due to the use of potent immunosuppressants, organ failure due to chronic allograft dysfunction (CAD) remains a leading cause of organ loss and death. Furthermore, chronic immunosuppression is associated with an increased risk of infections, malignancy, cardiovascular morbidity and target organ damage [1, 2].

Improvements in long-term outcomes in terms of allograft survival and function are sorely needed in light of the acute organ shortage [3], and new therapeutic strategies are clearly required. One such strategy is that of cellular therapy: regulatory T cells (Treg), a suppressive T cell population that is able to protect allograft in both murine and humanised mouse models, are currently being introduced into the clinic in an attempt to generate donor-specific tolerance; thus avoiding the wide-spread side-effects of global immunosuppressive reagents [4]. However, the effector immune response is multi-faceted and relies on a wide range of different cells to induce graft rejection [5]; there is now increasing evidence that a similarly multi-armed approach will be required to effectively regulate this profoundly destructive immune response [6, 7].

In transplantation, B cells and alloantibodies have generally been considered to be pathogenic: $CD20^+$ B cells can be found in kidney transplants undergoing rejection [8], alloantibodies produced by B cells are known to cause (hyper) acute graft rejection and B cells may be an important cause of CAD and late graft loss [9]. However, novel clinical data suggest an important role for specific subsets of B cells in allograft survival and development of operational transplantation tolerance. B cell depletion from naive individuals appears to enhance acute T cell-mediated rejection [10, 11]. B cell gene expression patterns associated with transitional B cells, have been described in immunosuppressive drug-free kidney transplant recipients (KTRs) [12-15]; findings mirrored in a non-human primate model of islet transplantation [16].

This growing body of evidence highlights the importance of a recently characterised regulatory population of B cells, or 'Breg,' in the context of transplantation.

The existence of B cells with regulatory function first emerged as early as 1974, with the observation that B cell-depleted splenocytes, through the administration of cyclophosphamide, were unable to suppress delayed-type hypersensitivity reactions (DTH) in guinea pigs upon adoptive transfer [17, 18]. This finding led to the conclusion that DTH responses and T cell function could be regulated by suppressor B cells that were distinct from antibody-producing cells. However, the phenotypic and functional characteristics of this putative regulatory population could not be identified at that time, and so the field was largely abandoned. It was not until the late 1990s when it was found that B-cell-deficient mice were unable to recover from experimental autoimmune encephalitis (EAE), that interest in a potential Breg population resurfaced [19]. Although identification of regulatory B-cell subsets and their mechanisms of action has taken over 3 decades, regulatory B cells are now recognised as an important new component of the immune system.

Breg-mediated cellular therapy may be an additional tool by which long-term outcomes can be markedly improved, not only in transplantation, but also in other diseases of chronic inflammation. However, before such a possibility can be considered, a much greater understanding of human Breg subsets is required in terms of identity, stability and mechanisms of suppression. The use of transgenic mouse models have revealed much about mouse Breg, yet human Breg are proving much more difficult to study. This is largely due to the rarity of such human B cell populations in peripheral blood [20], and the current inability to generate a substantial population of human Breg *ex vivo*. In this project, methods of *in vitro* generation of human Breg are explored, and the role of human Breg is examined within the field of transplantation. Current knowledge and avenues in Breg research are outlined in this section, both in the mouse and human, followed by an outline of hypotheses to be investigated within this project.

1.1 Breg in animal models of inflammation

Mizoguchi and colleagues were the first to coin the term 'regulatory B cells,' when they observed that adoptive transfer of peripheral B cells could alleviate colitis in TCR$\alpha^{-/-}$ mice [21]. Seminal studies at the turn of this century provided conclusive evidence that B cell could suppress inflammation, largely through the provision of the immunosuppressive cytokine, interleukin-10 (IL-10), in murine models of colitis, EAE and arthritis [22-24]. Using such models of chronic inflammation, the function and phenotype of mouse Breg has been probed in depth, forming the foundation upon which later studies into Breg have been based.

The therapeutic potential of Breg was first demonstrated in landmark studies by the Londei laboratory, which utilised an agonistic monoclonal antibody (mAb) to CD40, to effectively induce mouse Breg *in vivo* and treat mice with collagen induced arthritis (CIA) [24, 25]. CIA is a type 1 T helper cell (Th1) –dependent chronic arthritis, which develops after immunisation of DBA/1-TCRβ transgenic mice with type II collagen in complete Freund's adjuvant (CFA) [26]. Activation of arthritogenic splenocytes with antigen and agonistic anti-CD40 generated a B cell population that produced high levels of IL-10 and low levels of interferon gamma (IFNγ). Adoptive transfer of these B cells into mice at the time of CIA induction, inhibited T helper type 1 differentiation, prevented arthritis development, and was also effective in ameliorating established disease. IL-10 proved to be essential for the regulatory function of this B cell subset, as the B cells population isolated from IL-$10^{-/-}$ mice failed to alleviate inflammation. Furthermore, mouse B cells that were isolated from arthritogenic splenocytes and subsequently treated *in vitro* with blocking mAbs to IL-10 and the receptor to IL-10 (IL-10R), were then unable to protect recipient mice from developing arthritis.

Following on from these initial experiments, the CIA model has also been used to illustrate that IL-10^{+} Breg are able to skew T cell differentiation in favour of Treg induction, alongside suppression of pro-inflammatory immune responses [27, 28]. A chimeric mouse in which only B cells are IL-10 deficient, develops an exacerbated form of CIA when compared to chimeric wild type B cell mice (WT). Chimeras

deficient in IL-10^{+} B cells went on to demonstrate a significant decrease in absolute numbers of FoxP3^{+} Treg and a marked increase in Th1 and Th17 cells. Reconstitution of arthritic B cell deficient (μMT) mice with various B cell subsets illustrated that this regulatory function was mediated solely by transitional 2 marginal zone precursor Breg (T2-MZP). Transfer of WT T2-MZP B cells to arthritic IL-10$^{-/-}$ mice further increased the number of Treg and reduced frequencies of Th1 and Th17 cells to levels seen in arthritic WT mice; inflammation was inhibited.

In an alternate model of CIA, the administration of apoptotic thymocytes up to 1 month before the clinical onset of disease also resulted in a protective effect against severe joint inflammation and bone destruction [29]. Splenic B cells produced IL-10 in response to apoptotic cell treatment, in turn inducing T cells to secrete IL-10. In addition, adoptive transfer of B cells from mice that had been treated with apoptotic cells, provided significant protection from arthritis. Inhibition of IL-10 *in vivo* reversed the tolerising effect of apoptotic cell treatment, thereby reinforcing the IL-10-dependency of Breg in this model.

EAE is a Th1-mediated autoimmune disease of the central nervous system (CNS) that models multiple sclerosis in humans [30, 31]. Inflammation is induced by immunising mice with myelin proteins, such as myelin oligodendrocyte glycoprotein (MOG); the pathology is characterised by acute CNS inflammation, demyelination, and paralysis, and is largely mediated by antigen-specific CD4^{+} T cells. In a series of elegant experiments, Fillatreau et al. used a bone marrow chimera system to generate mice in which IL-10 deficiency was restricted to B cells [23]. In the absence of IL-10 production by B cells, a pro-inflammatory Th1 immune response persisted to drive EAE and the mice did not recover. Moreover, disease recovery was dependent on the presence of auto-antigen-reactive B cells, and B cells isolated from mice with disease produced IL-10 in response to auto-antigen stimulation.

Later work has revealed a much more complex role for B cells in this model [32]: Tedder's group found that timing of CD20^{+} B cell depletion greatly influenced alleviation or exacerbation of EAE. CD20 mAb-mediated B cell depletion before EAE induction substantially exacerbated disease symptoms and increased encephalitogenic T cell infiltration into the CNS. Adoptive transfer of splenic IL-10^{+}

B cells before EAE induction normalised EAE in B cell-depleted mice, demonstrating that increased symptom severity had resulted from the depletion of this subset prior to the onset of disease. However, although transfer of this B cell population was maximally suppressive during early EAE initiation, IL-10^+ B cells had no such ameliorating effect during disease progression. Instead, $CD20^+$ B cell depletion during EAE disease progression dramatically suppressed symptoms. Specifically, during the inflammatory course, B effector cells were required for the generation of $CD4^+$ T cells specific for CNS auto-antigen and the entry of Th1 cells into the CNS. These results are important as they highlight that the therapeutic effect of B cell depletion for the treatment of autoimmunity may depend on the relative contributions and timing of opposing B cell activities during the course of disease initiation and pathogenesis.

The role of Breg in animal models of transplantation has only been more recently established. In 2007, Yan et al. were among the first to demonstrate a role for B cell-mediated tolerance in a rat kidney allograft model [33]. Intravenous injection of donor B cells resulted in long-term graft acceptance (median kidney graft survival MST >300 days) in rats transplanted with fully MHC-mismatched kidneys. This tolerance was dependent on B cells and did not occur when donor T cells were injected into the recipients (MST of 17 days). Levels of IL-2, IFN-γ and IL-10 were significantly increased within tolerated grafts compared with rejected grafts. In a rat model of long-term cardiac allograft tolerance, Le Texier and colleagues observed that tolerant allografts were infiltrated by B cells organised in germinal centres, and what is more, that IgG alloantibody responses were profoundly suppressed [34]. Importantly, B cells from tolerant recipients could transfer allograft tolerance. An induction immunotherapy regimen consisting of rabbit anti-thymocyte globulin (rATG) and the depleting mAb to CD20, rituximab, promoted long-term islet allograft survival in non-human primates maintained on rapamycin monotherapy [16]. B lymphocyte reconstitution after rituximab-mediated depletion was characterised by a preponderance of immature and transitional cells, whose persistence was associated with long-term islet allograft survival.

Work by Rothstein's group has established the IL-10-dependency of some Breg subsets associated with allograft tolerance. In a mouse model of allograft tolerance

dependent on the low-affinity antibody ((RMT10) to the cell surface molecule T cell Ig domain and mucin domain protein 1 (TIM-1), long-term allograft survival was found to be dependent on B cells enriched for IL-10 [35]. IL-10^{+} Breg were induced by TIM-1 ligation and could transfer long-term acceptance of islet allografts to otherwise-untreated recipients in an IL-10–dependent fashion, and could also inhibit allergic airway disease. Further characterisation of Breg in transplantation has been achieved using a cardiac allograft mouse model, whereby tolerance is induced by a blocking mAb to CD45RB (anti-CD45RB) [36]. Tolerance could not be achieved in the absence of B cells; moreover, adoptive transfer of B cells restored anti-CD45RB-induced tolerance. Subsequent studies with this model revealed that in contrast to other Breg subsets, B cell-mediated tolerance in this context was in fact IL-10-independent, and relied on cognate interactions involving molecules such as CD40, CD80 and CD86 and ICAM-1 [37, 38]. Work from the same group has recently demonstrated the antigen-specific nature of Breg-mediated tolerance in this model, as well as a role for the immunosuppressive cytokine TGFβ and Breg-induced Treg [39].

Since these initial investigations, the suppressive function of mouse Breg has been described in a multitude of *in vivo* models, including those of autoimmune uveitis [40], diabetes [41], listeria infection [42] and *salmonella* sepsis [43] and tumour immunity [44]. The development of such transgenic models has been fundamental to the elucidation of mechanisms by which mouse Breg are induced and can mediate suppression, and these are discussed in detail later in this chapter. However, whilst mouse models are valuable in establishing the basis of such regulatory populations, it is the identification and characterisation of Breg within human subjects that is needed before any clinical application is possible.

1.2 Human Breg in health and disease

Human Breg has only been identified as recently as 2010, with the isolation of the transitional $CD24^{hi}CD38^{hi}$ human B cell subset found to be enriched with IL-10^{+} B cells in peripheral blood of healthy individuals [20]. This naïve subset, after isolation from the circulation, was able to inhibit pro-inflammatory cytokine production by $CD4^{+}$ T cells stimulated by CD3 mAb. Following *in vitro* stimulation with the co-

stimulatory molecule CD40 as well as brief activation with PMA and ionomycin, more than 12% of human $CD24^{hi}CD38^{hi}$ B cells could express IL-10. This was a marked increase compared to $CD24^{hi}CD38^{-}$ and $CD24^{int}CD38^{int}$ B cells, of which no more than 4% expressed IL-10, and furthermore demonstrated no suppressive activity *in vitro*. Importantly, $CD24^{hi}CD38^{hi}$ human B cells isolated from patients suffering from systemic lupus erythematosus (SLE), were refractory to CD40 stimulation, produced significantly less IL-10 upon stimulation and lacked the suppressive function of those $CD24^{hi}CD38^{hi}$ human B cells from healthy human subjects. Subsequent analysis of this putative Breg subset has demonstrated that healthy $CD24^{hi}CD38^{hi}$ human B cells can inhibit naïve T cell differentiation into Th1 and Th17 cells and promote Treg differentiation by IL-10-dependent mechanisms *in vitro* [45], similar to mouse Breg [27, 28].

Further work by Mauri and colleagues has demonstrated that the IL-10-enriched $CD24^{hi}CD38^{hi}$ human B cell subset is responsible for the activation and maintenance of invariant natural killer T cells (iNKT) through lipid presentation via the expression of CD1d [46]. However, in patients with SLE, this B cell-mediated stimulation of iNKT cells was defective, and was associated with altered CD1d recycling. iNKT cell frequency and function was later restored in patients upon rituximab-mediated B cell depletion and subsequent re-population of the B cell compartment with $CD24^{hi}CD38^{hi}$ human B cells that expressed CD1d comparable to levels of those in healthy subjects. Furthermore, $CD24^{hi}CD38^{hi}$ human B cells were found to be markedly reduced in number in patients with active rheumatoid arthritis (RA), compared with either patients with inactive disease or indeed healthy individuals [45]. Lemoine et al. went on to demonstrate that this same subset, when isolated from tonsillar tissue, was still suppressive but were functionally defective in patients with SLE or RA [47].

The identification of $IL\text{-}10^{+}$ Breg in the transitional B cell subset within human peripheral blood was closely followed in 2011 by the characterisation of another IL-10-enriched human B cells subset, that of memory $CD24^{hi}CD27^{+}$ B cells [48]. It should be noted however, that the fraction of human $CD24^{hi}CD27^{+}$ B cells in peripheral blood that expressed IL-10+ B cells following 5 hours of PMA and ionomycin stimulation was small (approximately 0.6%) and could not suppress an

immune response without prior stimulation. As these B cells express IL-10 upon such stimulation, Tedder has described this subset as human 'B10' cells. A larger population of approximately 5% of peripheral blood B cells, termed progenitor B10 (B10pro) cells, required more extensive stimulation for 48 hours in order to express IL-10. Following stimulation, the human B10 subset was able to suppress pro-inflammatory cytokine production by monocytes through IL-10-dependent mechanisms.

In contrast to $CD24^{hi}CD38^{hi}$ B cells in patients with RA [45], human B10 cells were identified at increased frequencies in patients with autoimmune diseases such as RA, SLE, primary Sjogren syndrome and multiple sclerosis. However regulatory function of these B10 subsets was not tested. A subsequent study by Tedder's group has demonstrated an inverse correlation of $CD24^{hi}CD27^{+}$ B cell frequency and disease progression in systemic sclerosis [49]. This has been reinforced by similar findings in SLE and Graft vs. Host Disease (GVHD), highlighting the possibility of $CD24^{hi}CD27^{+}$ B cells being used as a biomarker for disease progression [50-52]. Together, these findings support the view that altered Breg function may have a significant impact on the natural history of autoimmune disease and can play a vital role in the regulation of inflammation in humans as well as mice.

Whole-genome expression profiling of human IL-10^{+} B cells stimulated with the agonist to toll-like receptor (TLR) 9, CpG, has identified $CD25^{+}CD71^{+}CD73^{-}$ B cells in peripheral blood as those most enriched for IL-10 [53]; interestingly this B cell subset comprised both naïve $CD27^{-}$ and memory $CD27^{+}$ human B cells, indicating that IL-10 production by human B cells upon TLR9-stimulation is not restricted to a developmentally defined B cell subset. Moreover, this IL-10-enriched subset was able to significantly inhibit proliferation of $CD4^{+}$ T cells *in vitro*, at ratios as low as 1:25 B cells to peripheral blood mononuclear cells (PBMC). In addition, production of IgG_4, an immunoglobulin widely believed to possess anti-inflammatory properties [54-56], was selectively confined to $CD25^{+}CD71^{+}CD73^{-}$ B cells. By examining peripheral blood of immunologically tolerant bee-keepers, it was found that B cells specific for the major bee-venom allergen, Phospholipase A_2 (PLA), demonstrated increased expression of IL-10 as well as IgG_4. Excitingly, the frequency of IL-10^{+} PLA-specific B cells increased in allergic patients actually increase in allergic patients

receiving allergen-specific immunotherapy. Van de Veen et al. postulated that IL-10^{+} B cells would predominantly produce anti- or non-inflammatory antibodies. These findings complement mouse studies which demonstrate an altered homeostasis in IL-10^{+} Breg in models of allergic airway inflammation [57, 58]. Furthermore, the memory CD24^{hi}CD27^{+} B cell subset has also been reported to be decreased in human patients with allergic rhinitis and asthma when compared to healthy controls [59].

Both naïve CD24^{hi}CD38^{hi} and memory CD24^{hi}CD27^{+} human IL-10-enriched Breg have also been identified in context of infection and cancer respectively. Serum levels of IL-10 and frequencies of CD24^{hi}CD38^{hi}IL-10^{+} Breg were increased temporarily during hepatic flares in patients with chronic hepatitis B infection [60]. Patients who were diagnosed with gastric cancer and were subjected to a first-line chemotherapy regimen demonstrated differential response to treatment: those patients who showed no progression of the disease also had decreased frequencies of CD24^{hi}CD27^{+} B cells in peripheral blood, whilst those patients who did not respond to chemotherapy, maintained levels of this putative Breg population [61]. No other immune cell population was affected. The identification of human Breg in both healthy individuals as well as in an array of pathological and often chronic inflammatory situations reinforces the importance of this cell-type in maintaining balance within the broader immune system.

Clatworthy and colleagues were the first to uncover the possible involvement of Breg in the maintenance of allograft in transplantation [10]. In 2009, this group reported the early suspension of a randomised controlled trial of induction therapy using the rituximab versus the anti-CD25 monoclonal antibody daculizumab after recruitment of 13 human KTRs: a high incidence of acute cellular rejection (ACR) had occurred in the rituximab group (83% compared with 35% in the daculizumab group). The importance of Breg in transplantation was sharply highlighted by the identification of a B cell signature of tolerance in human KTRs across two multi-centre and continent-specific studies that cross-validated each other's findings [12, 13]. The authors identified two independent cohorts of tolerant renal transplant recipients who were defined by stable graft function and with no immunosuppression for over 1 year. Gene expression profiles and peripheral blood lymphocyte populations were compared to that of KTRs with stable graft function who were receiving

immunosuppression, KTRs who were undergoing chronic rejection as well as healthy controls. Tolerant KTRs were found to have increased percentage ratios of $CD19^+$ B cells to $CD3^+$ T cells in peripheral blood when compared to all control groups other than healthy subjects, in whose levels were equivalent. This increased ratios was due to an increase in total number of $CD19^+$ B cells rather than a decrease in number of $CD3^+$ T cells. Further analysis revealed that the enlarged B cell compartment in tolerant KTRs predominantly comprised transitional $CD24^{hi}CD38^{hi}$ B cells, with a decrease in $CD27^+$ memory B cell subsets. Incidence of donor-specific antibody (DSA) levels was significantly decreased in tolerant patients, and frequencies of activated $CD4^+CD25^+$ T cells were also reduced. In addition, use of gene microarrays and RT-PCR revealed a bias towards differential expression of B cell-related genes and their associated molecular pathways. Although no significant increase in IL-10 production by B cells in any patient group was observed, a significant proportion of B cells from tolerant KTRs did produce TGFβ on *in vitro* stimulation. Combination of some of these and other parameters resulted in an extremely specific and sensitive cross-platform biomarker signature that could predict tolerance in human KTRs. Subsequent analysis of peripheral blood in an alternate tolerant KTR cohort also revealed an increase in memory B cells with inhibitory characteristics including a decreased FcγRIIA/FcγRIIB ratio and an increase in expression of B-Cell Scaffold Protein With Ankyrin Repeats 1 (BANK1), which serves as a preventative signal of hyperactive B cell responses, when compared to KTRs undergoing chronic rejection [14].

Following on from these pivotal studies, a comprehensive analysis has been undertaken to elucidate the role of human B cells in the alloimmune response [62-64]. Our group has observed a transient increase in $CD19^+CD24^{hi}CD38^{hi}$ B cells with regulatory activity, and long-term dominance by naive B cells in peripheral blood during the first year post-transplant, in human KTRs who have been treated with the CD52-depleting monoclonal antibody, alemtuzumab [64]. This B cell subset produced high levels of IL-10 and was capable of inhibiting autologous T cell differentiation towards Th1 cells *in vitro*. Similar increases in transitional B cell subset frequency have also been reported during B cell repopulation following rituximab treatment in human KTRs [65]. Silva et al. analysed a small cohort of operationally tolerant KTRs and found that these patients had a diverse BCR repertoire similar to that of healthy

controls [63]. This observation mirrors findings in the mouse, providing a reassuring link between the mechanisms underlying human and mouse Breg function [66, 67]. Importantly, similar to healthy subjects assessed by Mauri and colleagues two years earlier [20], operationally tolerant patients retained the ability to activate CD40 and signal transducer and activator of transcription 3 (STAT3) signalling pathways in the $CD24^{hi}CD38^{hi}$ B cell subset enriched for human Breg. This ability was significantly decreased in $CD24^{hi}CD38^{hi}$ B cells of patients undergoing chronic rejection as well as GVHD [52, 63], much like in those patients suffering from SLE [20]. These observations together reinforce the importance of this signaling pathway in human Breg function in both an autoimmune and transplant setting.

Whilst this data provides convincing evidence for the role of Breg in animal models of transplantation and the existence of Breg in human transplant recipients, as well as in diseases of chronic inflammation, the dual role of B cells should not be discounted. Similar to work with mouse models of EAE, models of transplantation tolerance have also highlighted the divergent roles for pro-inflammatory and regulatory B cells. Examining the effects of depleting mAbs to CD20 and CD19, Tedder et al. have shown that B cells can either positively or negatively regulate allograft rejection depending on the nature of the allograft and the intensity of the rejection response [68]. It is vital to understand the exact nature, phenotype and methods by which Breg can be induced, before therapeutic strategies can be contemplated.

1.3 Breg Phenotype

Extensive work in mouse models has revealed a diverse and varied range of B cell phenotypes attributed to mouse Breg. The majority of mouse Breg have been identified through their secretion of IL-10, although there are several reports of IL-10-independent Breg, which are later discussed. B cells can be induced to produce IL-10 by a multitude of different stimuli [69-71], and so whilst the expression of IL-10 has been helpful in characterising populations of B cell that can regulate an immune response, the many different cell surface markers attributed to these subsets may also simply be a result of immune activation rather than of suppressive function. The assortment of Breg studies now published has used different means of stimulation by

which to identify IL-10-producing B cells with regulatory function, amongst different experimental settings. This has naturally led to inherent problems when trying to identify a unifying Breg marker similar to FoxP3 in Treg [72], and in so far, these attempts have been unsuccessful [43, 53]. It may be that there are many different subsets of Breg that are developmentally distinct, accounting for this disparity. Alternatively, the microenvironment in which most B cells of many different lineages are activated may be primarily responsible for the induction of suppressive activity. Finally, the identity of Breg may be a culmination of both of these theories: B cells with the capacity to become regulatory (as defined by a currently unknown factor) may be present across the many B cell compartments, but it is the microenvironment which determines whether regulatory or effective function is elicited in these cells.

Currently therefore, several phenotypically distinct subsets of both mouse and human Breg have been described. Mauri's group were the first to identify IL-10^+ B cells with regulatory function enriched in the immature, splenic T2-MZP B cell subset in mice, which displays a $CD19^+CD21^{hi}CD23^{hi}CD24^{hi}CD1d^{hi}$ phenotype [73]. This subset increased in frequency during the remission phase of CIA; moreover adoptive transfer of these cells significantly prevented new disease as well as ameliorating established disease. These developments were associated with an inhibition of antigen-specific T cell activation and a reduction in Th1 functional responses [27, 69, 73]. The transitional $CD24^{hi}CD38^{hi}$ human B cell subset enriched for human IL-10^+ Breg has been likened to the immature T2-MZP mouse subset [74]; prior to CD40-mediated stimulation, human $CD24^{hi}CD38^{hi}$ human B cells were naïve ($CD27^-$), expressed increased levels of IgM, IgD, CD5 and CD1d, and was also $CD10^+$ [20]. $CD21^{hi}CD23^-$ marginal zone (MZ) splenic B cells with IL-10-dependent regulatory function have also been identified in mouse models of infection and autoimmunity, and have been shown upon stimulation by TLR9 agonists or apoptotic cells to express IL-10, to induce Treg and to suppress both $CD4^+$ and $CD8^+$ T cell responses [29, 75, 76].

Tedder was the first to functionally define IL-10^+ B cells in mice, as those B cells that could express IL-10 upon 5 hour stimulation with PMA and ionomycin – these mouse Breg were termed 'B10' cells [32]. This definition was later extended to human B cells that could also produce IL-10 under these same stimulatory conditions [48].

B10pro cells were subsequently defined as those B cells in both mice and humans which could only express IL-10 upon more prolonged activation over 48 hours with CD40 stimulation as well as the 5 hour incubation period with PMA and ionomycin [32, 48, 77]; the theory being that any B cells which did not have almost immediate capacity to express IL-10 but could do after intense stimulation, must be a predecessor to the fully formed B10 cell. The generalised phenotype of B10 cells is $IgM^{hi}IgD^{lo}CD1d^{hi}CD5^{+}CD19^{hi}CD23^{lo}$ [66, 78-80]. Therefore splenic B10 cells share surface markers with multiple, phenotypically defined B cell subsets, including transitional, marginal zone, marginal zone precursor, memory, and B1 B cells [81]. Upon stimulation, 15–20% of splenic $CD1d^{hi}CD5^{+}$ B cells are B10 cells, and up to 50% are collectively B10 and B10pro cells. Similar to mouse B10 cells, the human $CD24^{hi}CD38^{hi}$ human B cell subset enriched for IL-10^{+} B cells express the $CD5^{+}CD1d^{+}$ phenotype [20]. However human B10 cells were conversely identified as enriched within memory cells, specifically the $CD24^{hi}CD27^{+}$ B cell subset described earlier [48]. Whilst this method of Breg identification does reliably detect IL-10-producing B cells, it is limited in that B cells which produce IL-10 under different forms of stimulation (i.e. human $CD24^{hi}CD38^{hi}$ or $CD25^{+}CD71^{+}CD73^{-}$ B cells and mouse T2-MZP cells already discussed) cannot be identified by this technique. Furthermore, the majority IL-10^{+} B cells fall outside of the $CD1d^{hi}CD5^{+}$ population: although strongly enriched for IL-10 expression, the $CD1d^{hi}CD5^{+}$ population is very small and actually accounts for less than 25% of all IL-10^{+}B cells in spleen [35]. A marker that can identify the majority of IL-10^{+} B cells is critical for improved understanding of Breg biology, including the relationships among Breg exhibiting different phenotypes.

The advent of TIM-1^{+} B cells with regulatory function, for a brief period appeared to represent the elusive unifying IL-10^{+} Breg subset. TIM-1, a phosphatidylserine receptor, was identified by Ding et al. as a putative universal marker of mouse Breg [35]. Adoptive transfer of TIM-1^{+} B cells was able to significantly prolong islet allograft survival in an IL-10-dependent and antigen-specific manner. TIM-1^{+} B cells obtained from un-transplanted mice were unable to prolong allograft survival upon adoptive transfer, consistent with previous reports indicating that Breg must be activated to be effective [82]. Furthermore, the low affinity agonistic TIM-1 antibody RMT10 was able to induce IL-10 expression by B cells as well as *de novo* TIM-1^{+} B

cells when in the presence of allograft, resulting in prolonged allograft survival. IL-10 expression was greatly enriched in TIM-1^+ B cells compared to TIM-1^- cells, and this percentage of IL-10^+TIM-1^+ B cells increased in the presence of allograft. In addition, the authors also showed that within multiple B cell sub-populations both splenic and peripheral, mouse TIM-1^+ B cells were enriched 8- to 20-fold for IL-10 expression compared with their TIM-1^- counterparts. Indeed, when splenic B cells were first gated on IL-10 expression, TIM-1 encompassed more than 70% of the IL-10^+ population [35].

Transgenic mouse models have recently demonstrated a role for TIM-1 in the induction and maintenance of IL-10 production by mouse Breg to achieve tolerance in models of islet transplantation, EAE and allergic airway disease [83-85]. Mouse B cells with TIM-1 defects have impaired IL-10 production but increased pro-inflammatory cytokine production, including IL-1 and IL-6. Consequently, TIM-1-deficient B cells promote Th1 and Th17 responses and inhibit the generation of Treg. TIM-1 has also been recently described in human B cells in peripheral blood of subjects infected with Human Immunodeficiency Virus-1 (HIV-1). TIM-1 was enriched in IL-10^+ B cells; moreover, TIM-1^+ B cells from HIV-1-infected, untreated patients were also able to inhibit pro-inflammatory cytokine responses of autologous T cells *in vitro* in an antigen-specific and IL-10-dependent manner, unlike TIM-1^- B cells. Whether TIM-1^+ B cells from healthy individuals could also mediate suppressive function, however, was not explored.

In support of TIM-1 as a universal Breg marker, mouse TIM-1^+ B cells are also enriched within the CD1$d^{hi}$$CD5^+$ B10 compartment [35]. However, IL-10^+ B cells are also present in the TIM-1^- compartment, and TIM-1^+ B cells are present in the non-CD1$d^{hi}$$CD5^+$ compartment. Thereby, despite having a potentially critical role in the suppressive function of IL-10^+ Breg in mice, TIM-1 is not a unifying cell surface marker and intracellular IL-10 staining remains the only way to reliably visualise a purely IL-10^+ Breg population [81].

Reports in the literature also describe the existence of regulatory plasma cells and plasmablasts in mice and very recently, in humans [43, 52, 66, 86, 87]. Tedder's group initially demonstrated that a proportion of B10 cells, under certain conditions,

only transiently expressed IL-10 to then go on to develop into antibody-producing cells [66]. IL-10 reporter expression was used to track B10 cell induction and fates in Tiger and 10BiT mice, to find that B10 cells only transiently expressed IL-10 prior to their terminal differentiation into clonally diverse, antibody-secreting plasmablasts and plasma cells *in vivo* upon lipopolysaccharide (LPS) stimulation. The authors hypothesised that these plasma cells may have the capacity to clear their inducing antigens by producing polyreactive and/or antigen-specific antibody, in a 'second wave' of immunoregulation following on from IL-10 secretion. Soluble antibodies secreted by these B10-derived plasma cells could quickly reduce antigen load and promote antigen clearance by opsonisation or complement-mediated phagocytosis; however suppressive capacity of these antibody-producing cells was not determined.

Shen et al. in 2014 demonstrated that two distinct subsets of plasma cells which expressed either IL-35 or IL10, were able to regulate inflammatory responses through the secretion of these immunosuppressive cytokines in mouse models of *salmonella* sepsis and EAE [43]. Wang et al. in the same year described another IL-35^{+} Breg population, but it is unclear whether this population expressed a plasma cell phenotype [40]. In a study of human allogeneic stem cell transplantations, populations of IL-10-producing CD24^{hi}CD27^{+} and CD27^{hi}CD38^{hi} plasmablast B cells were identified [52]; patients with chronic Graft versus Host Disease (GVHD) had significantly lower frequencies of CD24^{hi}CD27^{+} B cells and IL-10–producing plasmablasts, suggesting a role for these B cell subsets in the regulation of GVHD. Whether all of these regulatory plasma cell populations are part of the same lineage as the more phenotypically immature IL-10^{+} and IL-35^{+} Breg subsets already described, or if these antibody-producing cells are in fact distinct Breg populations, remains to be determined.

Thus it remains unclear how to define a Breg phenotypically, other than by the suppressive function. To add further confusion to the field, there are several studies now documenting the existence of IL-10-independent populations of B cells with regulatory function. For the sake of clarity, it may be best to sub-divide Breg populations firstly by mechanisms by which they predominately suppress immune responses: for instance, those that mediate immunosuppression through IL-10

secretion, and those that mediate immunosuppression through alternate mechanisms. These different sub-classes of Breg are discussed below.

1.4 Suppressive mechanisms employed by Breg

The majority of B cells with regulatory function already mentioned appear to suppress immune responses through the secretion of IL-10. The immunosuppressive properties of IL-10 are well documented, and are utilised by many other regulatory subsets as well as Breg, such as Treg, Tr1 cells and regulatory dendritic cells (DCs) [88-90]. The exact molecular mechanisms of immunosuppressive effects of IL-10 on antigen presenting cells (APCs) and T cells are still a matter of debate despite extensive research in this field. There is however general agreement that STAT3 activation by IL-10 receptor engagement is essential for the anti-inflammatory effects of IL-10. This was demonstrated most convincingly in STAT3-deficient mice [91] and confirmed by several *in vitro* studies of both lymphoid and myeloid cells [92, 93]. Downstream effects of IL-10 include direct inhibition of proliferation and cytokine synthesis by $CD4^+$ T cells, such as the production of IL-2 and IFNγ by Th1 and of IL-4 and IL-5 by Th2 cells [94, 95]. Indeed, both mouse and human Breg are well documented to inhibit to proliferation, differentiation and pro-inflammatory cytokine production in a partially IL-10-dependent manner [20, 27, 28, 42, 45, 47, 48, 60, 73, 79].

As well as inhibiting pro-inflammatory immune responses, Breg are also thought to promote, or skew, T cell responses to Treg differentiation in both IL-10-dependent and IL-10-independent mechanisms. Mouse studies have shown that mice harbouring a B-cell-specific deletion of IL-10 also exhibit a deficiency of Treg, which is associated with worsening of CIA [27, 28]. Human *in vitro* work has demonstrated that IL-10-producing $CD5^+$ Breg, when co-cultured with autologous $CD4^+$ T cells, are associated with an increase in percentage of $FoxP3^+$ T cells, although suppressive function of this putative Treg population was not tested [47]. $CD24^{hi}CD38^{hi}$ human Breg from healthy individuals can induce $CD4^+CD25^+CD127^{lo}$ Treg *ex vivo,* unlike the same Breg subset when isolated from patients with RA [45]. A recent study of a mouse model of islet allograft transplantation has revealed a fundamental role for

Breg-secreted TGFβ in the induction of Treg and subsequent prolongation of allograft survival [39].

IL-10 is also known to regulate immune responses through the modulation of APCs such as DCs, monocytes and macrophages. Breg have been reported to indirectly suppress the differentiation of Th1 and Th17 cells by suppressing production of pro-inflammatory cytokines such as IL-12, by dendritic cells [87, 96]. Mouse B10 cells also suppress macrophage activation, cytokine and nitric oxide (NO) production, and phagocytosis in an IL-10–dependent manner [32, 42]. Human activated B cells with regulatory function have also been shown to inhibit maturation of DCs and the development of immature DCs from monocytes, thereby suppressing DC-induced T cell proliferation [97]. Human Breg appeared to decrease the density of HLA-DR on mature DCs, the expression of CD80 and CD86 co-stimulatory molecules and the production of IL-12p70 required for antigen presentation and Th1 differentiation. *In vitro* experiments established that direct, cognate interactions involving CD62L were required to control expression of CD80 and CD86, as well as a soluble factor for the control of IL-12 production.

Cognate interactions are indeed thought to be fundamental to the action of Breg. Yoshizaki et al. demonstrated that in *MHC*$^{-/-}$ and *CD40*$^{-/-}$ mice, B10 cells are unable to regulate a T cell-driven autoimmune response [98]. Through the use of elegant transwell experiments and blocking mAbs, several human studies showed that human Breg required both cognate interactions and specifically CD154-CD40 engagement to be able to inhibit a $CD4^+$ T cell response [20, 47, 99, 100]. Interestingly, suppression of human T cells appears to differentially mediated, with pro-inflammatory cytokine production suppressed by IL-10 and CD80/CD86 expression by Breg, whilst proliferation is regulated by CD154-dependent cell-cell contact [47]. Breg, much like Treg, may utilise multiple mechanisms to combat the many arms of the inflammatory response.

Indeed, several Breg subsets have been reported which appear to regulate inflammatory immune responses by entirely IL-10-independent means. As already mentioned, $TGF\beta^+$ Breg are able to induce Treg to protect allografts [39]. In addition, LPS-activated B cells can induce both apoptosis of $CD4^+$ and anergy in $CD8^+$ effector

T cells through the production of TGF-β [41, 101]. As mentioned earlier in this chapter, IL-35 has recently been identified as being produced by IL-10$^-$ Breg to suppress autoimmune responses. Chimeric mice lacking expression of IL-35 sub-units develop exacerbated EAE and are protected against *Salmonella* sepsis; IL-35-stimulated B cells secrete IL-35 and inhibit autoimmune uveitis upon adoptive transfer in mice [40, 43]. Thus Breg appear to supress immune responses through the secretion of multiple different immunosuppressive cytokines, rather than simply representing IL-10-producers.

IL-10-independent Breg populations with the capacity to kill lymphoid cells as well as promote activation-induced cell death (AICD) have also been described in recent years. Activated B cells have been reported to up-regulate FAS-L [102], and its expression has been correlated to B cell–mediated T-cell apoptosis *in vivo* [41, 103, 104]. Following BCR-mediated stimulation, Tretter et al. found that activated, CD25$^+$ human B cells were able to suppress *in vitro* CD4$^+$ T cell responses in the presence of exogenous IL-2, through induction of apoptosis by a FAS-independent pathway [99]. Human Breg that regulate immune responses through the secretion of Granzyme B have now also been identified by several groups as being present in peripheral blood of healthy individuals, patients with HIV, RA and multiple sclerosis, as well as within solid tumours [105-107].

The immunosuppressive arsenal apparently available to Breg is growing, illustrative of the potential value of such populations to the maintenance of a tightly regulated and multi-faceted immune system. The question still remains as to whether the Breg subsets described here represent one multi-suppressor Breg population which is able to utilise different mechanisms dependent on the biological context, or whether many different Breg populations exist, with varied regulatory roles across the immune system, much like the different pro-inflammatory T cell populations which are well established today.

1.5 Induction and expansion of Breg

As outlined above, there are multiple phenotypes and mechanisms by which Breg can be identified and mediate immunosuppression. What the majority of these Breg subsets appear to share is a need to be activated prior to function [74, 81]. Most Breg populations so far described are capable of expressing IL-10, and IL-10 itself can be induced on B cells by several different stimuli, as outlined in fig. 1.1.

As early as 2003, the Londei laboratory demonstrated the requirement of the CD154-CD40 interaction for the induction of mouse Breg and the production of IL-10 [24]. CD40 is a member of the TNF superfamily and is expressed mainly on B cells and DCs, as well as endothelial cells, macrophages, T cells, and fibroblasts [108, 109]. The CD40-CD154 interaction is crucial for the production of soluble factors such as cytokines (e.g., IL-6, TNF-α, IL-12, IL-10, IL-1), and for the up-regulation of other co-stimulatory molecules such as CD80 and CD86 [108]. Furthermore, this pathway plays an important role in B cell activation, inducing proliferation and isotype switching [110, 111]. *In vitro* stimulation with an agonistic anti-CD40 mAb and antigen resulted in generation of B cells capable of inhibiting Th1 differentiation and preventing as well as ameliorating CIA [24]. Short-term co-culture of agonistic CD40 mAb with mouse splenic B cells resulted in generation of IL-10-producing Breg with a transitional 2 (T2) phenotype; this generated B cell subset went on to reverse autoimmunity *in vivo* upon injection, suppress Th1 responses and induce the differentiation of $IL\text{-}10^{+}CD4^{+}$ T cells which were also suppressive [69]. The immunosuppressive effect was abrogated by the *in vivo* neutralisation of IL-10. *In vivo* administration of anti-CD40 mAb also reversed nephritis and enhanced survival in mice with new onset disease by increasing the number of T2 B cells.

Several studies have gone on to exploit this interaction and induce Breg function [20, 47, 98]. Both Lemoine and Blair stimulated human B cells with membrane-bound CD154 to induce IL-10 production and Breg function [20, 47]. Co-culture of mouse splenic B cells with NIH3T3 cells that expressed CD154 and BAFF, the B cell survival factor in the presence of cytokines, resulted in greater than $4x10^{6}$ fold expansion of mouse B10 cells *in vitro* [98]. Using *CD40*$^{-/-}$ and *MHC*$^{-/-}$ mice in a

model of EAE, Tedder's group illustrated that B10pro cell maturation into functional IL-10-secreting Breg that could inhibit *in vivo* autoimmune disease, was critically dependent on CD40 engagement and cognate interactions with T cells [98]. The interaction of prospective Breg cells appears to be dependent on T cell-derived signals.

However, there are also reports of Breg induction and expansion by innate signals. CpG oligonucleotides are widely recognised to stimulate mouse and human B10 cells to produce IL-10 [48, 112]. LPS can also induce mouse B10pro cells to mature and become functional B10 cells [70]. TLR signals, infection, and apoptotic cells can all significantly expand B10 and B10 effector cell numbers *in vivo* [42, 66, 76].

Chimeric mice in which B cells were deficient in the signalling molecule downstream of TLR, MyD88, developed a chronic form of EAE as well as an enhanced control of *Salmonella typhimurium* replication [70]. MyD88-sufficient mice, however, express IL-10 and develop an inhibitory function to suppress neutrophil, NK cell and effector T cell activation, leaving mice more susceptible to *S. typhimurium* infection [86]. The release of IL-10 by TLR-stimulated B cells is significantly increased upon CD40 engagement, suggesting that *in vivo* the optimal production of IL-10 occurs after TLR-activated B cells interact with CD154-expressing $CD4^+$ T cells [113, 114]. It has been hypothesised that there is in existence both innate and adaptive Breg. Alternatively, the same Breg are responding to different stimuli to exert suppressive mechanisms including the secretion of IL-10 [81].

Cytokines have also proven critical to the induction and expansion of Breg subsets. Yoshizaki et al. identified that *in vitro* co-culture of mouse B cells with exogenous IL-21 and no other stimulation for 48-72 hours, was able to induce up to a x5.3 fold increase in mouse B10 frequencies [98]. Both mouse B10 and non-B10 cells up-regulated expression of IL-21R after 5 hours of stimulation with PMA and ionomycin, but only the percentage frequency of B10 cells increased *in vivo* following MOG immunisation of wild type mice to induce EAE. Through the use of transgenic mouse models, Yoshizaki et al. were able to demonstrate that B10 cells require IL-21 in order to function or expand *in vivo*. The authors were also able to expand mouse B10 cells *in vitro* by greater than $4x10^6$ fold through co-culture of mouse B cells with IL-

21, IL-4 and joint exposure to both CD154 and BAFF through incubation with NIH-3T3-CD154/BLyS cells. Rosser et al. have identified a role for pro-inflammatory cytokines IL-1β and IL-6, which are produced after the induction of antigen-induced arthritis [71]. The production of these cytokines in arthritic mice is controlled by the community of bacteria in the gut, collectively termed microbiota; in the absence of IL-1R1 or IL-6R expression on B cells, mice housed in non-sterile conditions developed exacerbated arthritis. It may be that Breg expand in response to inflammation, in order to restrict its effects. In support of this view, Breg increase in number during the inflammatory phase of several autoimmune disorders, and in their absence, mice develop exacerbated disease [24, 27, 28, 48].

Contrary to this hypothesis, however, the addition of immunosuppressive cytokine IL-35 has also been recently identified to induce mouse Breg and to be produced by Breg to mediate suppressive affects. IL-35 profoundly suppressed inflammation in mouse models of autoimmunity, whereas its absence in B cells enhanced survival after *Salmonella* infection in mice [40, 43]. IL-10 itself is a pleotropic cytokine that can convey immunosuppressive function and can also serve as a growth factor that drives differentiation of B cells and antibody class switching [111, 115-117]. Mouse Breg studies report conflicting results on whether Breg require IL-10 during development. The use of IL-$10^{-/-}$ mice has demonstrated that IL-10 is not required for the development or differentiation of mouse B10 cells [66]. However in early experiments in mouse models of CIA, Mauri et al. found that IL-10 was critical to the generation of mouse Breg with a T2-MZP phenotype [24]. Breg generated through the *in vitro* co-culture of mouse splenocytes with antigen and agonistic CD40 mAb in the presence of a neutralising mAb to IL-10R, were unable to confer protection against CIA upon adoptive transfer, unlike those Breg generated in the absence of IL-10-blockade. A recent paper has suggested an autocrine role for IL-10 in the induction of IL-10^{+} mouse Breg [118]. LPS stimulation of splenic B cells *in vitro* resulted in the generation of IL-10^{+} Breg which were enriched in $CD40^{hi}CD5^{+}$ B cells. The addition of exogenous IL-10, CD40L or BAFF increased both IL-10 and CD40 expression in splenic B cells, indicating that CD40 expression is associated with IL-10 expression, and is in part controlled by IL-10. LPS stimulation in the presence of IL-10 blockade or of IL-$10^{-/-}$ B cells abrogated the induction of CD40

expression. Furthermore an inhibitor of the JAK/STAT pathway blocked the autocrine effect of IL-10 on CD40 expression. These results were replicated *in vivo*, supporting the view that IL-10 plays a critical autocrine role in the development of IL-10^{+} Breg in mice.

Evidence also suggests that BCR recognition is important in Breg induction. In MD4 mice, where the BCR is fixed for an irrelevant antigen, Breg activation is impaired and there are lower frequencies of Breg [23, 70]. Bone marrow chimeras that have MD4 B cells are also unable to resolve EAE. Similar findings are observed in mice with decreased BCR signalling, such as in $CD19^{-/-}$ mice; these mice display exacerbated inflammation and symptoms of disease during contact hypersensitivity, EAE and IBD [32, 78, 119]. In contrast, CD19 overexpression actually expands B10 cell numbers [70, 78]. Increasing CD40 signalling by CD22 deficiency and ectopic B cell CD154 expression in transgenic mice also elevates B10 and B10pro cell numbers by at least 10 fold *in vivo* [120]. The calcium sensors, stromal interaction molecule 1 (STIM-1) and STIM-2, which are involved in facilitating intracellular calcium influx following antigen recognition by the BCR, are also required for B cell IL-10 production after BCR stimulation, as demonstrated by experiments in mice with a B cell-specific deletion in these molecules: B cells produce less IL-10 after stimulation with MOG and anti-CD40 [121]. Therefore BCR signalling pathways may drive the capacity of B cells to express IL-10, thereby selecting for antigen-specific Breg *in vivo*. Selection by antigen may also account for the rapid proliferation of primed B10 cells in response to mitogenic stimulation *in vitro* when compared to other B cell populations [70, 79]. In addition, despite antigen stimulation *in vivo*, B10 cells express diverse germline BCRs with only rare mutations [66, 79]. Tedder has hypothesised that BCR specificity and signalling intensity can act as developmental checkpoints which dictate the differentiation of potential Breg to an antigen: low-affinity or chronic antigen–BCR stimulation may drive IL-10 competence *in vivo*, and strong BCR signals may in fact divert intracellular signalling and induce B10pro cells to progress down a different functional pathway [81].

It appears that the CD154-CD40 interaction is critical for Breg function and expansion, with other innate signals, cytokines and antigen-driven stimulation acting as parameter by which Breg responses may be regulated and fine-tuned. Crucially,

whilst mouse Breg can be expanded *in vitro* [98], human Breg as of yet have not been successfully generated *ex vivo*. Human Breg are rare in peripheral blood, and so isolation and study of these regulatory populations is difficult. Without means to generate or expand large numbers of human B cells with regulatory function, it will be hard to draw further parallels or indeed to understand important differences between mouse and human Breg populations. Successful expansion or induction of human Breg *in vitro* could allow for in depth study of these cells, to establish phenotype, mechanisms of function as well as cell stability and fate. Manipulation of some the parameters for mouse Breg expansion discussed here, may offer avenues by which to further explore this possibility.

1.6 The interaction of CD40 and CD154

As described above, engagement of CD40 expressed by a B cell by CD154 usually expressed by an activated T cell, is fundamental to the generation of B cells with regulatory function and also to the secretion of B cell-derived IL-10. It is important to understand the nature of this engagement before one can consider exploiting such an interaction to expand or induce a population of human Breg.

CD40 is a 50kDa membrane glycoprotein, constitutively expressed on B cells. Development of mAb specific to human CD40 in the 1980s led to the demonstration that ligation of CD40 could mediate a wide range of activities on human B cells, including proliferation and differentiation [122-124]. However, several different forms of agonistic CD40 mAb and recombinant CD40L (CD154), both soluble and membrane-bound, have been developed over the last two decades. It has become evident that these different forms of CD40 mAb and CD40L orchestrate different biological outcomes, largely dependent on epitope specificity, valency, receptor density and oligomerisation. The way in which receptor ligation occurs is critical to the subsequent biological consequences of signalling through CD40 and these different permutations are summarised in table. 1.1. Crosslinking of receptors on the B cell surface is a prerequisite for entry into the cell cycle following CD40 ligation. Furthermore, co-stimulation with different cytokines can direct differentiation in various ways [125-129].

Extensive work in the 1990s established that different soluble CD40 (sCD40) mAbs bound to different epitopes in and around CD40, resulting in differing affinities, differential coupling to intracellular signal transduction pathways and diverse functional outcomes e.g. proliferation vs. apoptosis [126, 127, 130]. mAbs such as G28-5 and TNFRSF5 bind closest to the CD40L binding site on CD40 and so promote stronger clustering than those mAbs which bind outside this binding site [108, 126, 131]. Biotinylation increases crosslinking, but expansion however, remains minimal in comparison to larger complexes of CD40L, such as soluble trimeric CD40L (sCD40L-T) [125, 132].

The development of immortalised cell lines that had been transfected with CD154 resulted in maximum crosslinking of CD154 on engagement with CD40. These cell lines have been shown by several groups to promote rapid expansion of human CD19+ B cells, by up to a factor of $x10^9$ [133, 134]. Extensive work by Neron [127, 135] has also demonstrated that varying ratios of $CD154^+$ cells to human $CD19^+$ B cells in such cell cultures can determine the expansion factor and proportions of naive and memory B cells which expand i.e. lower $CD154^+$ cells to $CD19^+$ B cell ratios correlated with greater expansion of $CD19^+$ B cells and increased proportions of $CD27^-$ naive B cells compared to $CD27^+$ memory B cells. Indeed, an immortalised cell line expressing $CD154^+$ has been used with considerable success to expand mouse B10 cells [98]. Incubation of human $CD19^+$ B cells with such a cell line may be the prudent first step in an attempt to induce or expand human Breg. Since the majority of human Breg have been characterised through the secretion of IL-10, for the purposes of this project we can define an expanded or induced human Breg as a human B cell that expresses IL-10 and exert regulatory function upon *ex vivo* manipulation.

1.7 The humanised mouse model of skin transplantation

If human Breg can be expanded or generated *ex vivo*, as well as confirming *in vitro* regulatory function it will be important to investigate whether this suppressive population can also regulate an immune response *in vivo*. Highly activated Breg

populations may behave very differently in a complex biological environment in comparison to the carefully controlled, more one-dimensional conditions of *in vitro* culture. As outlined above, clinical data and mouse models have demonstrated an important role for Breg in the field of transplantation [12, 13, 39], and Treg cellular therapy is already being introduced into human KTRs as part of the ONE study collaboration. The advent of Breg cellular therapy could be important in the success of any multi-armed cellular approach to antigen-specific induced tolerance, given that Breg are able to suppress multiple cell types and can also induce and promote Treg function. A cocktail of regulatory cell types may increase potency, antigen specificity, cell stability and thus importantly decrease dosage requirements by generating a more robust tolerant environment in the human subject.

The humanised mouse model of transplantation provides an ideal basis by which to study *in vivo* the function of Breg populations in regulating a T cell-driven alloresponse. Our group has demonstrated that human PBMC can propagate T cell-mediated rejection of engrafted, allogeneic human internal mammary artery or a split-thickness skin graft, after reconstitution in a Balb/c $Rag2^{-/-}$ $c\gamma^{-/-}$ mouse devoid of mouse T cells, B cells and NK cells [136, 137]. The development of transplant arteriosclerosis and neointimal thickening in arterial grafts are consistent with that which occurs in clinical human kidney transplants and are known to lead to chronic allograft dysfunction [136] (fig. 1.2a). Human skin transplants undergo both macroscopic and microscopic rejection, with histological analysis demonstrating profuse infiltration of the graft by human $CD4^+$ and $CD8^+$ T cells as well as destruction of the underlying tissue architecture with necrosis, in keeping with the BANFF criteria for solid organ transplant rejection [137] (fig. 1.2b). Using such models, our group has demonstrated that human *ex vivo*-expanded Treg are able to control or prevent allograft rejection in a dose-dependent manner. In depth study has also revealed that different Treg subsets have varying capacity to protect the allograft [136, 138]. In addition, we have used this model to illustrate that ischaemia-reperfusion injury can exacerbate human antibody-mediated allograft transplant vasculopathy [139].

The use of such models is invaluable in exploring the potential behavior of human cellular therapy *in vivo* as well as the consequences of pharmaceutical manipulation

of such human cell populations, before translation to the clinic. It will be extremely important to test any burgeoning human Breg therapy in such a system in order to better comprehend the inherent nature of these cells before manipulating suppressive B cell populations at the bedside.

1.8 Hypotheses

Evidence of IL-10$^+$ B cells with regulatory function increasingly demonstrates that this regulatory population has a vital role in maintaining homeostasis within the immune system and may offer novel means by which to control unwanted inflammatory responses. Despite the therapeutic potential of Breg, this possibility is so far limited; human Breg are rare in peripheral blood in human subjects, the subsets are heterogenous and can only be reliably defined by secretion of IL-10, and there are currently no means by which human Breg can be generated or induced in large numbers *ex vivo*.

This project will investigate the following hypotheses:

- **Human CD19$^+$IL-10$^+$ B cells with regulatory function can be generated *ex vivo*: 'EXPB10 cells'**
- **EXPB10 cells express an endogenous human Breg phenotype**
- **EXPB10 cells can regulate a CD4$^+$ T cell response by cognate interactions and the secretion of IL-10 *in vitro***
- **EXPB10 cells can prolong allograft survival in a humanised mouse model of skin transplantation**

As the majority of murine models identify Breg as those which can express IL-10 and B cells with regulatory activity identified in human KTRs appear to mediate regulation principally through IL-10 production, I have defined human B cells with regulatory activity as a human B cell capable of expressing IL-10. Thus the expanded population of human B cells with regulatory activity is termed 'EXPB10.'

Fig. 1.1

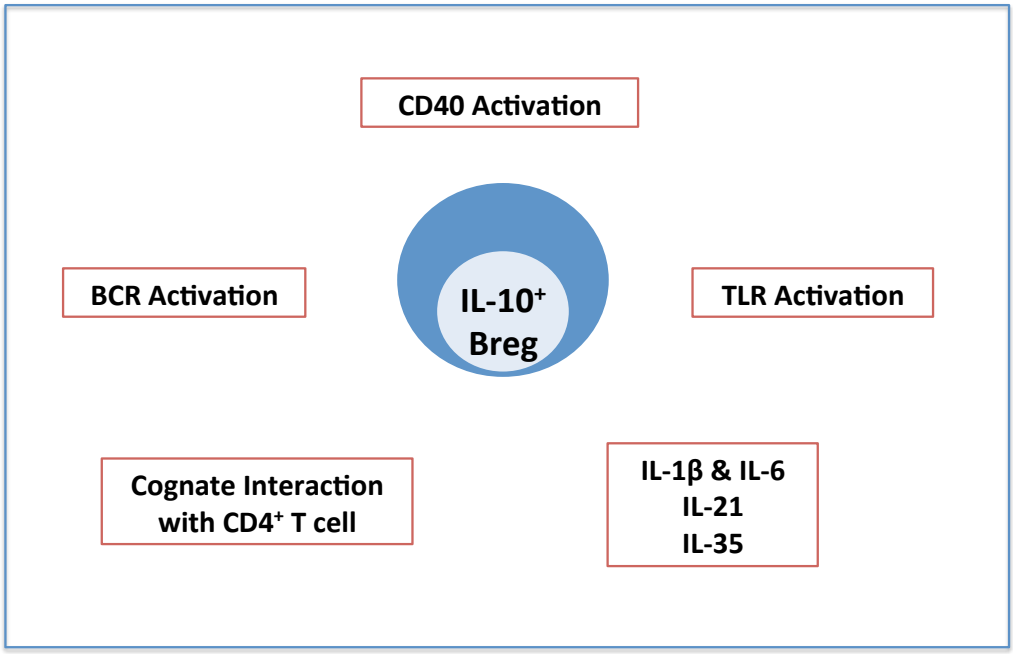

CD40 Activation
BCR Activation
IL-10+ Breg
TLR Activation
Cognate Interaction with CD4+ T cell
IL-1β & IL-6
IL-21
IL-35

Fig. 1.1 Signals required for Breg induction

IL-10^+ Breg can be induced by multiple signals including activation of CD40, Toll-like receptors (TLR) and B cell receptor (BCR) as well as by cognate interactions with $CD4^+$ T cells and both pro-inflammatory and immunosuppressive cytokines.

Table. 1.1

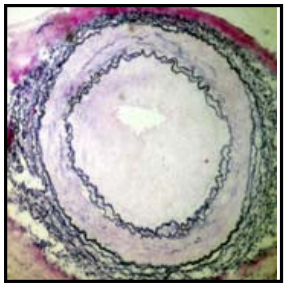
STIMULUS	*ACTIVATION	PROLIFERATION		**DIFFERENTIATION
		No co-stimulus	ŦPlus co-stimulus	
Membrane CD40L	++	+++	++++	++++
Soluble dimeric CD40L	+	+	+++	++++
Immobilised CD40 mAb	+	++	++++	++++
Soluble CD40 mAb	+	-	+++	++++

Table. 1.1 Summary of human $CD19^+$ B cell responses to different forms of CD40 ligand (CD40L) and CD40 monoclonal antibody (mAb)

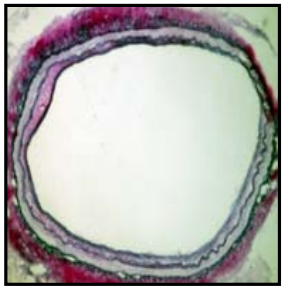
Modified from *Fanslow, W.C., et al., Structural characteristics of CD40 ligand that determine biological function. Semin Immunol, 1994. 6(5): p. 267-78.*
*Activation defined by some or all of the following: increased cell size, enhanced expression of CD23, MHC II or CD80.
**Differentiation defined as secretion of IgE with IL-4 as co-stimulus, IgM, IgG and IgA with IL-2 or IL-10 as co-stimuli
[Ŧ]Co-stimuli include anti-IgM, CD20 mAb, PMA and SAC.

Fig. 1.2

A



Vessel + Allogeneic PBMC



Vessel + Allogeneic PBMC + Treg

B



Skin + Allogeneic PBMC



Skin + Allogeneic PBMC + Treg

Fig. 1.2 Humanised mouse models of transplantation

(a) In a humanised mouse model of transplant rejection, allogeneic PBMC mediates rejection of transplanted human arterial graft, propagating transplant arteriosclerosis and neointimal hyperplasia. Introduction of ex-vivo expanded $CD4^{+}CD25^{+}CD127^{lo}$ regulatory T cells (Treg) regulates rejection by potently reducing transplant arteriosclerosis within the allogeneic human vessel graft. **(b)** A similar system has been developed to study the rejection of human skin transplantation *in vivo.* Human $CD4^{+}CD25^{+}CD127^{lo}$ Treg are able to significantly prolong survival of human skin allograft. This humanised model is extremely useful as it allows study of *in vivo* human immune responses without the need for direct patient participation thus enabling proof of concept studies before clinical translation. Figures obtained from *Nadig, S.N., et al., In vivo prevention of transplant arteriosclerosis by ex vivo-expanded human regulatory T cells. Nat Med, 2010. 16(7): p. 809-13.* and *Issa, F., et al., Ex vivo-expanded human regulatory T cells prevent the rejection of skin allografts in a humanized mouse model. Transplantation, 2010. 90(12): p. 1321-7.*

2.0 MATERIALS AND METHODS

2.1 *In vitro* cell isolation and culture

All cellular isolation and culture techniques were performed with sterile precautions in a laminar flow cabinet.

2.1.1 Human PBMC isolation

Human PBMC were isolated from leucocyte cones provided by the National Blood Service by Ficoll-Paque (GE Healthcare) gradient centrifugation. Whole blood was carefully layered above Ficoll solution and then centrifuged for 25 minutes at 2200rpm at 20°C. Isolated PBMC were pelleted and red cells lysed with Pharmlyse™ buffer (BD Pharmingen, UK). PBMC were washed, enumerated and were either used immediately in subsequent experiments or were frozen in liquid nitrogen for later use.

2.1.2 Negative selection of human $CD19^+$ B cells

Human $CD19^+$ B cells were isolated from PBMC by negative selection using an EasySep® Human B Cell Enrichment Kit (Stem Cell Technologies), in accordance with manufacturer's guidelines. Purified human B cells were >96% $CD19^+$ as determined by flow cytometry.

2.1.3 Selection of human $CD4^+$ T cells

Human $CD4^+$ T cells were isolated from PBMC by positive or negative selection using MACS cell separation methods (Miltenyi). Human $CD4^+$ T cell Microbeads and Human $CD4^+$ T Cell Isolation Kits were used to isolate human $CD4^+$ T cells

according to manufacturer's guidelines. Purified human T cells were >95% $CD4^+$ as determined by flow cytometry.

2.1.4 Culture of Chinese Hamster Ovary (CHO) cells

$CD154^+$ and $CD154^-$ CHO cell lines were kindly donated by Professor Claudia Mauri, University College London. CHO cells were cultured in DMEM containing 4500µg/L glucose, 110µg/L sodium pyruvate and L-glutamine (Sigma-Aldrich) with 100U/ml penicillin and streptomycin (Life Technologies, Carlsbad, USA) and 5% foetal calf serum (FCS) (Gibco, UK). CD154 expression was routinely checked by flow cytometry analysis after staining with CD154-PE mAb (expression >98%). All CHO cell lines were cultured in Corning ® T75 flasks and were passaged using standard operating procedure with Trypsin solution from porcine pancreas (2.5g/L, Sigma, UK). Unless otherwise stated, CHO cells were split once cell culture had reached 60% confluence. Before being used in EXPB10 cell expansion cultures, CHO cells were γ-ray irradiated (65 Gy, 6500 rad) in 50ml corning tubes for 30 minutes using a Caesium 137 irradiator.

2.1.5 Expansion of EXPB10 cells

For human $CD19^+$ B cell expansion experiments, $CD19^+$ B cells were cultured in 'complete media' (c.RPMI): RPMI-1640 (Sigma-Aldrich, St. Louis, USA) supplemented with 100 U/µg/ml penicillin/streptomycin (Life Technologies, Carlsbad, USA), and 10% FCS (Gibco, UK). 0.06 $x10^6$ Human $CD19^+$ B cells per well were seeded in 96-well U-bottom plates for 7 days. Irradiated CHO cells were co-cultured with human $CD19^+$ B cells at ratios of 1:1, 1:5 or 1:10 CHO cells to $CD19^+$ B cells in a total volume of 200ul per well. In initial expansion experiments, different human cytokine combinations were added: the combination of 50U/ml of IL-2, 100U/ml of IL-4 and 25U/ml of IL-10, or 100U/ml of IL-4 only (all PeproTech, Rocky Hill, NJ). Control conditions included no cytokines, and these plates were instead supplemented with complete media only.

Cultures were fed by replacing culture medium and cytokines every 2–3 days, while irradiated CHO-CD154 cells were renewed on day 3 of expansion. Ratios of CHO-CD154 cells to CD19 were kept constant throughout the culture period. Unless otherwise stated, viable $CD19^+$ B cells were harvested on day 7 of co-culture, stained with appropriate antibodies (CD19 APC Cy7 and 7AAD viability dye PerCp) and sorted to obtain purities of >97% using a BD FacsARIA cell sorter (BD). Both Trypan-Blue exclusion and Calibrite counting beads (BD Biosciences) were used to determine absolute numbers of viable EXPB10 cells at time-points analysed.

2.1.6 Isolation of IL-10^+ B cells

The isolation of human IL-10^+ B cells was attempted using a Human IL-10 Secretion Assay - Cell Enrichment and Detection Kit (Miltenyi), in accordance with manufacturer's guidelines.

2.2 *In vitro* functional assays

All *in vitro* suppression assays were set up with sterile precautions in a laminar flow cabinet. Responder cells were invariably stained with either Carboxyfluorescein Succinimidyl Ester (CFSE) or Violet Proliferaton Dye (VPD) as outlined below, in order to monitor proliferation.

2.2.1 Suppression assays

To assess affect of EXPB10 cells or Treg cells on proliferation of autologous $CD4^+$ T cells, 0.05 x 10^6 EXPB10 cells or Treg cells were co-cultured with $CD4^+$ T cells or PBMC for 5 days in 96-well U-bottom plates in a total volume of 200ul per well. $CD4^+$ T cells and PBMC (responder cells) were stained with either CFSE or VPD. Dye-stained responder cells were stimulated with anti-CD3/CD28 beads at a ratio of 1:10 beads to responder cells. Stimulated $CD4^+$ T cells or PBMC cultured without EXPB10 (0:1), or instead with NON-EXPB10 cells, served as control conditions in EXPB10 cell functional experiments. On day 5 of culture, cells were harvested,

stained for cell-surface markers and analysed by flow cytometry. Counting beads were used to determine absolute numbers of remaining $CD4^+$ T cells at day 5.

To assess affect of EXPB10 cell on intracellular cytokine production by $CD4^+$ T cells, EXPB10 cells were co-cultured with $CD4^+$ T cells and anti-CD3/CD28 beads for 72 hours. 2% PMA, 1% ionomycin and 1% brefeldin (PIB) were added to cultures for the last 6 hours of culture; cells were then harvested, surface-stained, permeabilised and stained intracellularly for Th1 cytokines interferon-γ (IFNγ) and tumour necrosis factor α (TNFα).

2.2.2 Mechanistic *in vitro* blockade

To determine mechanisms by which EXPB10 cells suppressed proliferation or cytokine production by autologous $CD4^+$ T cells, different inhibitory reagents were added to *in vitro* suppression co-cultures on day 0 of the assay, unless otherwise stated. Inhibitory agents were added to both control and experimental conditions and EXPB10-mediated suppression calculated relative to the control condition which had also been exposed to the inhibitory reagent. Inhibitory concentrations were determined by titration in excess of the ND_{50} provided by the relevant manufacturers. When necessary, appropriate isotype mAbs were also used in control conditions. The different reagents and concentrations used are summarised in Table 2.1.

2.2.3 CFSE staining

EXPB10 cells, $CD4^+$ T cells, Treg or PBMC were incubated at 37°C for 10 minutes with Carboxyfluorescein Succinimidyl Ester (CFSE) at 5μM in RPMI-1640. Cells were then washed and spun with c.RPMI for 5 minutes at 1500rpm before enumeration using Trypan-Blue exclusion. Labelling was confirmed by flow cytometry.

2.2.4 VPD staining

30 x 10^6 cells/ml EXPB10 cells, $CD4^+$ T cells, Treg or PBMC were incubated at 37°C for 10 minutes with Violet Proliferation Dye (VPD) at 1μM in phosphate-buffered

saline (PBS). Cells were then washed and spun twice with PBS and 5% FCS for 5 minutes at 1500rpm before enumeration using Trypan-Blue exclusion. Labelling was confirmed by flow cytometry.

2.2.5 Trogocytosis assay

Trogocytosis was measured by methods previously described and validated [140]. Expression of the lipophilic membrane dye, 1,1'-Dioctadecyl (Dil) by both $CD154^+$ CHO cells and $CD19^+$ B cells was measured after1 hour and 5 hours of co-culture by flow cytometry.

2.3 Measurement of human cytokine and immunoglobulin levels

Levels of human cytokines and immunoglobulin in culture supernatants and/or mouse sera were measured by both ELISA and cytokine bead arrays (CBA) as described below.

2.3.1 ELISA

Levels of human cytokines IL-35 and IL-10 in *in vitro* culture supernatants were measured using a Human IL-35 Heterodimer ELISA Kit (Biolegend) and a Human IL-10 ELISA kit (BD Biosciences) respectively, in accordance with manufacturers' guidelines.

2.3.2 Cytokine bead array

Levels of human cytokines IL-2, IL-6, IL-10, IL-17a, IFNγ and TNFα as well as human immunoglobulin IgG (total) and IgM in *in vitro* culture supernatants and humanised Balb/c $Rag2^{-/-}$ $c\gamma^{-/-}$ mouse sera were measured using a Human Cytokine Bead Array Enhanced Sensitivity Flex Set system (BD Biosciences), in accordance with manufacturer's guidelines. All data was processed using FCAP v1.0 analysis software (BD Biosciences).

2.4 Fluorescence-activated cell sorting (FACS) and flow cytometry

Flow cytometric data were acquired using a FACSAria or BD Canto II (BD Biosciences, UK), and analysed using FlowJo software (Flowjo Enterprise, USA). All fluorochrome-coupled mAbs used are summarised in Table 2.2.

2.4.1 Cell-surface marker staining

Prior to analysis by flow cytometry, cells were first incubated with human sera for 15 minutes at 4°C to reduce non-specific antibody binding. Cells were then incubated with the appropriate fluorochrome-coupled mAbs for 30-45 minutes at 4°C in the dark. Cells were then washed with PBS and spun for 5 minutes at 1500rpm at 4°C before flow cytometry analysis. Mean fluorescence minus 1 (mf-1) controls were used as standard controls to permit accurate gating of positive and negative populations.

2.4.2 Intracellular marker staining

Following cell-surface staining, cells which were to undergo intracellular staining were permeabilised using a BD Cytofix/Cytoperm^{TM} Kit (BD Biosciences, UK) in accordance with manufacturer's guidelines. Cells were then incubated with either the fluorochrome-coupled mAb under interrogation or the appropriate isotype control for 1 hour at 4°C in the dark. Cells were subsequently washed with PBS and spun for 5 minutes at 1500rpm at 4°C before flow cytometry analysis. In order to measure expression of IL-10, TNFα or IFNγ, cells were first incubated for 6 hours with PMA, ionomycin and brefeldin (PIB), or PMA, ionomycin and monensin (PIM) in c.RPMI before staining.

2.4.3 pSTAT3 staining

In order to stain for phosphorylated STAT3 (pSTAT3), 0.1 x10^6 EXPB10 cells were incubated in 100µl c.RPMI for 10 minutes at 37°C, with exogenous human IL-10 at 25U/ml (or c.RPMI as a control) in 96-well, v-bottom plates. 100µl/well of Cytofix™ fixation Buffer (BD Biosciences, UK) was added to cell culture for an additional 10 minute incubation period at 37°C. Cells were subsequently spun for 5 minutes at 1500rpm at 4°C and supernatants decanted. 100µl/well of Phosflow™ Perm Buffer (BD Biosciences, UK) was added to cell culture and cells were incubated for 30 minutes on ice. Cells were then washed twice with PBS at 1500 rpm for 5 minutes at 4°C before staining with pSTAT3-Alexa Fluor 450® 647 antibody (BD Pharmingen) for 1 hour at 4°C. Cells were once more washed with PBS at 1500 rpm for 5 minutes at 4°C before immediate FACS analysis. mf-1 controls were used to assist accurate gating. $CD4^+$ T cells, NON-EXPB10 cells and unstained EXPB10 cells were included as relevant controls.

2.5 Animal procedures

Balb/c $Rag2^{-/-}$ $c\gamma^{-/-}$ mice were obtained from Charles River Laboratories and were housed and bred under specific pathogen-free conditions in the Biomedical Services Unit of the John Radcliffe Hospital (Oxford, UK). Mice were housed in individually ventilated cages and handled with gloves. All experiments were performed using protocols approved by the Committee on Animal Care and Ethical Review at the University of Oxford and in accordance with the UK Animals (Scientific Procedures) Act 1986, under project license PPL 30/2550 and personal license PIL 30/9202. At the time of the first experimental procedure, mice were between the ages of 6 and 12 weeks. Any mice developing signs of GvHD or weight loss of >20% from starting weight were euthanized according to local animal welfare regulations. Graft survival experiments using human skin were performed as described in the literature [137] as well as in *Chapter 5.0*.

2.5.1 Pre- and post-operative care

Stock concentrations of medetomidine hydrochloride (Domitor®, Pfizer Pharmaceutical Ltd.) were diluted with normal saline to provide a working concentration of 100µg/ml. This solution was then mixed with a 7.6µg/ml solution of ketamine hydrochloride (Ketaset®, Fort Dodge Animal Health Ltd.). Recipient mice received a subcutaneous injection of 200µl of the combined Domitor/Ketaset solution along with 200µl of 10µg/ml buprenorphine (Vetergesic®, Reckitt Benckiser Healthcare Ltd.), to provide general anaesthesia during surgical procedures. For terminal anaesthesia, mice received 500µl of Domitor/Ketaset solution. Post-operatively, mice received 200µl of 100µg/ml atipamezol hydrochloride (Antisedan®, Pfizer Pharmaceutical Ltd.) as anaesthetic reversal, and were subsequently placed on a warm recovery cabinet for 2 hours.

2.5.2 Procurement of human skin

Split-thickness human skin at between 8-10/1000 inches thick were harvested with the use of an air-driven dermatome from the anterior abdominal wall of live donors undergoing plastic surgery procedures in the Department of Plastic and Reconstructive Surgery, John Radcliffe Hospital, Oxford. Exclusion criteria included donors who were taking immunosuppressive medication, donors with a primary inflammatory condition or donors with any current or past history of malignancy. Donor age ranged from 28 to 53 years (median age 43). Skin was procured with the assistance of Mr. Fadi Issa and other members of the Plastic Surgery team. Tissue was stored in RPMI-1640 on ice and was transplanted within 12 hours of procurement. Unused skin was destroyed. Tissue was obtained with full patient consent and ethical approval from the Oxfordshire Research Ethics Committee, study number 07/H0605/130. Blood samples were obtained with consent from skin donors for HLA-typing.

2.5.3 Skin Grafting

Skin grafting was performed with full sterile precautions. Mice were placed prone and a 1x1cm piece of skin removed from the left dorsal thorax over the costal margin. A 1x1cm piece of human skin was then fashioned and its edges sutured to the mouse recipient skin with a non-absorbable 8-0 Prolene suture (Ethicon, UK). Grafts were fenestrated and covered with a povidone-iodine mesh and pressure dressing which was secured with circumferential tape. Bandages were left in place for 7-10 days and then removed under general anaesthetic. Skin grafts were monitored every 1-2 days until complete loss.

2.5.4 Adoptive transfer of cells

Cells were suspended in RPMI-1640 and were injected intraperitoneally (ip) or intravenously (iv) via the lateral tail veins as a 300µl suspension.

2.5.5 Procurement of tissue from experimental mice

Mie were sacrificed at the stated time-points. Animals received terminal doses of anaesthetic for tissue procurement. Human skin allografts were initially excised and stored in c.RPMI on ice, before incubation with 1.6mg/ml collagenase (Roche) for 60 minutes at 37°C. Cells from skin grafts were subsequently obtained by moralisation though a 70µm cell strainer followed by washing with PBS supplemented with 5mM EDTA and 2% FCS, in order to inhibit the collagenase. Analysis was performed by flow cytometry. A midline laparotomy incision was then performed and a 26-gauge needle used for inferior venal cava puncture with up to 1ml of blood harvested. Serum was obtained after centrifugation and stored at -80°C. The remaining cell pellet was used for flow cytometry analysis after red blood cell lysis and filtration. Splenectomy was performed, with harvested spleens placed in c.RPMI on ice. Cells from mouse spleens were obtained by moralisation though a 70µm cell strainer followed by washing, red blood cell lysis and filtration, before flow cytometry

analysis. In some experiments, cells from bone marrow were also harvested by flushing the medullary cavities of both femurs with c.RPMI; the cells procured were subsequently analysed by flow cytometry. For monitoring of peripheral blood over time, serial tail bleeds were performed. Total blood volume obtained did not exceed 10% of the total blood volume on any one occasion.

2.5.6 Tissue typing

5ml of whole blood was obtained from skin donors and leucocyte cones. These samples were analysed by the Oxford Transplant Centre Histocompatibility and Genetics Laboratory. Full tissue-typing was performed for HLA-A, -B, -Cw, -DR and –DQ (Table 2.3).

2.6 Statistical analysis

Survival data were only analysed from mice that achieved human leucocyte chimerism levels of >1% in the spleen or 0.1% in the peripheral blood in the absence of GvHD. Log-rank tests, non-parametric Mann-Whitney U tests, parametric Student t-tests and 2-way ANOVA tests were applied as appropriate and as detailed in figure legends. Statistical analysis was performed using Graphpad Prism v6.0 software. *p* values under 0.05 were considered significant.

Table 2.1

Reagent	Supplier	Concentration	Function
6-*N*, *N*-Diethyl-D-β,γ-dibromomethylene ATP trisodium salt (ARL 67156)	R&D Systems	0.1mM/L	EctoATPase inhibitor
AMT Hydrochloride	Enzo Science	0.002mM/L	iNOS inhibitor
S-(2-boronoethyl)-L-cysteine (BEC)	Cambridge Bioscience	1.0mM/L	Arginase II inhibitor
L-Arginine	Sigma Aldrich	1-4mM/L	Substrate for Arginase II
SB 431542	R&D Systems	10μM/L	TGFβ inhibitor
Anti-FAS-L	R&D Systems	10μM/L	Blockade of FAS-L
Anti-PD-1 mAb	R&D Systems	10μg/ml	Blockade of PD-1
Anti-CD80 mAb	R&D Systems	10μg/ml	Blockade of CD80
Anti-CD86 mAb	R&D Systems	10μg/ml	Blockade of CD86
Anti-CD154 mAb	R&D Systems	10-50μg/ml	Blockade of CD154
Anti-CD40 mAb	R&D Systems	10-50μg/ml	Blockade of CD40
Anti-IL-10 mAb	R&D Systems	10-50μg/ml	Blockade of IL-10
Anti-IL-10R mAb	R&D Systems	10-50μg/ml	Blockade of IL-10R
Anti-CD25 mAb	R&D Systems	10μg/ml	Blockade of CD25
Anti-CD122 mAb	R&D Systems	10μg/ml	Blockade of CD122
Anti-TIM-1 mAb	Biolegend	10μg/ml	Blockade of TIM-1
$IgG_{1\kappa}$ mAb	R&D Systems	10-50μg/ml	Isotype control
$IgG_{2a\kappa}$ mAb	R&D Systems	10-50μg/ml	Isotype control

Table 2.1 Mechanistic *in vitro* blockade

Inhibitors used during *in vitro* suppression assays to investigate potential mechanisms of suppression utilised by EXPB10 cells. Reagents are listed together with supplier, concentrations used in experiments as well as the expected function of each reagent.

Table 2.2

Specificity	Conjugation	Clone	Species of Origin	Supplier
7-AAD	PerCP	N/A	N/A	eBioscience
CD19	APC-Cy7	SJ25C1	Mouse	BD Pharmingen
CD20	PE-Cy7	2H7	Mouse	eBioscience
CD4	PE-Texas Red	RPA-T4	Mouse	eBioscience
CD8	FITC	RPA-T8	Mouse	eBioscience
CD80	FITC	2D10.4	Mouse	eBioscience
CD86	PE	IT2.2	Mouse	eBioscience
FAS-R	PE	DX2	Mouse	eBioscience
γCR	PE	TUGh4	Rat	eBioscience
CD122	APC	TU27	Mouse	Biolegend
CD25	PE-Cy7	M-A251	Mouse	BD Pharmingen
IgD	PE-Cy7	IA6-2	Mouse	BD Pharmingen
IgM	eFluor® 450	SA-DA4	Mouse	eBioscience
CD27	eFluor® 450	O323	Mouse	eBioscience
CD138	eFluor® 450	HB7	Mouse	eBioscience
BAFF-R	FITC	8A7	Mouse	eBioscience
TACI	PE	11H3	Mouse	eBioscience
BCMA	APC	19F2	Mouse	Biolegend
CD1d	APC	51.1	Mouse	eBioscience
CD10	PE-Texas Red	ALB1	Mouse	Beckman Coulter
CD5	FITC	L17F12	Mouse	eBioscience
CD21	eFluor® 450	HB5	Mouse	eBioscience
CD23	APC-eFluor® 780	EBVCS2	Mouse	eBioscience
CD24	PE	ML5	Mouse	BD Pharmingen
CD38	FITC	HIT2	Mouse	BD Pharmingen
CD71	APC	OKT9	Mouse	eBioscience
CD73	eFluor® 450	AD2	Mouse	eBioscience
TIM-1	PE	1D12	Mouse	Biolegend
IL-21R	PE	2G1-K12	Mouse	Biolegend
LAP	PE	FNLAP	Mouse	eBioscience
CD22	APC	S-HCL-1	Mouse	eBioscience
B7-H4	APC	H74	Mouse	eBioscience
CD39	PE-Cy7	eBioA1	Mouse	eBioscience
Arginase II	FITC	P78540	Rabbit	BIOSS USA
FAS-L	PE	NOK-1	Mouse	eBioscience
PD-L1	PE-Cy7	MIH-1	Mouse	eBioscience
PD-L2	APC	MIH18	Mouse	eBioscience
Granzyme B	FITC	GB11	Mouse	BD Pharmingen
TRAIL	PE	RIK-2	Mouse	eBioscience
IL-10	eFluor® 660	JES3-9D7	Rat	eBioscience
TNFα	FITC	MAb11	Mouse	eBioscience
IFNγ	PE	4S.B3	Mouse	eBioscience
CD45	APC	HI30	Mouse	Invitrogen
CD127	PE	H1L-7R-M21	Mouse	BD Pharmingen
CD154	PE	24-31	Mouse	eBioscience
CD40	APC	5C3	Mouse	eBioscience
IL-10R	APC	3F9	Rat	Biolegend
pSTAT3	Alexa Fluor 450® 647	pY705	Mouse	BD Pharmingen

Table 2.2 Fluorescence-activated cell sorting (FACS) and flow cytometry

The various fluorochrome-coupled mAbs used to detect both extracellular and intracellular cell markers. Antibody specificities together with the conjugated fluorochrome, clone, species of origin and supplier are included.

3.0 EXPANSION OF HUMAN CD19$^+$IL-10$^+$ B CELLS WITH REGULATORY FUNCTION

The study of IL-10$^+$ B regulatory cells (Breg) in both animal models and human peripheral blood has demonstrated the growing importance of this suppressive population in the regulation of the immune system [12, 13, 20, 24, 32, 78]. Similar to T regulatory cells (Treg), adoptive transfer of mouse Breg can ameliorate autoimmune disease and prevent rejection of allograft [4, 39, 68, 98, 141]. However, unlike with human Treg, it is not currently possible to expand or generate a population of human Breg *in vitro* to facilitate either the further study of human Breg or the development of a Breg cellular therapy. The aim of *Chapter 3.0* was to investigate the first hypothesis outlined in *section 1.8:*

- **Human CD19$^+$IL-10$^+$ B cells with regulatory function can be generated *ex vivo***

Induction of IL-10 expression by human CD19$^+$ B cells has been previously demonstrated to be dependent on CD154 (CD40 ligand), a ligand which is up-regulated on early-activated CD4$^+$ T cells [47]. To recreate this stimulatory environment, an immortalised Chinese Hamster Ovary (CHO) cell line, which has been transfected to express human CD154 was utilised (kind gift of Professor Claudia Mauri). This particular cell line is highly stable, can be transfected with relative ease and grows well in culture [142]. Previous work in mice has demonstrated that anti-CD40 stimulation of whole CD19$^+$ B cells yielded higher numbers of viable transitional 2 (T2) immature B cells with regulatory function when compared to anti-CD40 stimulation of purified T2-like B cells [69]. Therefore the co-culture of CD154$^+$ CHO cells with whole human CD19$^+$ B cells, rather than with individual B cell subsets was explored initially.

RESULTS

3.1 Expansion of human $CD19^+IL\text{-}10^+$ B cells

$CD19^+$ B cells were initially isolated from peripheral blood mononuclear cells (PBMC) of healthy individuals by negative selection (purities > 96%), as outlined in *Chapter 2.0 Materials and Methods*. Human $CD19^+$ B cells were then co-cultured for up to 7 days with varying ratios of $CD154^+$ CHO cells to $CD19^+$ B cells, and under different cytokine conditions (fig. 3.1.1a) in round bottomed, 96-well plates. A non-transfected, $CD154^-$ CHO cell line and/or complete media served as controls. Each condition was set up in triplicate and was performed three times using three different human donors. In order to vary the ratio of CHO cells to $CD19^+$ B cells, CHO cell numbers were varied in accordance with the ratio under test whilst B cell numbers were kept constant. CHO cells were co-cultured with human $CD19^+$ B cells at ratios 1:1, 1:5 and 1:10, to investigate whether the intensity of stimulation by CD154 influenced either rate of B cell expansion or IL-10 production. Cytokine combinations were also investigated to ascertain whether proliferation of $IL\text{-}10^+$ B cells would benefit from exposure to different microenvironments: media alone; IL-4 only; IL-2, IL-4 and IL-10.

It is important to note that the only obvious difference between $CD154^+$ CHO cells and $CD154^-$ CHO cells was that of CD154 expression. Rate of growth during passages (*data not shown*) and cell size were not significantly different (fig. 3.1.1b). CHO cells were irradiated prior to co-culture with human $CD19^+$ B cells, in order to prevent proliferation of this immortalised cell line and possible impedance of B cell expansion. In accordance with similar protocols designed to expand human memory B cells [115], cultures were re-plated with fresh CHO cells after the first 3 days of expansion in order to maintain optimal stimulation, and media +/- cytokines was replaced on days 3 and 5. At daily time-points, cells were harvested from wells by gentle agitation using a pipette and washed with media twice before FACS analysis (fig. 3.1.1c). Cell counts at this stage were performed using counting beads during FACS acquisition. Expansion factor was calculated as the fold increase in $CD19^+$ B

cell number at day of harvest when compared to the starting cell number at day 0, with expansion factor at day 0 being normalised to the value of 1.

3.1.1 Human $CD19^+$ B cells can be expanded by co-culture with $CD154^+$ CHO cells

Co-culture with 1:1 or 1:5 ratios of $CD154^+$ CHO cells and $CD19^+$ B cells resulted in greatest rates of expansion under all cytokine culture conditions by day 7 (fig. 3.1.1d). Co-culture with the cytokine combination IL-2, IL-4 and IL-10, resulted in greatest rates of expansion in all co-culture ratios of $CD154^+$ CHO cells to $CD19^+$ B cells by day 7. CD19+ B cells did not expand in the presence of $CD154^-$ CHO cells with any cytokine combination or in complete media alone. Under all conditions, cell numbers initially decreased during the first few days of co-culture of human B cells and CHO cells, predominantly due to cell death (fig. 3.1.1e). Under conditions of CD154 stimulation (with the exception of 1:10 co-culture in the absence of cytokines), $CD19^+$ B cells began to proliferate by day 5 and continued to expand up to day 7 of co-culture. A similar pattern of proliferation was seen during co-culture of B cells with $CD154^-$ CHO cells when in the presence of cytokines; however the pattern of proliferation appeared to be much more variable in the absence of both cytokines and CD154 stimulation (fig. 3.1.1d).

Stimulation by $CD154^+$ CHO cells results in greater percentage frequencies of $IL\text{-}10^+$ B cells compared to $CD19^+$ B cells co-cultured with $CD154^-$ CHO cells

As co-culture of $CD19^+$ B cells with $CD154^+$ CHO cells at a 1:1 ratio results in greatest rate of expansion under all cytokine conditions tested, IL-10 expression of $CD19^+$ B cells stimulated under these conditions was investigated (fig. 3.1.1f). $CD19^+$ B cells were harvested at day 3 and day 7 of co-culture; following 6 hours of stimulation with PMA, ionomycin and monensin (PIM), $CD19^+$ B cells were stained intracellularly for IL-10. Gating was performed against isotype controls. IL-10 expression was compared to that of freshly isolated $CD19^+$ B cells from the same human donor, which had undergone only the stimulation step with PIM, as well as

$CD19^+$ B cells that had been co-cultured with $CD154^-$ CHO cells and were also stimulated with PIM.

Expanded $CD19^+$ B cells were able to express IL-10 at all time-points taken, under all cytokine conditions (fig. 3.1.1f). The highest percentage of $CD19^+$ $IL\text{-}10^+$ B cells was achieved after co-culture with $CD154^+$ CHO cells in the absence of cytokines i.e. in complete media only. IL-10 expression was greatest under all cytokine conditions when B cells were cultured with $CD154^+$ CHO cells rather than $CD154^-$ CHO cells (fig. 3.1.1f, $^{¥}p<0.0001$). There was no significant difference in percentage of IL-10 expression between $CD19^+$ B cells harvested at day 3 and day 7, except under conditions of CD154 stimulation and cytokines IL-2, IL-4 and IL-10, where a significantly greater percentage of $CD19^+$ $IL\text{-}10^+$ B cells was generated at day 7 compared to day 3 (fig. 3.1.1f, $*p<0.05$). Although IL-10 expression was significantly greater in the presence of CD154 stimulation under all conditions, IL-10 levels decreased when $CD19^+$ B cells were cultured in the presence of cytokines when compared to those B cells cultured with complete media alone.

Rate of $CD19^+IL\text{-}10^+$ B cell expansion correlates with ratio of $CD154^+$ CHO cells co-cultured with human $CD19^+$ B cells

EXPB10 cells were co-cultured at varying ratios of $CD154^+$ CHO cells to human $CD19^+$ B cells: 1:10, 1:5 and 1:1. Across all cytokine conditions, it was found that increased rates of $CD19^+$ B cell expansion correlated with this ratio (fig. 3.1.1d), demonstrating that the degree of CD154 stimulation dictated, in part, the rate of proliferation of $CD19^+$ B cells in this *in vitro* system.

Lower levels of $CD19^+$ B cell death are associated with duration of co-culture

The number of $CD19^+$ B cells fell dramatically on day 1 of co-culture with CHO cells, and then gradually increased during the remaining 6 days of culture. FACS analysis on days 1, 3 and 7 illustrated that the % of dead $CD19^+7\text{-}AAD^+$ B cells significantly decreased with time, such that by day 7, >85% of $CD19^+$ B cells present in culture were live cells (fig. 3.1.1e, $**p<0.007$).

Highest rate of CD19$^+$IL-10$^+$ B cell expansion is achieved with the cytokine combination IL-2, IL-4 and IL-10

Although human CD19$^+$ B cells could be expanded when co-cultured with CD154$^+$ CHO cells in the absence of cytokines, this expansion rate was enhanced when exogenous IL-4 was added to the co-cultures, and was further increased when the cytokine combination IL-2, IL-4 and IL-10 was used (fig. 3.1.1d). It should be noted that this increase in expansion rate did not correspond to an increase in percentage frequencies of IL-10 expression by expanded CD19$^+$ B cells (fig. 3.1.1f).

Highest rate of expansion of CD19$^+$IL-10$^+$ B cells generated on day 7 of co-culture with IL-2, IL-4, IL-10 culture conditions

Although the highest percentage of CD19$^+$IL-10+ B cells was generated after 7 days of co-culture with complete media, expansion factor of these CD19$^+$ B cells remained poor (fig. 3.1.1d). Thus the actual rate of expansion of CD19$^+$IL-10$^+$ B cells under these conditions was low. The highest rate of expansion of CD19$^+$IL-10$^+$ B cells was achieved when CD19$^+$ B cells were cultured for 7 days with CD154$^+$ CHO cells at 1:1 ratio, with a combination of cytokines IL-2, IL-4, IL-10 (fig. 3.1.1g, $^¥p<0.0001$). Culture under these conditions consistently resulted in greater than 200 fold expansion of CD19$^+$IL-10$^+$ B cells. CD19$^+$ B cells cultured by this method will be referred to as 'EXPB10.' If CD19$^+$ B cells were expanded by a modified protocol, a suffix has been added to the term EXPB10 i.e. if CD19$^+$ B cells co-cultured with CD154$^+$ CHO cells at a 1:1 ratio were expanded with IL-4 only, and not the cytokine combination IL-2, IL-4 and IL-10, these expanded CD19$^+$ B cells have been described as 'EXPB10-IL-4.'

3.1.2 Sustained proliferation of EXPB10 cells requires repeated stimulation by CD154$^+$ CHO cells

One of the putative goals of *ex vivo* human Breg expansion is that of cellular therapy. Although human CD19$^+$IL-10$^+$ B cells could be generated and expanded successfully by *in vitro* co-culture for 7 days, it would be exigent to understand whether such a

technique could be used to generate much larger numbers of EXPB10 cells by increasing the length of *in vitro* co-culture. By day 7 of co-culture, the majority of irradiated $CD154^+$ CHO cells that had been plated on day 0 and day 3, were dead (*data not shown*). We investigated whether further plating of fresh, irradiated $CD154^+$ CHO cells on day 7 could improve proliferation of EXPB10 cells, compared to EXPB10 cells which did not receive fresh CHO cells but which were otherwise maintained until day 14. Media and cytokines were again replaced on days 7, 10 and 12 in both conditions.

Re-plating cultures with fresh $CD154^+$ CHO cells on day 7 did indeed significantly increase the rate of $CD19^+$ B cell proliferation (fig. 3.1.2a, $^{¥}p<0.0001$). EXPB10 cells which did not receive further $CD154^+$ CHO cells on day 7 actually decreased in number until day 9, when total $CD19^+$ B cell number again started to increase albeit at a lower rate than EXPB10 cells which had been re-stimulated at day 7.

Re-stimulation of EXPB10 cells with $CD154^+$ CHO cells results in an increased percentage frequency of $IL\text{-}10^+$ B cells at day 14

IL-10 expression of EXPB10-d14 cells was examined at day 14, again following further stimulation by 6-hour co-culture with PIM. EXPB10-d14 cells that had received repeated CD154 stimulation at day 7, demonstrated a significant increase in percentage of $CD19^+IL\text{-}10^+$ B cells when compared to those EXPB10-d14 cells that had just undergone media and cytokine replacement (fig. 3.1.2b, $^{¥}p<0.0001$).

Sustained stimulation with $CD154^+$ CHO cells in the presence of cytokines IL-2, IL-4 and IL-10, can achieve greater than 800 fold expansion of $CD19^+IL\text{-}10^+$ B cells

Combining the increase in total number of $CD19^+$ B cells with the increase in IL-10 expression seen when human $CD19^+$ B cells are co-cultured with $CD154^+$ CHO cells in the presence of cytokines IL-2, IL-4 and IL-10 for 14 days with repeated stimulation by CD154, resulted in greater than 800 fold expansion of EXPB10 cells (fig. 3.1.2c). These findings demonstrate that EXPB10 cells can be generated in large numbers by extending both duration of co-culture and exposure to CD154.

3.2 EXPB10-mediated suppression of $CD4^+$ T cell responses

Although we have demonstrated that human $CD19^+$ B cells which express IL-10 can be both generated and expanded, it does not necessarily follow that EXPB10 cells exert suppressive function. To determine whether *ex vivo* generated $CD19^+IL\text{-}10^+$ B cells are able to suppress an immune response, live EXPB10 cells were first sorted from $CD154^+$ CHO cells (fig. 3.2.1a) after the 7-day expansion co-culture outlined above. As it was not possible to sort IL-10-producing cells easily, the whole $CD19^+$ B cell population was isolated based on expression of CD19. Sorted EXPB10 cells were then co-cultured with autologous $CD4^+$ T cells that had been stained with CFSE, and anti-CD3/CD28 beads for 5 days. Stimulated $CD4^+$ T cells cultured without EXPB10 (denoted as either 'STIM $CD4^+$ T CELL ONLY' or '0:1') as well as $CD4^+$ T cells co-cultured with anti-CD3/CD28 beads and freshly isolated, un-manipulated $CD19^+$ B cells ('NON-EXPB10') served as controls. At day 5 of the suppression assay, cells were harvested, stained with cell surface antibodies and analysed by FACS. Doublets and dead cells were excluded prior to analysis of $CD4^+$ T cell responses (fig. 3.2.1b).

In some experiments outlined below, expression of pro-inflammatory cytokines produced by $CD4^+$ T cells was measured. In these studies, EXPB10 cells were co-cultured with anti-CD3/CD28 bead-stimulated $CD4^+$ T cells for 3 days. On day 3, cells were harvested, washed and then stimulated with PMA, ionomycin and brefeldin (PIB) for 5 hours as described in *Chapter 2.0,* prior to intracellular staining for cytokine expression.

The number of $CD4^+$ T cells at day 5 has been expressed as a percentage relative to that of the stimulated $CD4^+$ T cell only control at day 5 of culture. Proliferation of $CD4^+$ T cells could be measured by analysing the number of cell divisions generated over the 5-day duration of the suppression assay. This analysis could then be expressed as either the proliferation index or division index. The proliferation index is defined as the total number of divisions divided by the number of cells that went into division; therefore only cells that have undergone at least one cell division i.e. the cell population capable of responding to stimulation present in the culture, would be

analysed. The division index is the average number of cell divisions that a cell in the original population has undergone; therefore all cells including those which have not responded to stimulation, would be included in the analysis.

It was not yet clear how or indeed if EXPB10 cells could suppress activated $CD4^+$ T cell responses; suppression may have been through apoptosis and cell death, inhibition of division in cells which are already proliferating, or in fact inhibition of cell stimulation thus preventing the initiation of proliferation. Furthermore the stimulation itself was polyclonal (anti-CD3/CD28 beads), thus all $CD4^+$ T cells in the co-culture should have been able to respond, as opposed to an antigenic stimulus where only a limited proportion of $CD4^+$ T cells would have been alloreactive [143]. Therefore under such *in vitro* conditions, it was considered to be more accurate to measure division index, as this calculation included all cells and therefore took into account suppression of the entire $CD4^+$ T cell population. Suppression was calculated as a measure of division index of $CD4^+$ T cells when co-cultured with B cells relative to that of $CD4^+$ T cells when co-cultured with anti-CD3/CD28 beads only [144]. Using this calculation, the STIM $CD4^+$ T CELL ONLY condition was always normalised to 0% suppression.

3.2.1 EXPB10 cells significantly suppress proliferation of polyclonally stimulated, autologous $CD4^+$ T cells in a dose-dependent manner

EXPB10 cells significantly suppressed proliferation of autologous $CD4^+$ T cells *in vitro* in a dose-dependent manner (fig. 3.2.2c, $^{¥}p<0.0001$). Significant suppression of proliferation was achieved at ratios as low as 0.5:1 EXB10 cells to $CD4^+$ T cells, and was no longer apparent at ratios as low as 0.1:1. Suppression was calculated as a measure of division index relative to the stimulated $CD4^+$ T cell only control. NON-EXPB10 cells were not able to suppress $CD4^+$ T cell proliferation *in vitro*.

EXPB10 cells significantly suppress absolute numbers of autologous $CD4^+$ T cells when stimulated *in vitro*

EXPB10 cells significantly suppressed the absolute number of $CD4^+$ T cells, including at ratios as low as 0.1:1 EXPB10 to $CD4^+$ T cells (fig. 3.2.1d, $^{¥}p<0.0001$). In contrast, non-expanded $CD19^+$ B cells from the same donor as EXB10 cells, did not suppress the absolute number of $CD4^+$ T cells, at any ratio, and may have resulted in increased numbers of $CD4^+$ T cells when compared to stimulated $CD4^+$ T cells alone.

EXPB10 cells inhibit expression of pro-inflammatory cytokine production by polyclonally stimulated autologous $CD4^+$ T cells *in vitro*

EXPB10 cells significantly inhibited expression of both interferon-γ **(**IFNγ) and tumour necrosis factor α (TNFα) by $CD4^+$ T cells, including at a ratio of 0.5:1 EXPB10 to $CD4^+$ T cells, when compared to either STIM $CD4^+$ T CELL ONLY or NON-EXPB10 cells (fig. 3.2.1e & f, $^{¥}p<0.0001$). NON-EXPB10 cells did not exert any suppressive effect on pro-inflammatory cytokine production by $CD4^+$ T cells.

EXPB10 suppressive or co-stimulatory function may be determined by $CD4^+$ T cell activation *in vitro*

EXPB10 cells were cultured with CFSE-stained $CD4^+$ T cells in the presence or absence of anti-CD3/CD28 beads for 5 days. As previously demonstrated, EXPB10 cells suppressed $CD4^+$ T cell proliferation in the presence of anti-CD3/CD28 beads. In the absence of such stimulation however, EXPB10 cells appeared to promote $CD4^+$ T cell proliferation, relative to the un-stimulated $CD4^+$ T cell control. Indeed, $CD4^+$ T cell proliferation in EXPB10 co-culture was also significantly greater than that of co-culture with non-expanded $CD19^+$ B cells (fig. 3.2.1g).

EXPB10 cells significantly suppress proliferation of allogeneic $CD4^+$ T cells with equal potency as suppression of autologous $CD4^+$ T cells *in vitro*

Although it has been established that EXPB10 cells are able to suppress proliferation of autologous $CD4^+$ T cells *in vitro***,** this does not necessarily predict suppression of allogeneic $CD4^+$ T cells in this system. Thus EXPB10 cells were co-cultured with either autologous or allogeneic $CD4^+$ T cells (isolated from a different, HLA-mismatched human donor, see *Chapter 2.0*) in the suppression assay described above. It was found that EXPB10 cells were able to significantly suppress proliferation of allogeneic $CD4^+$ T cells *in vitro*, that too at an equal potency to that of autologous $CD4^+$ T cells (fig. 3.2.1.h).

EXPB10 cells significantly suppress proliferation of autologous $CD8^+$ T cells with equal potency as suppression of autologous $CD4^+$ T cells *in vitro*

Given that inflammatory responses, particularly those surrounding transplant rejection, are multi-factorial and can involve several arms of the adaptive immune system [145], EXPB10 cell-mediated suppression of autologous $CD8^+$ was also explored. EXPB10 cells were co-cultured with autologous PBMC at a ratio of 1:1 EXPB10 cells to PBMC and anti CD3/CD28 beads for 5 days. EXPB10 cells were able to significantly suppress proliferation of $CD8^+$ T cells at an equivalent potency to that of $CD4^+$ T cell suppression (fig. 3.2.1i).

EXPB10 cells do not require isolation from $CD154^+$ CHO cells after harvest from expansion cultures and prior to co-culture with $CD4^+$ T cells

As mentioned earlier, analysis of cell cultures after harvest from expansion conditions had revealed that the majority of irradiated $CD154^+$ CHO cells were dead by day 7 of co-culture. Thus we asked whether FACS-sorting of EXPB10 cells prior to use in suppression co-cultures was a necessary step. EXPB10 cells that had not been sorted from $CD154^+$ CHO cells were able to significantly suppress proliferation of $CD4^+$ T cells at equal potencies as sorted EXPB10 cells (fig. 3.2.1j). Therefore in all subsequent experiments, the FACS-sorting step was omitted and EXPB10 cells used in suppression assays directly after harvest and washings as outlined in *Chapter 2.0*.

3.2.2 Suppressive potency of *ex vivo* expanded CD19$^+$ B cells can vary depending on different parameters of expansion co-culture

Given that CD19$^+$IL-10+ B cells could be generated by co-culture with CD154$^+$ CHO cells at several different ratios, under several different cytokine conditions and following co-culture of different durations, we investigated whether these different parameters could influence the suppressive function of EXPB10 cells.

Different cytokine combinations used in expansion co-cultures do not affect suppressive potency of EXPB10 cells

CD19$^+$IL-10+ B cells generated by co-culture with CD154$^+$ CHO cells at a 1:1 ratio and with cytokines IL-4 only, IL-2, IL-4 and IL-10, or complete media only were tested to compare suppressive potency. EXPB10 cells generated by all three conditions outlined were able to significantly suppress proliferation of polyclonally stimulated, autologous CD4$^+$ T cells. There was no difference in suppressive potency between the three differentially expanded EXPB10 cell populations (fig. 3.2.2a).

Suppressive potency of EXPB10 cells does correlate with ratio of CD154$^+$ CHO cells to CD19$^+$ B cells in expansion co-cultures

CD19$^+$IL-10+ B cells were generated by co-culture with CD154$^+$ CHO cells at different ratios of CHO to B cells: 1:1 (EXPB10 1:1), 1:5 (EXPB10 1:5) and 1:10 (EXPB10 1:10). All expansion cultures were incubated with the cytokines IL-2, IL-4 and IL-10, as this combination had already been demonstrated to result in the greatest rate of CD19$^+$ B cell expansion (fig 3.1.1d). The resulting expanded CD19$^+$ B cells were then co-cultured with autologous CD4$^+$ T cells and anti-CD3/CD28 beads for 5 days. Suppressive potency correlated with the ratio of CD154 CHO cells co-cultured with CD19$^+$ B cells, such that EXPB10 1:1 cells were significantly more suppressive than EXPB10 1:5 cells, which in turn were significantly more suppressive than EXPB10 1:10 cells (fig. 3.2.2b, $^{¥}p<0.0001$, $^{**}p<0.007$).

EXPB10 cells are most suppressive when generated by co-culture with CD154$^+$ CHO cells and cytokines IL-2, IL-4 and IL-10 for 7 to 10 days

CD19$^+$IL-10+ B cells were generated by co-culture with CD154$^+$ CHO cells at a 1:1 ratio with cytokines IL-2, IL-4 and IL-10, given that these conditions result in optimal expansion of CD19$^+$IL-10$^+$ B cells with optimal suppressive potency (fig. 3.1.1d & 3.2.2b). EXPB10 cells were harvested at days 3, 7, 10 or 14 and suppression tested by 5-day co-culture with polyclonally stimulated CD4$^+$ T cells. EXPB10 cells harvested at day 7 or day 10 were significantly more suppressive than EXPB10 cells harvested at either day 3 or day 14 (fig. 3.2.2c, ¥¥$p<0.01$, ¥¥¥$p<0.02$). There was no significant difference in suppressive potency between EXPB10 cells harvested on day 7 or day 10.

3.2.3 Suppressive potency of *ex vivo* expanded CD19$^+$ B cells can vary depending on different parameters of CD154$^+$ CHO cell passage

EXPB10 cells differ in suppressive potency depending on the ratio of CD154$^+$ CHO cells cultured with CD19$^+$ B cells. Expression of CD154 on CHO cells also significantly decreases with progressive passages (fig. 3.2.3a, **$p<0.007$). Therefore we asked whether levels of CD154 expression on CHO cells, as well as other parameters of CHO cell passage, could subsequently affect suppressive potency of EXPB10 cells.

CD154^{hi} CHO cells generate significantly more suppressive EXPB10 cells than CD154^{lo} CHO cells

CHO cells were FACS-sorted according to levels of CD154 expression. CD154^{hi} CHO and CD154^{lo} CHO cells were defined as those CHO cells expressing the highest 10% or lowest 10% of CD154 cell surface levels respectively (fig. 3.2.3b). CD154^{hi} CHO and CD154^{lo} CHO cells were subsequently cultured with human CD19$^+$ B cells at a 1:1 ratio with the cytokines IL-2, IL-4 and IL-10 for 7 days. The resulting EXPB10 cells were cultured in the 5-day suppression assay with polyclonally stimulated CD4$^+$ T cells. EXPB10 cells which had been expanded with CD154^{hi}

CHO cells were significantly more suppressive than EXPB10 cells which had been expanded with $CD154^{lo}$ CHO cells (fig. 3.2.3c, ¥¥¥$p<0.02$). There was no difference in rate of EXPB10 expansion (*data not shown*). These observations indicate that not only did the ratio of $CD154^+$ CHO cells to $CD19^+$ B cells affect EXPB10 cell-mediated suppression, but that the actual CD154 expression levels on CHO cells also had significant effects on EXPB10 suppressive potency.

Confluence of $CD154^+$ CHO cell culture affects potency of EXPB10 cells despite equal levels of CD154 expression

CD154 expression on CHO cells did not vary with confluence during CHO cell culture (fig. 3.2.3d). However, it was observed that suppressive potency of EXPB10 cells significantly decreased when $CD154^+$ CHO cell confluence exceeded 60%; that is, the more confluent the $CD154^+$ CHO cells when used in expansion co-cultures (when confluence was greater than 60%), the less suppressive the resulting EXPB10 cells. EXPB10 cells remained potently suppressive when $CD154^+$ CHO cells were less than or equal to 60% confluent. No change was seen in $CD19^+$ B cell expansion rates (*data not shown)*. In addition, there appeared to be no difference in survival of irradiated $CD154^+$ CHO cells by day 7 of expansion co-culture with human $CD19^+$ B cells, regardless of confluence (*data not shown*).

Confluence was calculated based on the consistent finding that in a T75 flask (which has a surface area of $75cm^2$), confluence yielded 10 $x10^6$ $CD154^+$ CHO cells. In these experiments and indeed all expansions of human $CD19^+$ B cells, $CD154^+$ CHO cells were grown and harvested in T75 flasks immediately prior to use in the expansion protocol. $CD154^+$ CHO cells were at the 5^{th} passage (P-5) in this experiment; cells were cultured for 5 days and were split at different times in order to achieve the different degrees of confluence. Given that CD154 expression and CHO cell survival remained unchanged with confluence, these findings suggest that other parameters within the CHO cell are affected by confluence, and which thereby impact upon EXPB10 cell function.

Duration of CD154$^+$ CHO cell culture per passage affects potency of EXPB10 cells despite equal levels of CD154 expression

We have observed that EXPB10 cells remain potently suppressive when CD154$^+$ CHO cells remain less than or equal to 60% confluent (fig. 3.2.3d). To explore whether duration of CHO cell passage can also affect EXPB10 suppressive potency, CD154$^+$ CHO cells were passaged to maintain less than 60% confluence whilst varying duration of the last passage prior to use in CD19$^+$ B cell expansion co-cultures. CD154$^+$ CHO cells were split 1:20 and cultured at P-5 for 2, 3, 4, or 6 days. CD154 expression levels remained stable with passage duration (fig. 3.2.3e). Resulting CD154$^+$ CHO cells were then co-cultured with CD19$^+$ B cells at a 1:1 ratio with cytokines IL-2, IL-4 and IL-10 for 7 days before EXPB10 harvest and use in suppression assays. It was found that EXPB10 suppressive potency varied with time of CD154$^+$ CHO cell passage prior to use in the expansion protocol. Whilst there was no difference in EXPB10 cell potency when CD154$^+$ CHO cells had been passaged for 2 or 3 days, suppressive potency significantly decreased when CHO cells had been cultured for 4 days, and even more so when cultured for 6 days (fig. 3.2.3e, $^{¥}p<0.0001$). Again, there was no difference in survival of irradiated CHO cells at day 7 of expansion co-cultures with CD19$^+$ B cells, regardless of passage duration (*data not shown*).

3.2.4 Proliferation and survival of EXPB10 cells within the CD4$^+$ T cell suppression assay

Human CD19$^+$ B cells were highly stimulated in expansion co-cultures before suppression function of EXPB10 cells was tested. It is important to ascertain how EXPB10 cells behave in the suppression assay with polyclonally stimulated CD4$^+$ T cells, and whether this behavior differs between EXPB10 cells of differing potency. Two such parameters to study include EXPB10 survival and proliferation – poor survival and poor proliferation of less suppressive EXPB10 cells or indeed NON-EXPB10 cells could account for the difference in putative suppressive potency observed. It may be that the suppressive effects seen on CD4$^+$ T cells are merely

artefact of improved survival and/or proliferation of highly activated $CD19^+$ B cells *in vitro*.

EXPB10 cells proliferate significantly more than NON-EXPB10 cells in suppression assays with polyclonally stimulated $CD4^+$ T cells

EXPB10 cells or NON-EXPB10 cells were stained with VPD before co-culture with CFSE-stained $CD4^+$ T cells at a 1:1 ratio with anti-CD3/CD28 beads for 5 days. Proliferation of $CD19^+$ B cells was then measured by both proliferation index and division index on day 5 (fig. 3.2.4a). Proliferation of EXPB10 cells was significantly greater than that of NON-EXPB10 cells at all ratios of $CD19^+$ B cells to $CD4^+$ T cells, as illustrated by both histograms of $CD19^+VPD^+$ expression and division index. It is worth noting, however, that proliferation index in this instance was grossly misleading; proliferation indices of NON-EXPB10 cells were significantly greater than that of EXPB10 cells, but proliferation histograms clearly demonstrated that the majority (>80%) of NON-EXPB10 cells remained as undivided. It was, as discussed earlier in this chapter, the division index that which gave a more accurate representation of proliferation profiles within the confines of this assay, and so the division index has been used to define proliferation within the *in vitro* suppression co-culture.

Proliferation and survival of EXPB10 cells does not vary with suppressive potency of EXPB10 cells

The proliferation and survival of EXPB10 1:1, EXPB10 1:5 or EXPB10 1:10 cells at day 5 of the $CD4^+$ T cell suppression assay were analysed. There was no difference in either division index or final cell number of $CD19^+$ B cells between any EXPB10 cell group (fig. 3.2.4b), despite significant differences in suppressive potency between these *ex vivo* generated Breg subsets (fig. 3.2.2b). These findings suggest that the differential potencies of EXPB10 cells when co-cultured with varying ratios of $CD154^+$ CHO cells to $CD19^+$ B cells, was not due to differential proliferation or survival of EXPB10 cells.

DISCUSSION

The interaction of CD40 and its ligand, CD154, is a complex and can have multiple effects on the CD40-expressing target cell [115, 135]. Human $CD19^+$ B cells in peripheral blood constitutively express CD40 and are able to interact with activated $CD4^+$ T cells through this interaction, usually in the presence of antigen, to receive 'T cell help' [146]. Two of the many outcomes of this interaction are thought to include proliferation and the induction of IL-10 expression by $CD19^+$ B cells [116, 117, 146]. The importance of IL-10 producing Breg in both health and disease, in particular in transplantation, is rapidly becoming evident [12, 13, 20, 48]; however, despite successful efforts to expand and study mouse Breg [98], the *ex vivo* generation and in depth understanding of expanded human Breg remains elusive, as does the possibility of a human Breg cellular therapy for application in patients.

The primary objectives of this first section of work were to develop a robust technique by which $CD19^+IL\text{-}10^+$ B cells could be generated or expanded, as well as to establish the suppressive capacity of these cells. Murine studies have demonstrated the important role of the CD154-CD40 interaction in mouse Breg induction [98]; human studies have identified a crucial role for this interaction in suppressive function by human Breg [47]. Extensive work in the 1990s has illustrated that the configuration of CD154-mediated stimulation required to achieve optimal $CD19^+$ B cell proliferation is in the membrane-bound form i.e. that which is expressed on the cell surface [115, 125]. Human $CD154^+$ CHO cells provide an ideal medium by which to mimic $CD4^+$ T cell help and the CD154-CD40 interaction *in vitro*, and so co-culture of this cell line with $CD19^+$ human B cells to generate or expand $IL\text{-}10^+$ B cells with regulatory function (EXPB10 cells) was explored.

Expansion co-cultures were initially performed in the absence or presence of cytokines, namely IL-4 alone or the combination of IL-2, IL-4 and IL-10. These different cytokine combinations were initially selected based on extensive work in the last decade looking at cytokine effects on human $CD19^+$ B cells. It has been reported that IL-4 in conjunction with CD40 stimulation, can promote the proliferation of naïve human $CD19^+CD27^-$ B cells whilst being unable to support proliferation of

memory $CD19^+CD27^+$ B cells [115, 147]. However after prolonged culture (greater than 7 days), naïve B cells up-regulated CD27 and switched from IgM to IgG production, demonstrating that phenotypic stability may be an issue under such conditions [115]. Stimulation via CD40 and IL-4 has also been reported to protect human B cells from susceptibility to apoptosis [147] and also to promote the expansion or induction of mouse B10 cells [98]. Several other common cytokines, either alone or in combination have been known to promote proliferation and differentiation of human $CD19^+$ B cells. As early as 1984, IL-2 was identified as a key growth factor involved in proliferation of polyclonally activated B cells [148-150]. Although shown to promote plasma cell development [151], this differentiation was regulated when B cells are also stimulated in conjunction with IL-4 [152]. In addition, IL-2, by means of signal transducer and activator of transcription (STAT) 5, has been reported to enhance IL-10 production, albeit in Treg [153, 154]. IL-10 can act as a positive regulator of itself [155] and is known to prevent B cell apoptosis, promote B cell proliferation, differentiation, and MHC II expression in addition to playing a positive role in immunoglobulin class switching. Moreover, the use of IL-2, IL-4 and IL-10 together with CD40 stimulation, has been demonstrated to establish rapid proliferation of an apparently stable, naïve B cell population [135].

Co-culture of $CD19^+$ B cells and $CD154^+$CHO cells at all ratios tested, and under conditions of no cytokines, IL-4 alone or the combination of IL-2, IL-4 and IL-10, resulted in expansion of $CD19^+IL\text{-}10^+$ B cells (fig. 3.1.1d & 3.1.1f). Co-culture of human B cells with $CD154^-$ CHO cells demonstrated that in this context, CD154 stimulation was largely responsible for IL-10 production. $CD19^+$ B cells co-cultured with $CD154^-$ CHO cells were able to express IL-10, but at significantly lower levels than those exposed to CD154 stimulation, and that too, only following PMA and ionomycin stimulation (fig. 3.1.1f). Although under conditions where no cytokines were used, EXPB10 cells expressed the highest frequency of $IL\text{-}10^+$ cells, expansion rates of these B cell populations was very low when compared to EXPB10 cells generated with exogenous cytokines (3.1.1d). These observations suggest that whilst CD154 was primarily responsible for optimal IL-10 expression in this system, the cytokines used may have actually dampened this response, in favour of B cell proliferation.

Curiously, although there appeared to be a significantly greater percentage of IL-10$^+$ B cells within the EXPB10-media subset when compared to EXPB10 cells generated with IL-4 only or the combination IL-2, IL-4 and IL-10, there was no apparent difference in suppressive potency amongst these Breg populations (fig. 3.2.3a). It is important to note however, that the suppressive potency of EXPB10 cells did correlate with both time exposed to CD154 stimulation (fig. 3.2.2c) and ratios of CD154$^+$CHO to CD19$^+$ B cells (fig. 3.2.2b), which in turn both correlated with IL-10 production (3.1.1f & 4.1.3a). This may indicate that IL-10 was not the main driver by which EXPB10 cells regulated an immune response and indeed may be even be irrelevant to Breg function in this system; it may also be a result of IL-10 saturation in the suppression assays, such that EXPB10 cells required only low levels of IL-10 in order to mediate suppression. Alternatively, it is possible that a technical error affected the ELISA and flow cytometry results documenting IL-10 levels and percentage frequencies associated with EXPB10 cells. In recent years, other cytokines and growth factors have been found to promote mouse Breg development, such as B cell survival factor BAFF, pro-inflammatory cytokine IL-21 and anti-inflammatory cytokine IL-35 [98]. It will be valuable to establish in future experiments whether the addition of all or some of these factors could improve either expansion rate or suppressive function of EXPB10 cells in this *in vitro* system.

Human CD19$^+$ B cells were initially isolated through negative selection to reduce any possibility of activation through the isolation process prior to co-culture with CHO cells. Irradiated CD154$^+$CHO cells were first co-cultured with CD19$^+$ B cells at different ratios. In depth work on memory B cells by Neron and colleagues has demonstrated that by varying the density or levels of CD154-mediated stimulation, it is possible to direct differentiation of human B cell fate [127, 135]. The authors found that low levels of CD40 stimulation maintained a relatively high proportion of memory CD27$^+$ B cells. However, increasing CD154 binding altered this dynamic and the proportion of CD27$^+$ B cells decreased, while the proportion of naïve CD27$^-$ B cells increased. It was also observed that that low CD40 stimulation increased IgG secretion by up to 5-fold, and led to a reversal of the IgG/IgM secretion ratio. These findings suggest that fine-tuning of the CD40–CD154 interaction could be a significant parameter in the regulation of B cell physiology [135].

Since it is the transitional $CD24^{hi}CD38^{hi}$ subset of naïve human B cells which comprises the highest percentage of IL-10^{+} B cells [20] and CD40 stimulation is known to induce B cells to express IL-10 [20, 24, 69, 156], we hypothesised that the higher the ratio of $CD154^{+}$CHO to $CD19^{+}$ B cells, the greater the proportion of naïve B cells generated and thus the greater likelihood of inducing human IL-10^{+} Breg. Indeed, higher ratios of $CD154^{+}$ CHO cells to $CD19^{+}$ B cells and higher levels of CD154 expression on CHO cells resulted in increased rates of B cell expansion as well as increased suppressive potency (fig. 3.1.1d, 3.2.2b, 3.2.3c).

EXPB10 cells appeared to gain suppressive potency with time exposed to CD154 stimulation i.e. from 3-day co-culture to 7-day co-culture with $CD154^{+}$ CHO cells (fig. 3.2.2c), and this correlated with IL-10 expression (3.1.1f). Suppressive potency did fall if EXPB10 cells were co-cultured with $CD154^{+}$ CHO cells for 14 days, but this may have been due to inadequate number of $CD154^{+}$ CHO cells available to rapidly proliferating EXPB10 cells. EXPB10 cells appeared to increase rapidly in number from day 9 of co-culture; however irradiated $CD154^{+}$ CHO cells were only added on days 3 and 7, and furthermore, only appeared to survive for 3-4 days following irradiation (fig. 3.2.2a). Given that we found that re-plating with additional $CD154^{+}$ CHO cells on day 7 made a significant difference to rate of expansion and IL-10 expression (fig. 3.2.1b-d) of EXPB10-14 cells, it is possible that a further re-stimulation on day 9 or 10 would have better maintained $CD154^{+}$ CHO to $CD19^{+}$ B cell ratios, thus providing adequate stimulation for the expanding EXPB10 cell population. Alternatively, EXPB10 cells may have been of a transient nature, and with prolonged stimulation this population may have lost suppressive function to either reach senescence or to develop effector function. However the observation that EXPB10-d14 cells expressed similar percentage frequencies of IL-10^{+} B cells to that of EXPB10-d7 cells, suggests that this is not the case (fig. 3.1.1f and 3.1.2b). These findings indicate that continuous stimulation by membrane-bound CD154 was required to maintain optimal EXPB10 cell suppressive function as well as proliferation. In addition, the intensity or degree of CD154-mediated stimulation also appeared to dictate expansion rates and suppressive potency.

Following co-culture in the expansion protocol, it is not yet clear whether the resulting B cell populations were a homogenous, regulatory population of which only

a proportion expressed IL-10, or whether the expanded B cells were in fact heterogeneous and consisted of both Breg and B effectors. To try and elucidate how $CD19^+$ B cells with regulatory function were being induced or expanded in this system, several parameters of the CD154-CD40 interaction should be considered.

1. CD154: a multitude of studies has demonstrated that different membrane-bound forms of CD40 ligands (CD40L) have different specificities, affinities and the capacity for oligomerisation whilst binding to CD40 epitopes. Stimulation by soluble $CD154^+$ membranes [157] or CD154-transfected cells [135] induces dose-dependent, differential physiological responses, including modulation of proliferation and differentiation; by doing so, this can influence the balance between naïve and memory cells [115]. Binding strength and occupancy of CD40 receptors could thus be related to the concentration and potency of ligands used, which could in turn differentially modulate B cells. It may be that this particular form of CD40L which has been transfected to be expressed on this CHO cell line, targets a specific CD40 epitope and engenders optimal oligomerisation; when the CD40 epitope is thus activated, this triggers Breg development and proliferation. The CHO cell itself may also have non-specific effects by virtue of cell contact with B cells; however this is less likely given the poor expansion and IL-10 production seen with $CD154^-$ CHO cells (fig. 3.1.1d & 3.1.1f).

2. The B cell: it is not clear whether Breg are a developmentally defined lineage of B cells, or whether all B cells when stimulated in the appropriate conditions, have the capacity to develop Breg function. If the latter, we can assume that all B cell lineages present in *in vitro* cultures can respond to this particular CD154 stimulation in the same way. Initial cell death (fig. 3.1.1.e) is arbitrary and simply the artefact of *in vitro* culture; such that these many different cell types present in peripheral blood are all strongly activated by CD154 and having undergone specific downstream signaling triggered by these conditions, develop suppressive capacity across all B cell lineages. However it seems unlikely that such a response is the case physiologically; in severe inflammation, both B effector and Breg cells are present, suggesting

that different B cell subsets react different to the same stimuli to create a much more nuanced immune response [71, 74].

Alternatively, a developmentally distinct lineage of progenitor Breg (pro-Breg) and Breg cells may exist. Only this subset is able to survive such a strong activation signal from intense CD154 stimulation, and B effector cells instead undergo apoptosis through activation-induced cell death [158]. Reduced CD154 levels encourage B effectors to survive and proliferate, whilst Breg are less able to survive or develop with a lower CD154 signal; thus resulting in reduced total $CD19^+$ B cell numbers, reduced EXPB10 cell numbers and a perceived reduction in EXPB10 cell suppressive potency (fig. 3.1.1d & 3.2.2b).

3. CD40: the CD154-CD40 interaction may elicit an all or nothing reaction by the B cell i.e. those B cells which are able to respond to this form of CD154 under these in vitro conditions are activated and are then directed to proliferate and/or differentiate as Breg.

   Alternatively, the interaction of CD154 and CD40 could instead determine a much more graded response. CD40 density on normal human B lymphocytes can be substantially modulated following activation or differentiation [127]. CD40 is known to become up-regulated on polyclonally stimulated B cells and in response to IL-4 [159]. Pro-Breg/Breg may up-regulate CD40 expression upon CD40 ligation and/or in response to cytokines present in culture, thus acquiring further capacity for additional activation, unlike B effectors. CD40 expressed by pro-Breg/Breg may also have a greater affinity for CD154 than B effectors or have a greater density of distribution on the cell surface, and so out-compete B effector cells for what is effectively T cell help. Gordon and collaborators have questioned the importance of CD40 cross-linking as a determinant of signal strength delivered to resting or germinal centre human B cells, using combinations of anti-CD40 mAbs, soluble CD154 and $Fc\gamma RIIb^+$ adherent cells [126]. The authors demonstrated that regulation of homotypic adhesion, entry into the active cell cycle and rescue from apoptosis by CD40 require differential binding of different CD40 epitopes. The work of

Arpin et al. further demonstrated that naïve and memory B cell populations are not equal towards CD40 activation, and are influenced by the intensity of the CD40–CD154 interaction [160].

Following the interaction of B cells with T cells, the engagement of CD40 by CD154 induces CD40 clustering and enables the recruitment of several adaptor proteins. Notably, CD40 receptors on unstimulated B cells represent relatively weak binding sites, whereas multimerised forms show distinct affinities for adaptor proteins [161]. The cytoplasmic portion of CD40 can recruit TNFR-associated factor (TRAF) proteins and can also interact with Janus family kinase 3 (Jak3) [162]. It is the variations in recruitment of TRAF1, TRAF2, TRAF3, TRAF5 or TRAF6, alone or in combination, to the cytoplasmic domain of CD40, which subsequently regulates specific signalling pathways and cellular fates [163]. Diverse functional outcomes following CD40 binding on human $CD19^+$ B cells probably reflect differential interactions with intracellular signal transduction pathways.

Confluence of $CD154^+$ CHO cells during passage as well as duration of $CD154^+$ CHO culture per passage (independent of confluence), both appeared to subsequently affect the suppressive potency of resulting EXPB10 cells, despite maintaining high levels of CD154 expression (fig. 3.2.3d-e). Although CHO cells themselves are known to comprise a very stable cell line, earlier work with these cells did highlight that periods of passage longer than 3-4 days and over-confluent culture could adversely affect the cells themselves [164]. Whilst not necessarily resulting in cell death, these more hostile conditions can predispose CHO cells to changes in cellular metabolism resulting in extracellular alanine accumulation, and enhanced rates of glucose and lactate utilisation. Such a microenvironment is thought to be able to modify cell surface protein structures including that of CD154. Given that the CD154-CD40 interaction and resulting downstream effects are known to be easily affected by changes in the CD40 epitope targeted, it is likely that such a phenomenon has occurred here; thus highlighting both the importance of careful culture as well as the selectivity involved in EXPB10 cell generation.

It should also be noted that EXPB10 suppressive potency correlated with expansion rate (fig. 3.1.1d). It may be that human $CD19^+$ B cells need to undergo a certain number of divisions to develop into Breg. However, different human donors expanded at variable rates such that $CD19^+$ B cells could expand poorly in these co-cultures by as little as 1.5 fold; but EXPB10 1:1 cells still consistently expanded relatively better than EXPB10 1:5 and EXPB10 1:10 cells, and were still maximally suppressive (*data not shown)*. Similar findings have also been found with *in vitro* expansion protocols of human Treg (*K. Millward, personal communication*), demonstrating the inherent variability of human lymphocytes across different human subjects. To further establish whether the number of B cell divisions is related to development of a suppressive function, study of telomere length may be warranted. Telomeres have been well documented to undergo shortening with cell division both *in vitro* and *in vivo* [165-167], and it may be that telomere length correlates with suppressive function, indicating a role for cell division in Breg development. It is possible that telomere length remains unchanged or even lengthens during human $CD19^+$ B cell division, but this is usually associated with telomerase activity found in germinal centre and memory B cells [168]. Further phenotyping will confirm whether EXPB10 cells are naïve (see *Chapter 4.0*), and thus whether such telomere studies will be helpful.

Thus it is not clear from our data how EXPB10 cells were being induced or indeed generated. It is evident that the strength of the CD154-CD40 interaction and the duration of stimulation were both important. However whether this interaction was targeting specific pro-Breg and Breg, or whether all B cell lineages were able to respond to these conditions such that EXPB10 cells were generated, is not apparent. The sorting of different starting B cell populations before expansion i.e. naïve vs. memory B cells, may help to answer this question. Furthermore, the use of different CD40 ligands in expansion co-cultures, either membrane-bound or soluble, may help to determine whether it is this particular form of CD154 expressed on CHO cells which is targeting a specific CD40 epitope, that in turn triggers downstream signalling to direct suppressive function. Identification of the targeted CD40 epitope by X-ray crystallography may also help elucidate this interaction. More in depth work to understand the signalling pathways triggered by the interaction of $CD154^+$ CHO cells

with $CD19^+$ B cells is warranted to determine how the resulting Breg are generated and exert function. This is investigated in part, in *Chapter 4.0*.

The suppressive function of EXPB10 cells was initially tested by co-culturing the *ex vivo* generated Breg population with polyclonally stimulated, autologous $CD4^+$ T cells. Although only approximately 30% of EXPB10 cells actually expressed IL-10 (fig. 3.1.1d), sorting of viable IL-10^+ B cells is not an easy process (*Chapter 4.0* fig. 4.2.6e*)*; it is also not necessarily the case that only the IL-10^+ B cell population is regulatory, given the growing evidence for highly activated, IL-10-independent Breg [99, 100]. Therefore the entire $CD19^+$ EXPB10 cell population generated was used in these initial studies of suppressive function.

In keeping with other studies of human Breg [20, 47, 99, 100, 169], EXPB10 cells were able to mediate dose-dependent suppression of both $CD4^+$ T cell proliferation (fig. 3.2.1c) and pro-inflammatory cytokine production (fig. 3.2.1e & f). This demonstrates that EXPB10 cells could not only reduce proliferative responses but that these regulatory cells could also modulate the differentiation of $CD4^+$ T cells to Th1-type cells. A profound decrease in the number of $CD4^+$ T cells at day 5 of the suppression assay was also observed when co-cultured with EXPB10 cells, relative to stimulated $CD4^+$ T cells cultured with NON-EXPB10 cells or stimulated $CD4^+$ T cells alone (fig. 3.2.1d). This may be reflective of inhibited proliferation, or indeed may be indicative of cell death by apoptosis or cell killing. It is possible that EXPB10 cells were anergic as they were highly stimulated in the absence of antigen, much like $CD4^+$ T cells when stimulated in the absence of co-stimulation. However B cell anergy is usually associated with antigen stimulation in the absence of other signals such as CD154, rather than the reverse approach [170, 171]. Moreover, the finding that EXPB10 cells were able to slightly but significantly promote proliferation of $CD4^+$ T cells in the absence of anti-CD3/CD28 bead-driven stimulation (fig. 3.2.1g), and that multiple studies have demonstrated that activation via CD154-CD40 in the absence of antigen resulted in highly activated, functional B cells, would suggest otherwise [127]. These possibilities are explored further in *Chapter 4.0*.

With any *in vitro* system the potential for artefact is a real one, and so it is important to consider that possibility here. EXPB10 cells would have been, by nature of the expansion, highly activated, much more so than autologous $CD4^+$ T cells or NON-

EXPB10 cells on day 0 of the suppression assay. This was reflected in the different proliferation profiles of EXPB10 and NON-EXPB10 cells by day 5 of the assay (fig. 3.2.4a). EXPB10 cells continued to proliferate within the suppression assay illustrated by the substantial dilution of VPD, whereas NON-EXPB10 cells demonstrated very little proliferation. This phenomenon, whilst not unexpected, does raise the possibility that what appeared to be active suppression mediated by EXPB10 cells, was actually just the result of highly activated cells depleting media of essential nutrients, thus impeding the $CD4^+$ T cell response. Whilst $CD4^+$ T cells appeared to be suppressed, in a more complex, *in vivo* environment such a depletion would be unlikely to occur, and so the *in vitro* suppression recorded may not have been a true observation. It is difficult to set up appropriate controls in light of this, to exclude such a possibility.

Indeed, in support of this view, EXPB10 1:5 and 1:10 cells, as well as EXPB10-d3 cells, which one can assume were less activated than EXPB10 cells generated at a 1:1 ratio with $CD154^+$ CHO cells for 7 days, were significantly less suppressive (fig. 3.2.2b & 3.2.2c). However, when the proliferation profiles were examined of these different Breg populations, it was evident that there was no significant difference in proliferation rates of these EXPB10 cell subsets (fig. 3.2.4b & 3.2.4c). Furthermore, there appeared to be no difference in EXPB10 cell survival capacity to account for this discrepancy in suppressive potency, as at day 5 of the suppression assay there was no difference in the total number of $CD19^+$ B cells remaining across any group. EXPB10 cells may have indeed depleted $CD4^+$ T cells of essential growth factors such as arginine as a legitimate mechanism of suppression [172], and this is explored to greater depth in *Chapter 4.0*; but given that EXPB10 cells of different suppressive potencies were able to proliferate and survive at the same rates, it is less likely that incidental suppression through depletion of an essential nutrient such as glucose (which would not be the case *in vivo*), was the principle means by which $CD4^+$ T cell responses were suppressed in this system. This is of course not conclusive; establishing the mechanism of suppression, which is explored in *Chapter 4*.0, and the interrogation of EXPB10 cell function *in vivo,* described in *Chapter 5.0,* will help to ratify these assumptions.

It was observed that in the absence of $CD4^+$ T cell stimulation, EXPB10 cells appeared in fact to promote $CD4^+$ T cell proliferation (fig. 3.2.1g). This may reflect the highly activated state of the EXPB10 cell compared to NON-EXPB10 cells. This activating potential of EXPB10 cells appeared to be overridden when $CD4^+$ T cells received polyclonal stimulation in the form of anti-CD3/CD28 beads. This suggests that this stimulation must have instigated a signal from the activated $CD4^+$ T cell, which then enabled the EXPB10 cell to suppress the subsequent T cell response.

The finding that EXPB10 cells were also able to suppress $CD8^+$ T cell responses is valuable in that it indicates that EXPB10 cells may be able to control multiple arms of the adaptive immune response (fig. 3.2.2i); an important consideration particularly in the field of transplantation, where $CD8^+$ T cell responses to allograft play a major role in acute rejection [5]. Mouse models of allogeneic bone marrow transplantation have demonstrated the need for both recipient B cells and dendritic cells (DCs) in order to maintain peripheral $CD8^+$ T cell tolerance [173, 174]. Although dendritic cells appeared to mediate this tolerance through MHC II and antigen presentation, the mechanism by which B cells achieved tolerance could not be elucidated, although both MHC classes, IL-10, CD80 and CD86 were all excluded as possible candidates. Both mouse and human Breg have also been reported to directly suppress DC responses [97, 175, 176]. Morva et al. demonstrated that, on activation by CD40L and CpG-oligodeoxynucleotides (ODN), human B cells inhibited the maturation and function of DCs [97]. In addition, this activated B cell population decreased density of HLA-DR on mature DCs, expression of CD80 and CD86, production of IL-12p70 required for antigen presentation and Th1 differentiation. Ultimately these effects resulted in inhibited dendritic cell-induced T-cell proliferation.

Given that EXPB10 cells may receive a signal from activated $CD4^+$ T cells in order to exert suppressive function, it may be worth examining EXPB10 cell-mediated suppression of other immune cell populations such as DCs and $CD8^+$ T cells in both the absence and presence of such $CD4^+$ T cells, to identify if this is indeed the case. It will be important to establish if and how EXPB10 cells exert suppression of these different lymphocyte populations, in order to have a greater understanding of human Breg in the context of complex disease systems.

The co-culture of EXPB10 cells with either autologous or allogeneic $CD4^+$ T cells resulted in equally potent suppression of T cell proliferation (fig. 3.2.2h). Since polyclonal stimulation was used in these suppression assays rather than antigen, these observations by no means preclude the possibility of EXPB10-mediated antigen-specific responses. Antigen specificity of mouse Breg has been recently documented in the literature. Lombardi's group have demonstrated that transfer of B cells from mice rendered tolerant to MHC class I mismatched skin grafts could prolong graft survival in a dose-dependent and antigen-specific manner; only transitional-2 (T2) B cells from tolerised mice, not naïve T2 nor alloantigen experienced T2 B cells, were able to prolong skin allograft survival and suppress T-cell activation [177]. IgV_H transcriptome analysis by Kleffel et al. revealed that islet-infiltrating, IL-10-enriched B cells in long-term normoglycaemic (Lnglc) NOD mice were more antigen-experienced and possessed more diverse B-cell receptor repertoires compared to those of hyperglycaemic (Hglc) mice [67]. Rituximab-mediated B-cell depletion followed by adoptive transfer of B cells from Hglc mice induced hyperglycaemia in Lnglc human CD20 transgenic NOD mouse models. Importantly, both murine and human IL-10^+ B cells significantly abrogated T-cell–mediated responses to self- or islet-specific peptides *ex vivo*, suggesting that antigen-matured Breg may maintain tolerance to islet autoantigens by selectively suppressing autoreactive T-cell responses. Work by our own group has demonstrated that not only were mouse IL-10^+ Breg antigen-specific when generated *in vivo*, but it was both the presence of tolerising conditions and antigenic stimulus, in this case heart allografts, which were responsible for this generation (*Stolp et al. manuscript under review*). It would be valuable to explore how human EXPB10 cells behave in the presence of antigen during the expansion protocol; in particular whether the BCR repertoire would narrow, and whether EXPB10 cells would have become polarised to gain antigen specificity, and importantly whether they retain regulatory function. A mixed leucocyte reaction (MLR) would also help clarify how EXPB10 cells behave *in vitro* in the presence of antigen.

SUMMARY

Co-culture of human $CD19^+$ B cells with $CD154^+$ CHO cells induced IL-10 expression by $CD19^+$ B cells and also promoted $CD19^+$ B cell proliferation. Suppressive potency of EXPB10 cells increased with increased ratios of $CD154^+$ CHO cells to human $CD19^+$ B cells as well as with time exposed to CD154 stimulation. The combination of exogenous cytokines IL-2, IL-4 and IL-10 promoted proliferation of this Breg population, possibly at the expense of IL-10 induction. It is not clear whether all $CD19^+$ B cells were able to develop suppressive function, or whether this *in vitro* system promoted the survival and induction of a particular Breg subset. The resulting EXPB10 cells were able to suppress both autologous and allogeneic $CD4^+$ T cell responses, specifically by inhibiting proliferation as well as Th1 differentiation. It may be that induction of cell death also had a role. EXPB10 cells were also able to suppress $CD8^+$ T cell responses, although whether this was possible in the absence of $CD4^+$ T cells was not established.

To establish the identity of EXPB10 cells and the means by which they are generated and function, extensive phenotypic analysis and in depth mechanistic studies are required. This process is started in *Chapter 4.0*, where the phenotype of EXPB10 cells is compared to that of known Breg subsets and associative links to suppressive function are investigated. EXPB10 suppressive function is interrogated to reveal, in part, aspects of how a human $CD19^+$ B cell may suppress a $CD4^+$ T cell response. Our understanding of how this regulatory cell population functions is fundamental to their future application, either as a cellular therapy or as a target for pharmaceutical manipulation *in vivo*.

Fig. 3.1.1

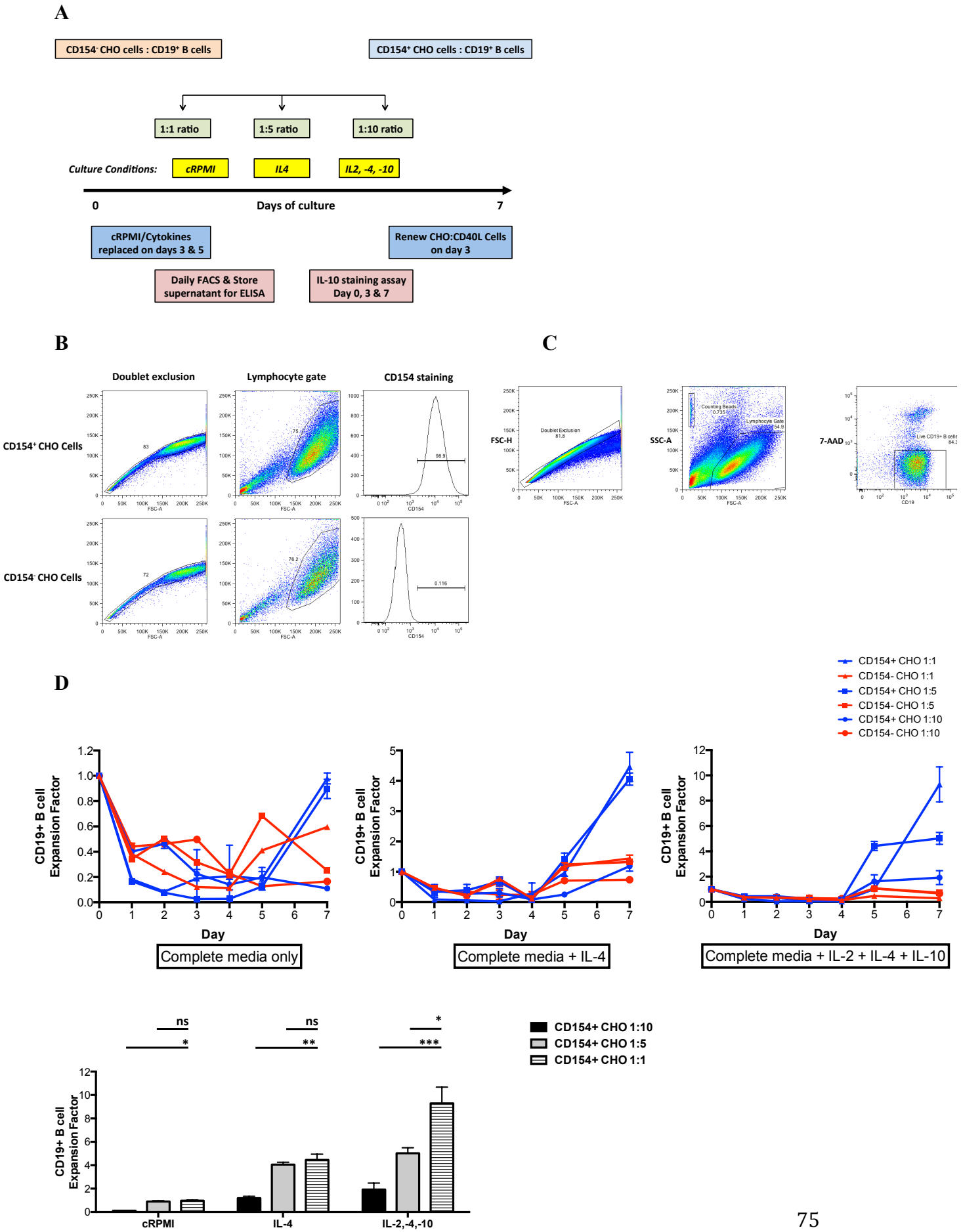
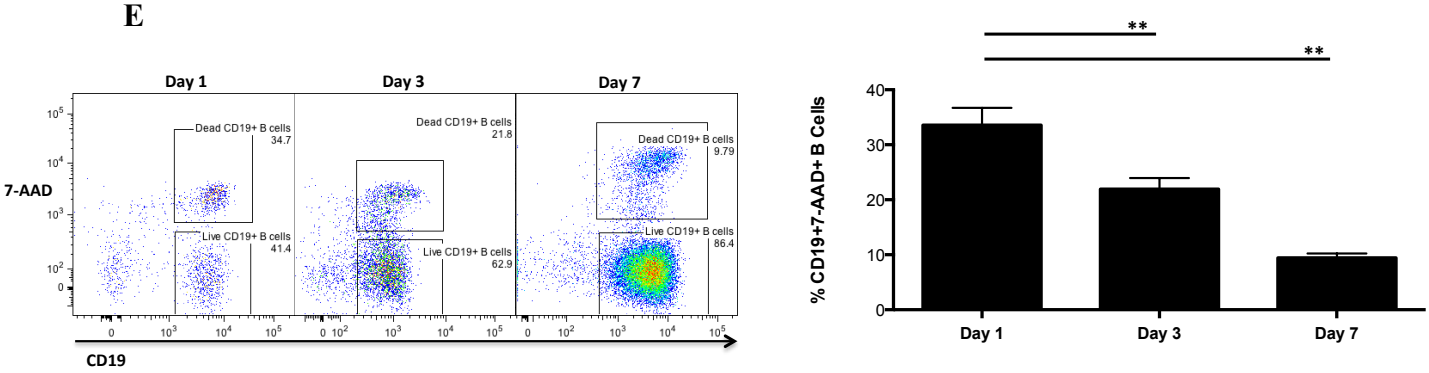
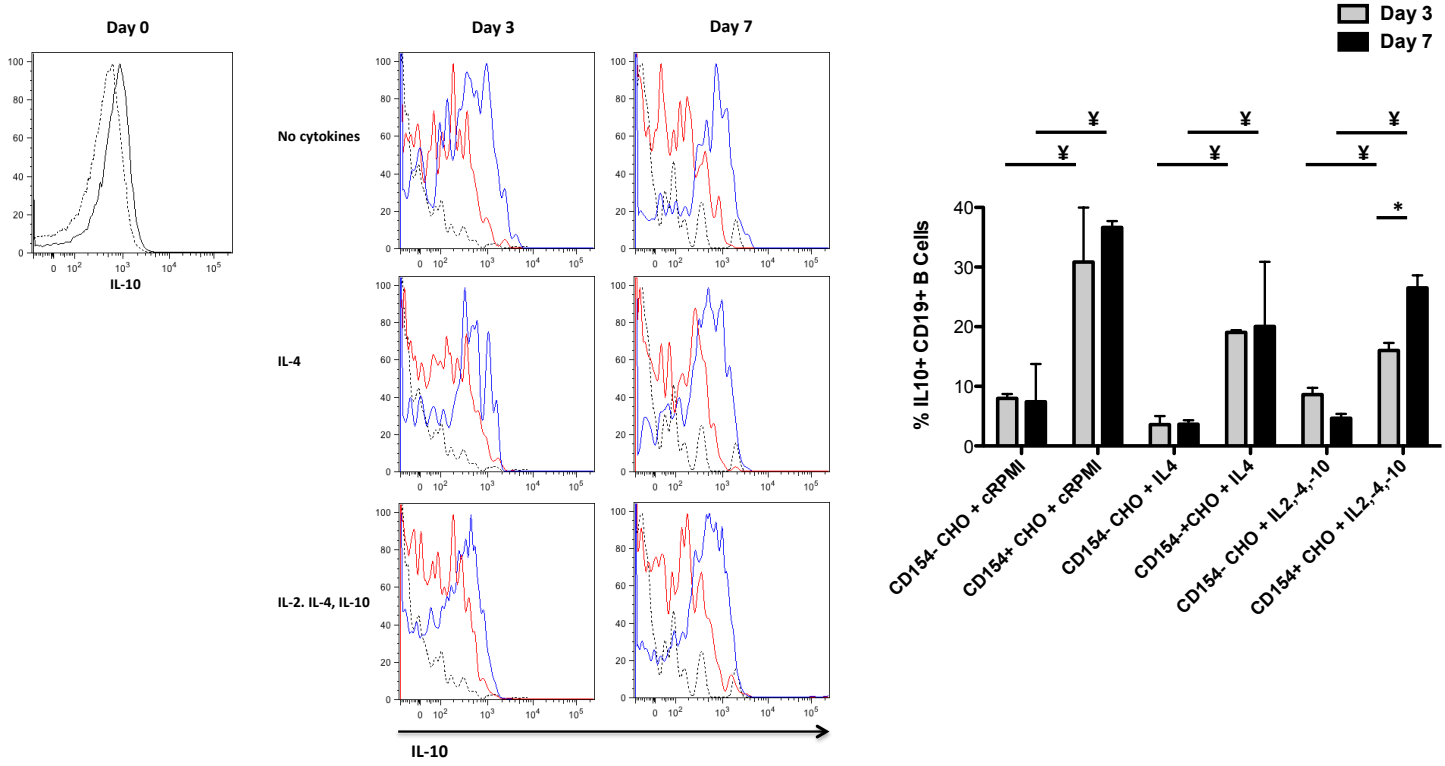

A
CD154- CHO cells : CD19+ B cells
CD154+ CHO cells : CD19+ B cells
1:1 ratio
1:5 ratio
1:10 ratio
Culture Conditions:
cRPMI
IL4
IL2, -4, -10
0
Days of culture
7
cRPMI/Cytokines replaced on days 3 & 5
Renew CHO:CD40L Cells on day 3
Daily FACS & Store supernatant for ELISA
IL-10 staining assay Day 0, 3 & 7
B
Doublet exclusion
Lymphocyte gate
CD154 staining
CD154+ CHO Cells
CD154- CHO Cells
C
FSC-H
SSC-A
7-AAD
D
CD154+ CHO 1:1
CD154- CHO 1:1
CD154+ CHO 1:5
CD154- CHO 1:5
CD154+ CHO 1:10
CD154- CHO 1:10
CD19+ B cell Expansion Factor
Day
Complete media only
Complete media + IL-4
Complete media + IL-2 + IL-4 + IL-10
ns
*
ns
**
*
***
CD154+ CHO 1:10
CD154+ CHO 1:5
CD154+ CHO 1:1
cRPMI
IL-4
IL-2,-4,-10

Fig. 3.1.1 (cont.)

E



F



G

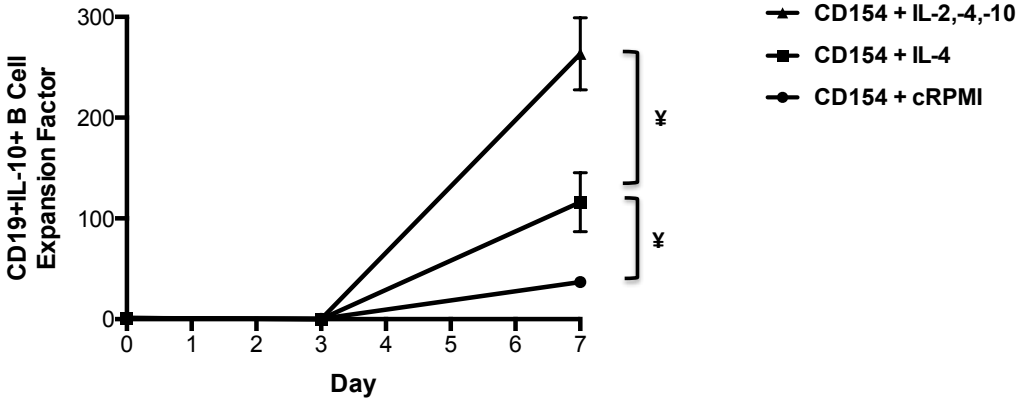


Fig. 3.1.1 Human $CD19^+$ B cells can be expanded by co-culture with $CD154^+$ CHO cells

(a) Schematic representation of Breg expansion protocol optimisation. $CD154^+$ CHO cells were irradiated and co-cultured with human $CD19^+$ B cells isolated from PBMC. Co-cultures were set up at with three different ratios of CHO cells to $CD19^+$ B cell: 1:1, 1:5 and 1:10. In each co-culture condition, three different media conditions were set up: with complete media alone (cRPMI), with cytokine IL-4 (100U/ml), or with cytokines IL-2 (50U/ml), IL-4 (100U/ml) and IL-10 (25U/ml). $CD154^-$ CHO cells co-cultured with human $CD19^+$ B cells served as controls. Co-cultures were incubated for up to 7 days. CHO cells were replenished on day 3. Media +/- cytokines were replaced on days 3 and 5 of co-culture. Cell cultures were harvested and analysed by FACS daily for cell counts, viability and phenotype. Supernatants were harvested for ELISA to detect human immunoglobulin. Intracellular IL-10 staining was performed on day 0, 3 & 7. **(b)** Representative FACS plots of $CD154^+$ and $CD154^-$ CHO cells demonstrating cell size and CD154 expression. CD154 staining was gated on a mean fluorescence-1 (mf-1) control. **(c)** Gating strategy for IL-10 staining of $CD19^+$ B cells after 7 days of expansion co-culture. After doublets exclusion, dead cells were excluded by 7-AAD staining and CD19 staining was gated on an mf-1 control. **(d)** Expansion factor of $CD19^+$ B cells when co-cultured with varying ratios of $CD154^+$ or $CD154^-$ CHO cells, in the presence of no cytokines, IL-4, or a combination of IL-2, IL-4 and IL-10. Expansion factor is the expression of number of live $CD19^+$ B cells at any given time-point relative to the original number of live $CD19^+$ B cells on day 0, where expansion factor at day 0 = 1. Starting purities of human $CD19^+$ B cells >96% in all experiments. Expansion factor is displayed for each media condition when $CD154^+$ CHO cells were co-cultured with human $CD19^+$ B cells at different ratios for 7 days. *$p<0.05$, **$p<0.007$, ***$p<0.003$, paired t-test. **(e)** Representative FACS plots and graph demonstrating increased % $CD19^+$ B cell death based on 7-AAD staining when CD19+ B cells were co-cultured with $CD154^+$ CHO cells at 1:1 ratio with cytokines IL-2, IL-4 and IL-10. All gating performed on mean fluorescence -1 (mf-1) controls. **$p<0.007$, paired t-test. **(f)** $CD19^+$ B cells were harvested on days 3 and 7 of expansion co-culture and stimulated with PIM for 6 hours, before undergoing intracellular staining for IL-10. Representative histograms of IL-10 expression of live $CD19^+$ B cells. Dashed line represents isotype controls; black line represents non-expanded $CD19^+$ B cells (NON-EXPB10) which express IL-10; red line represents CD19+IL-10+ B cells co-cultured with $CD154^-$ CHO cells; blue line represents CD19+IL-10+ B cells co-cultured with CD154+ CHO cells. % $CD19^+IL\text{-}10^+$ expression of B cells expanded with $CD154^+$ or $CD154^-$ CHO cells at a 1:1 ratio on days 3 and 7 is displayed graphically. *$p<0.05$, 2-way ANOVA. $^¥p<0.0001$, paired t-test. **(g)** Expansion factor of $CD19^+IL\text{-}10^+$ B cells when co-cultured with $CD154^+$ CHO cells at 1:1 ratio, +/- differing cytokine combinations. Expansion factor is the expression of number of live $CD19^+IL\text{-}10^+$ B cells at any given time-point relative to the original number of live $CD19^+IL\text{-}10^+$ B cells on day 0, where expansion factor at day 0 =1. $^¥p<0.0001$, paired t test. Error bars in each panel represent Mean +/- SD. Data are representative of 3 independent experiments using $CD19^+$ B cells from 3 different human donors.

Fig. 3.1.2

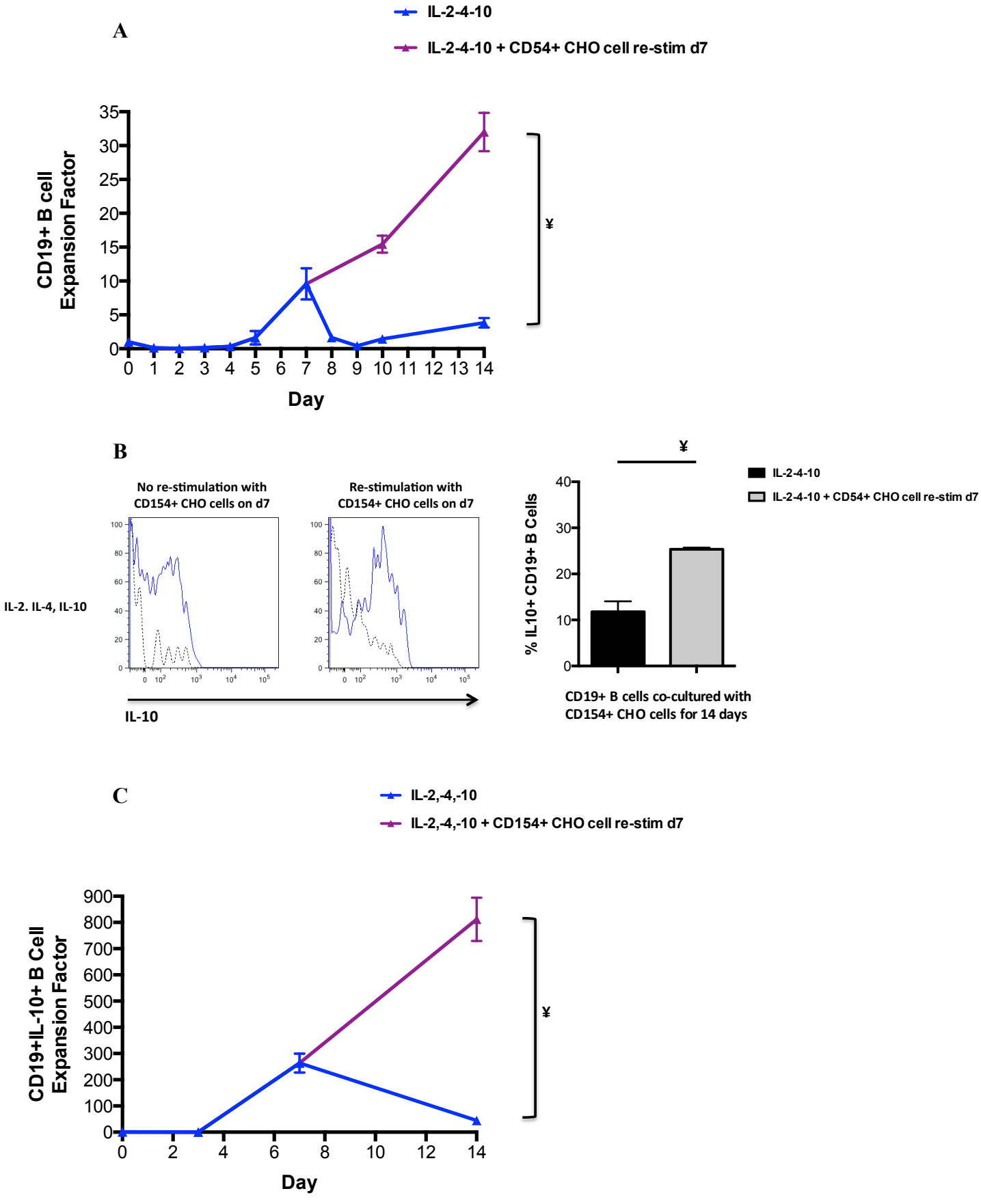

A
IL-2-4-10
IL-2-4-10 + CD54+ CHO cell re-stim d7
CD19+ B cell Expansion Factor
Day
¥
B
No re-stimulation with CD154+ CHO cells on d7
Re-stimulation with CD154+ CHO cells on d7
IL-2. IL-4, IL-10
IL-10
% IL10+ CD19+ B Cells
IL-2-4-10
IL-2-4-10 + CD54+ CHO cell re-stim d7
CD19+ B cells co-cultured with CD154+ CHO cells for 14 days
C
IL-2,-4,-10
IL-2,-4,-10 + CD154+ CHO cell re-stim d7
CD19+IL-10+ B Cell Expansion Factor
Day

Fig. 3.1.2 Sustained proliferation of EXPB10 cells requires repeated stimulation by CD154$^+$ CHO cells

(a) Human CD19$^+$ B cells were co-cultured with CD154$^+$ CHO cells at a 1:1 ratio with cytokines IL-2, IL4, IL-10, for 14 days. All co-cultures were re-stimulated with CD154$^+$ CHO cells on day 3, and then either incubated until day 14 (blue line) or were re-stimulated again on day 7 and incubated until day 14 (purple line). Expansion factor of human CD19$^+$ B cells was calculated at time-points during the two co-culture conditions. $^{¥}p<0.0001$, paired t-test. **(b)** Representative FACS plots of intracellular IL-10 expression by live CD19$^+$ B cells at day 14, comparing expression of IL-10 when CD19$^+$ B cells were either re-stimulated on not re-stimulated by CD154$^+$ CHO cells on day 7. Graph illustrates that re-stimulation with CD154$^+$ CHO cells resulted in increased IL-10 expression at d14. $^{¥}p<0.0001$, paired t-test. **(c)** Expansion factor of CD19$^+$IL-10$^+$ B cells to d14, when CD19$^+$ B cells were either re-stimulated or not re-stimulated at day 7 with CD154$^+$ CHO cells. $^{¥}p<0.0001$, paired t-test. Error bars in each panel represent Mean +/- SD. Data are representative of 3 independent experiments using CD19$^+$ B cells from 3 different human donors.

Fig. 3.2.1

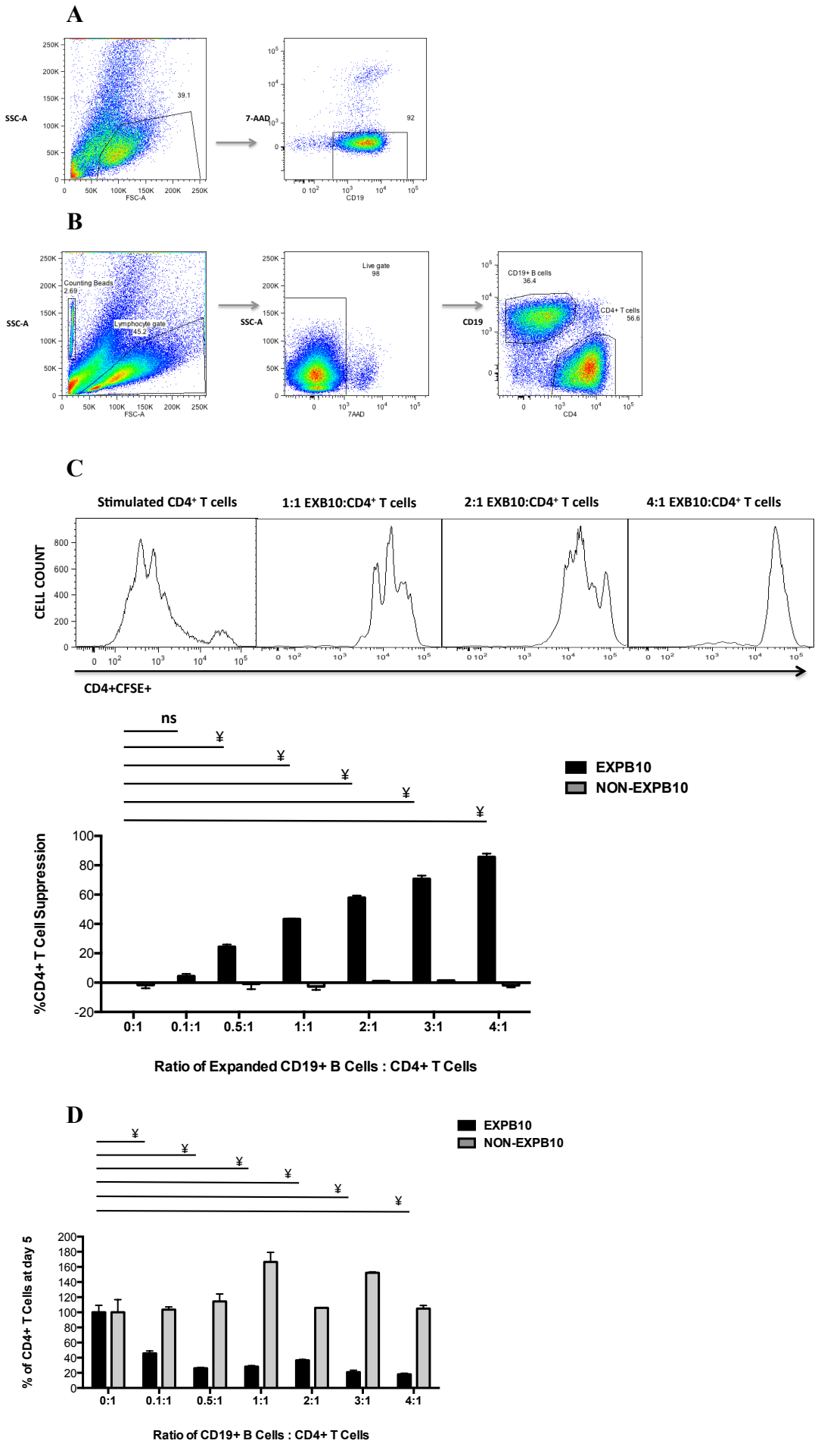
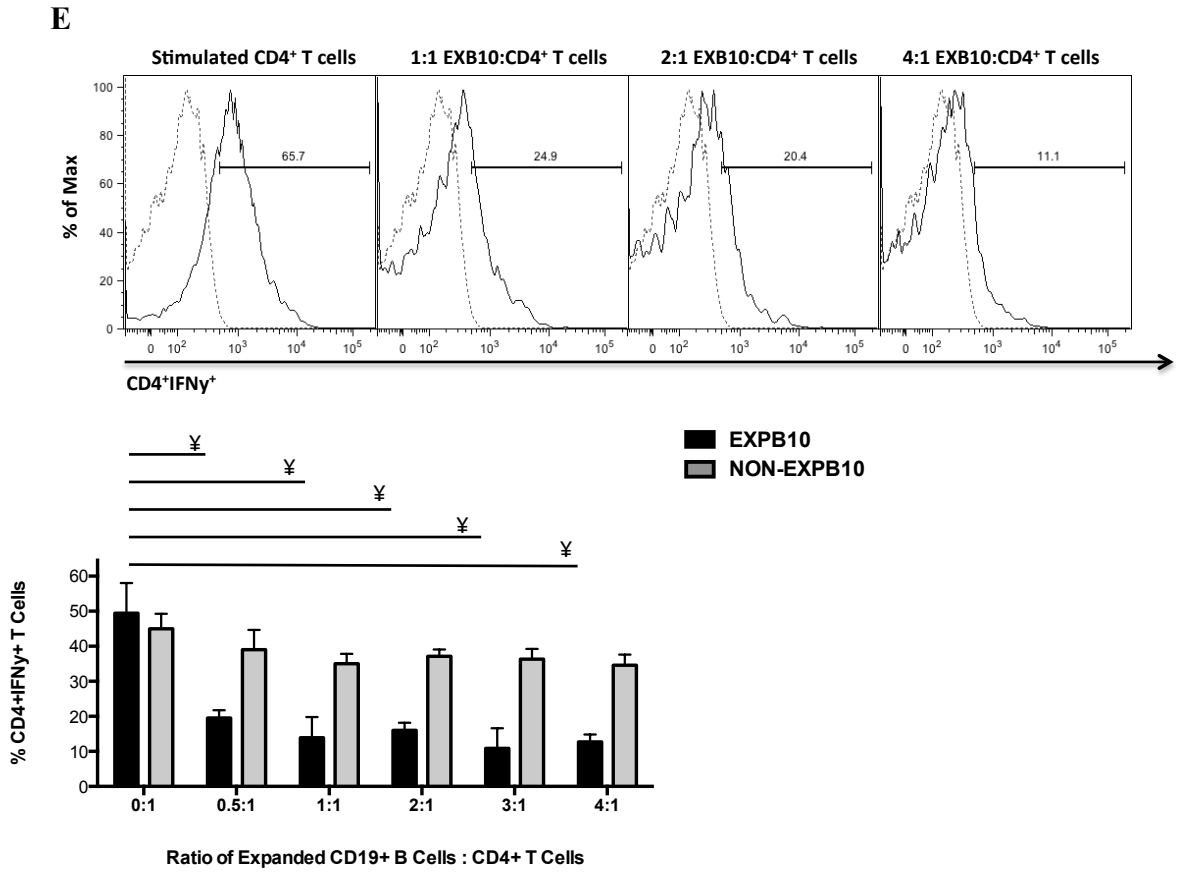

A
SSC-A
FSC-A
39.1
7-AAD
CD19
92
B
Counting Beads
2.69
Lymphocyte gate
45.2
Live gate
98
CD19+ B cells
36.4
CD4+ T cells
56.6
CD4
C
Stimulated CD4+ T cells
1:1 EXB10:CD4+ T cells
2:1 EXB10:CD4+ T cells
4:1 EXB10:CD4+ T cells
CELL COUNT
CD4+CFSE+
ns
EXPB10
NON-EXPB10
%CD4+ T Cell Suppression
0:1
0.1:1
0.5:1
1:1
2:1
3:1
4:1
Ratio of Expanded CD19+ B Cells : CD4+ T Cells
D
EXPB10
NON-EXPB10
% of CD4+ T Cells at day 5
Ratio of CD19+ B Cells : CD4+ T Cells

Fig. 3.2.1 (cont.)


E
Stimulated CD4+ T cells
1:1 EXB10:CD4+ T cells
2:1 EXB10:CD4+ T cells
4:1 EXB10:CD4+ T cells
% of Max
65.7
24.9
20.4
11.1
CD4+IFNγ+
EXPB10
NON-EXPB10
% CD4+IFNγ+ T Cells
0:1
0.5:1
1:1
2:1
3:1
4:1
Ratio of Expanded CD19+ B Cells : CD4+ T Cells

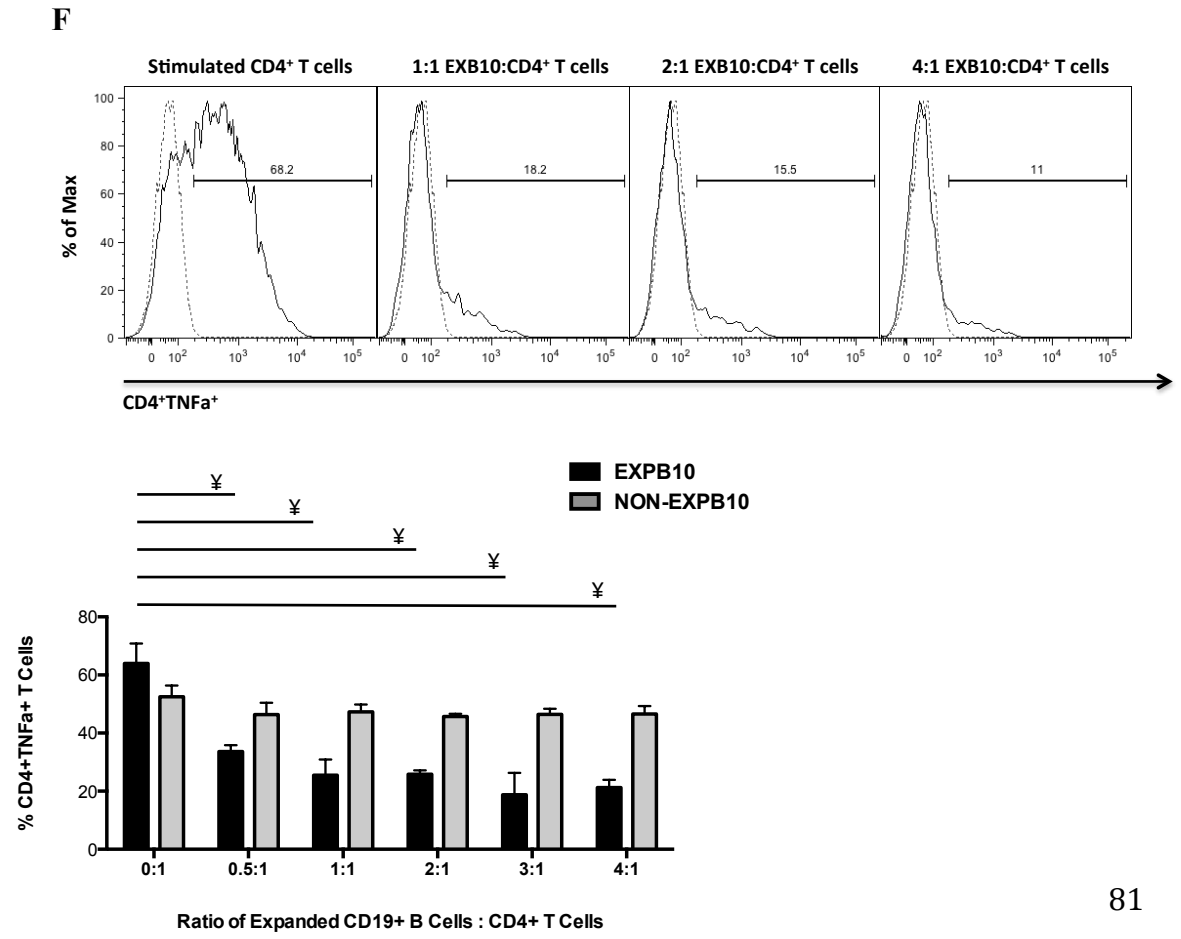
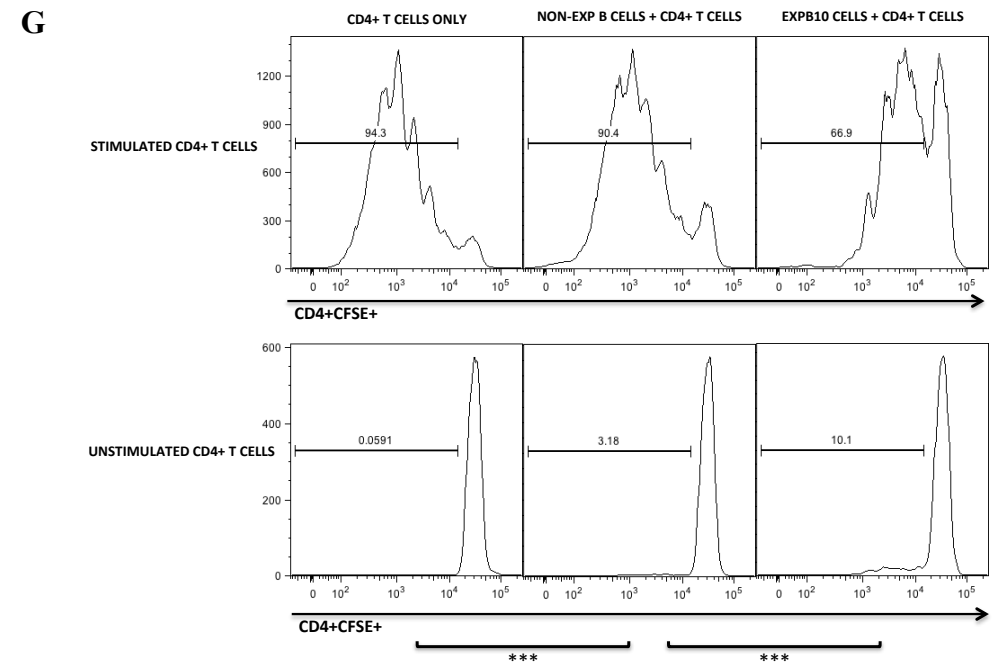
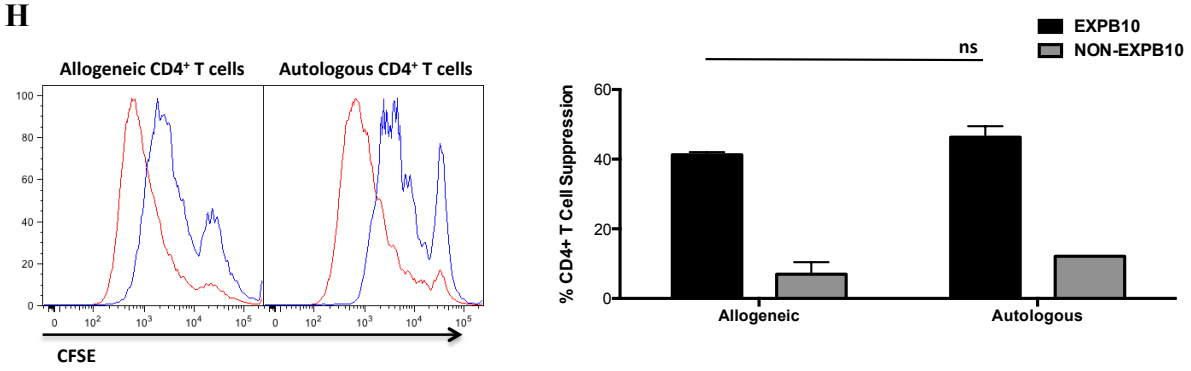
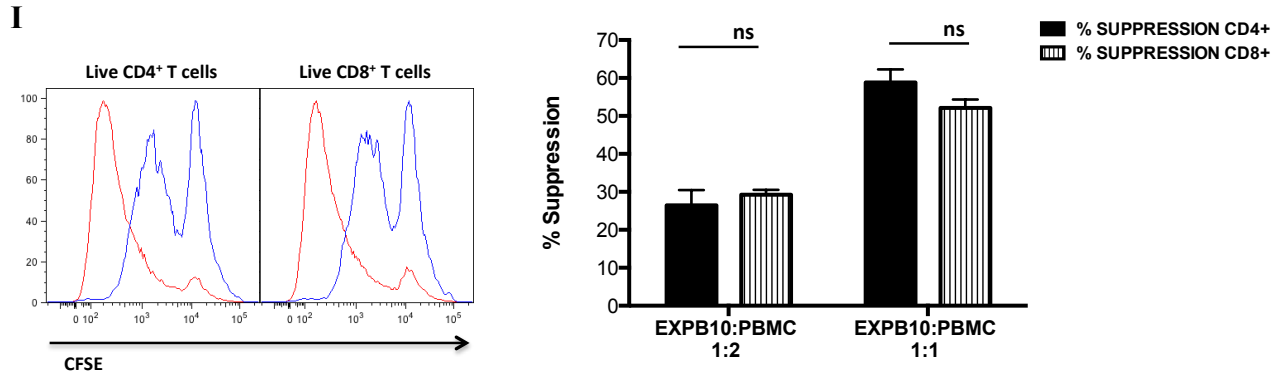

F
Stimulated CD4+ T cells
1:1 EXB10:CD4+ T cells
2:1 EXB10:CD4+ T cells
4:1 EXB10:CD4+ T cells
% of Max
68.2
18.2
15.5
11
CD4+TNFa+
EXPB10
NON-EXPB10
% CD4+TNFa+ T Cells
0:1
0.5:1
1:1
2:1
3:1
4:1
Ratio of Expanded CD19+ B Cells : CD4+ T Cells

Fig. 3.2.1 (cont.)


G
CD4+ T CELLS ONLY
NON-EXP B CELLS + CD4+ T CELLS
EXPB10 CELLS + CD4+ T CELLS
STIMULATED CD4+ T CELLS
94.3
90.4
66.9
CD4+CFSE+
UNSTIMULATED CD4+ T CELLS
0.0591
3.18
10.1
CD4+CFSE+
***
***


H
Allogeneic CD4+ T cells
Autologous CD4+ T cells
CFSE
EXPB10
NON-EXPB10
ns
% CD4+ T Cell Suppression
Allogeneic
Autologous


I
Live CD4+ T cells
Live CD8+ T cells
CFSE
% SUPPRESSION CD4+
% SUPPRESSION CD8+
ns
ns
% Suppression
EXPB10:PBMC 1:2
EXPB10:PBMC 1:1

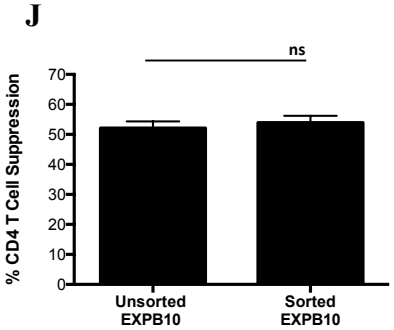

J
ns
% CD4 T Cell Suppression
Unsorted EXPB10
Sorted EXPB10

Fig. 3.2.1 EXPB10 cells significantly suppress proliferation of polyclonally stimulated, autologous $CD4^+$ T cells in a dose-dependent manner

a) Sort strategy to isolate EXPB10 cells from remaining $CD154^+$ CHO cells prior to suppression assay on day 7 of expansion co-culture. **(b)** Gating strategy for suppression assay analysis. Doublets and dead cells were excluded by gating and 7-AAD staining. T and B cells were then identified by CD4 and CD19 antibody staining. **(c)** EXPB10 cells suppress proliferation of autologous $CD4^+$ T cells in a dose-dependent manner *in vitro.* Autologous $CD4^+$ T cells were cultured in the presence of anti-CD3/CD28 stimulatory beads for 5 days +/- EXPB10 cells or NON-EXPB10 cells in increasing ratios. 0:1 represents stimulated $CD4^+$ T cell controls in absence of B cells. Representative histograms of live, $CD4^+CFSE^+$ T cells at day 5 of culture. Graph to illustrate % suppression of $CD4^+$ T cells compared to the stimulated $CD4^+$ T cell control 0:1. Non-expanded B cells in co-culture with stimulated $CD4^+$ T cells served as additional controls. % Suppression is an expression of Division Index of live, $CD4^+CFSE^+$ T cells at day 5 relative to that of the stimulated $CD4^+$ T cell control 0:1. $^¥p<0.0001$, paired t-test. **(d)** % $CD4^+$ T cells at day 5 is the measure of % $CD4^+$ T cells relative to that of the stimulated $CD4^+$ T cell control 0:1 at day 5. $^¥p<0.0001$, paired t-test. Data are representative of 5 independent experiments. **(e) + (f)** $CD4^+$ T cells were cultured in the presence of anti-CD3/CD28 stimulatory beads for 3 days, +/- EXPB10 cells in increasing ratios. PMA, ionomycin and brefeldin (PIB) were added to cultures for the last 6 hours of culture; cells were then harvested and stained intracellularly for Th1 cytokines IFNγ and TNFα. Representative histograms of live $CD4^+IFNy^+$ and live $CD4^+TNFa^+$ cells. Dashed line represents isotype controls; solid lines represent cytokine staining. $^¥p<0.0001$, paired t-test. **(g)** Representative histograms of live, $CD4^+CFSE^+$ T cells at day 5 of suppression assay when $CD4^+$ T cells were cultured alone, with NON-EXPB10 or EXPB10 cells, in the absence or presence of anti-CD3/CD28 bead stimulation. ***$p<0.003$, paired t-test. **(h)** Comparison of % suppression of autologous and allogeneic $CD4^+$ T cells by EXPB10 cells. Representative histograms of live $CD4^+$ T cells. red line represents the stimulated $CD4^+$ T cell control; blue line represents $CD4^+$ T cells when co-cultured with EXPB10 cells. **(i)** Representative histograms of live $CFSE^+CD4^+$ T cells and live $CFSE^+CD8^+$ T cells when PBMC were co-cultured with EXPB10 cells at a 1:1 ratio and with anti CD3/CD28 beads. Red line represents the stimulated PBMC control; blue line represents PBMC when co-cultured with EXPB10 cells. Each FACS plot represents $CD4^+$ or $CD8^+$ T cells as indicated. % Suppression of $CD4^+$ and $CD8^+$ T cells are represented on graph at both 1:1 and 1:2 ratios of EXPB10:PBMC. **(j)** % $CD4^+$ T cell suppression by EXPB10 FACS-sorted on CD19 expression from $CD154^+$ CHO cells vs. unsorted EXPB10 cells. Sort purity > 99%. Error bars in each panel represent Mean +/- SD. Data are representative of 5 independent experiments.

Fig. 3.2.2

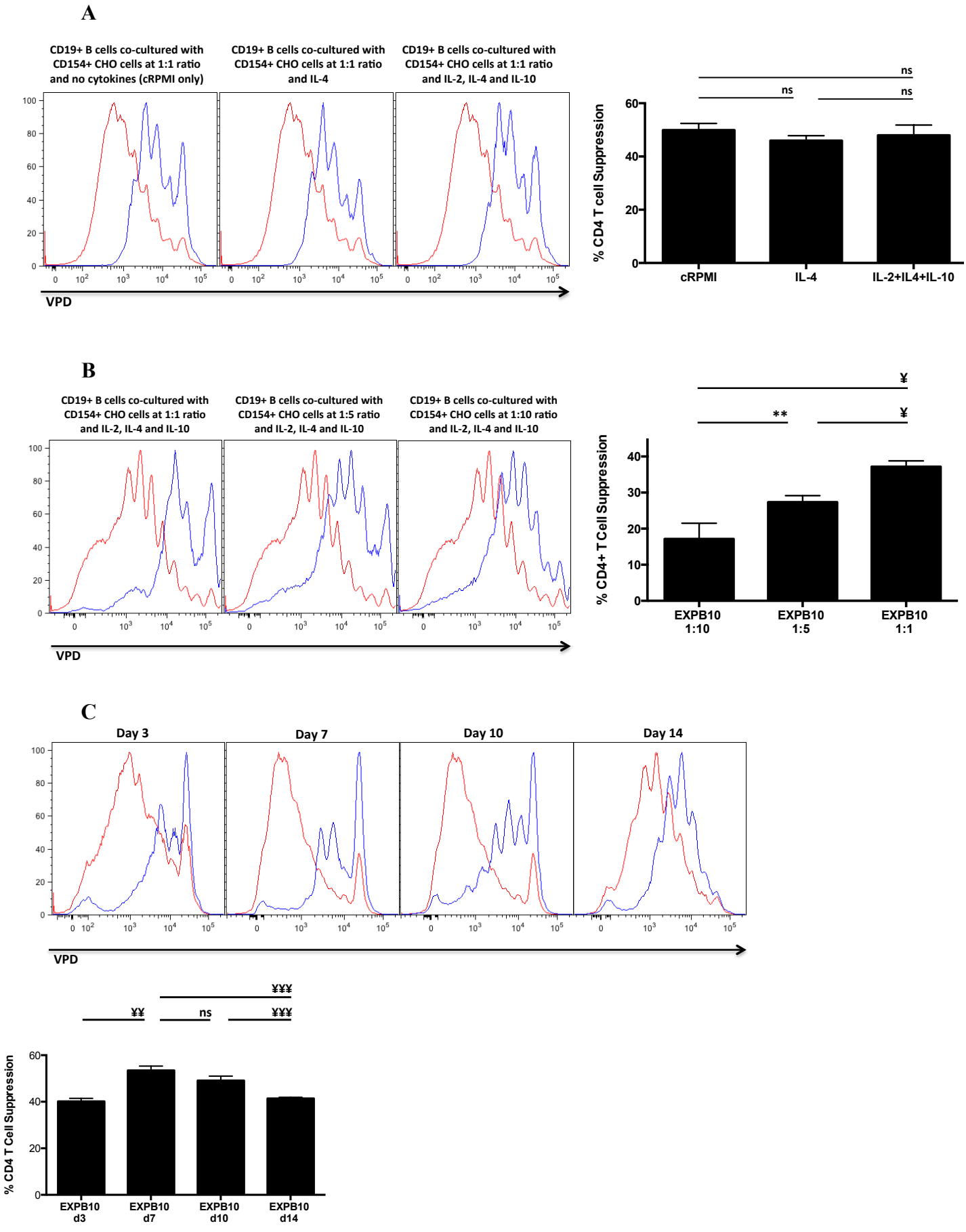

A
CD19+ B cells co-cultured with CD154+ CHO cells at 1:1 ratio and no cytokines (cRPMI only)
CD19+ B cells co-cultured with CD154+ CHO cells at 1:1 ratio and IL-4
CD19+ B cells co-cultured with CD154+ CHO cells at 1:1 ratio and IL-2, IL-4 and IL-10
VPD
% CD4 T cell Suppression
ns
ns
ns
cRPMI
IL-4
IL-2+IL4+IL-10
B
CD19+ B cells co-cultured with CD154+ CHO cells at 1:1 ratio and IL-2, IL-4 and IL-10
CD19+ B cells co-cultured with CD154+ CHO cells at 1:5 ratio and IL-2, IL-4 and IL-10
CD19+ B cells co-cultured with CD154+ CHO cells at 1:10 ratio and IL-2, IL-4 and IL-10
VPD
% CD4+ T Cell Suppression
¥
**
¥
EXPB10 1:10
EXPB10 1:5
EXPB10 1:1
C
Day 3
Day 7
Day 10
Day 14
VPD
¥¥¥
¥¥
ns
¥¥¥
% CD4 T Cell Suppression
EXPB10 d3
EXPB10 d7
EXPB10 d10
EXPB10 d14

Fig. 3.2.2 Suppressive potency of *ex vivo* expanded $CD19^+$ B cells can vary depending on different parameters of expansion co-culture

(a) Representative histograms of live $CD4^+VPD^+$ T cells on day 5 of suppression assay when autologous $CD4^+$ T cells were co-cultured with EXPB10 cells that had been generated with no cytokines, with IL-4 or with IL-2, IL-4 & IL-10. Red line represents the stimulated $CD4^+$ T cell control; blue line represents $CD4^+$ T cells when co-cultured with EXPB10 cells. % Suppression of $CD4^+$ T cells is illustrated graphically to demonstrate no significant difference in suppressive potency across the EXPB10 cell tested. Paired t-test. **(b)** Representative histograms of live $CD4^+VPD^+$ T cells on day 5 of suppression assay when autologous $CD4^+$ T cells were co-cultured with EXPB10 cells that had been generated with $CD154^+$ CHO cells at 1:1, 1:5 & 1:10 ratios of $CD154^+$ CHO:$CD19^+$ B cells in presence of IL-2, IL-4 & IL-10. Red line represents the stimulated $CD4^+$ T cell control; blue line represents $CD4^+$ T cells when co-cultured with EXPB10 cells. % Suppression of $CD4^+$ T cells is illustrated graphically to demonstrate significant differences in suppressive potency between the EXPB10 cell populations tested. **$p<0.007$, $^{¥}p<0.0001$, paired t-test. **(c)** Representative histograms of live $CD4^+VPD^+$ T cells on day 5 of suppression assay when autologous $CD4^+$ T cells were co-cultured with EXPB10 cells that have been generated with $CD154^+$ CHO cells for 3, 7, 10 or 14 days in presence of IL-2, IL-4 & IL-10. Red line represents the stimulated $CD4^+$ T cell control; blue line represents $CD4^+$ T cells when co-cultured with EXPB10 cells. % Suppression of $CD4^+$ T cells is illustrated graphically to demonstrate significant differences in suppressive potency between the EXPB10 cell populations tested. $^{¥¥}p<0.01$, $^{¥¥¥}p<0.02$, 2-way ANOVA for repeated measurements. Error bars in each panel represent Mean +/- SD. Data are representative of 3 independent experiments.

Fig. 3.2.3

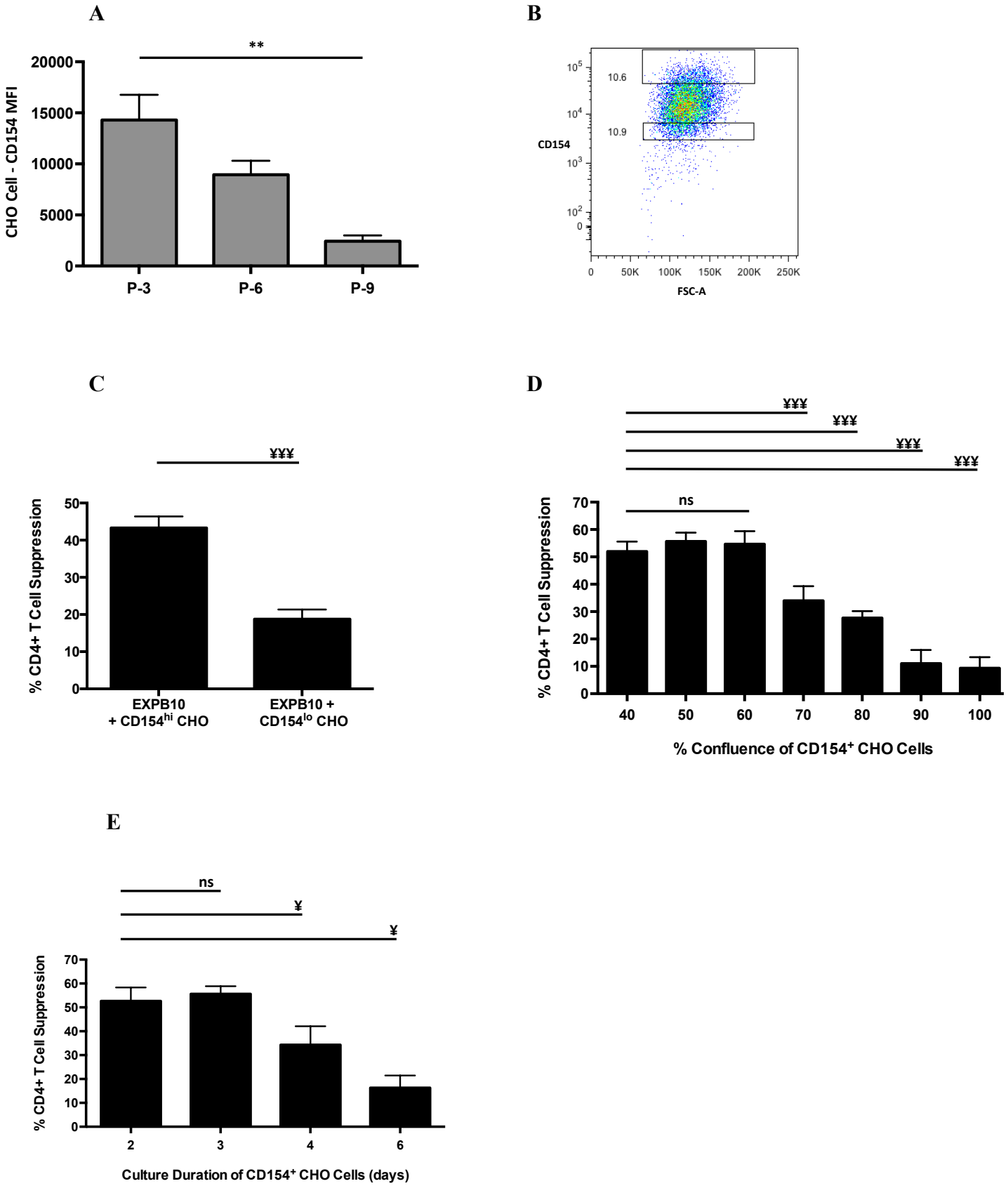
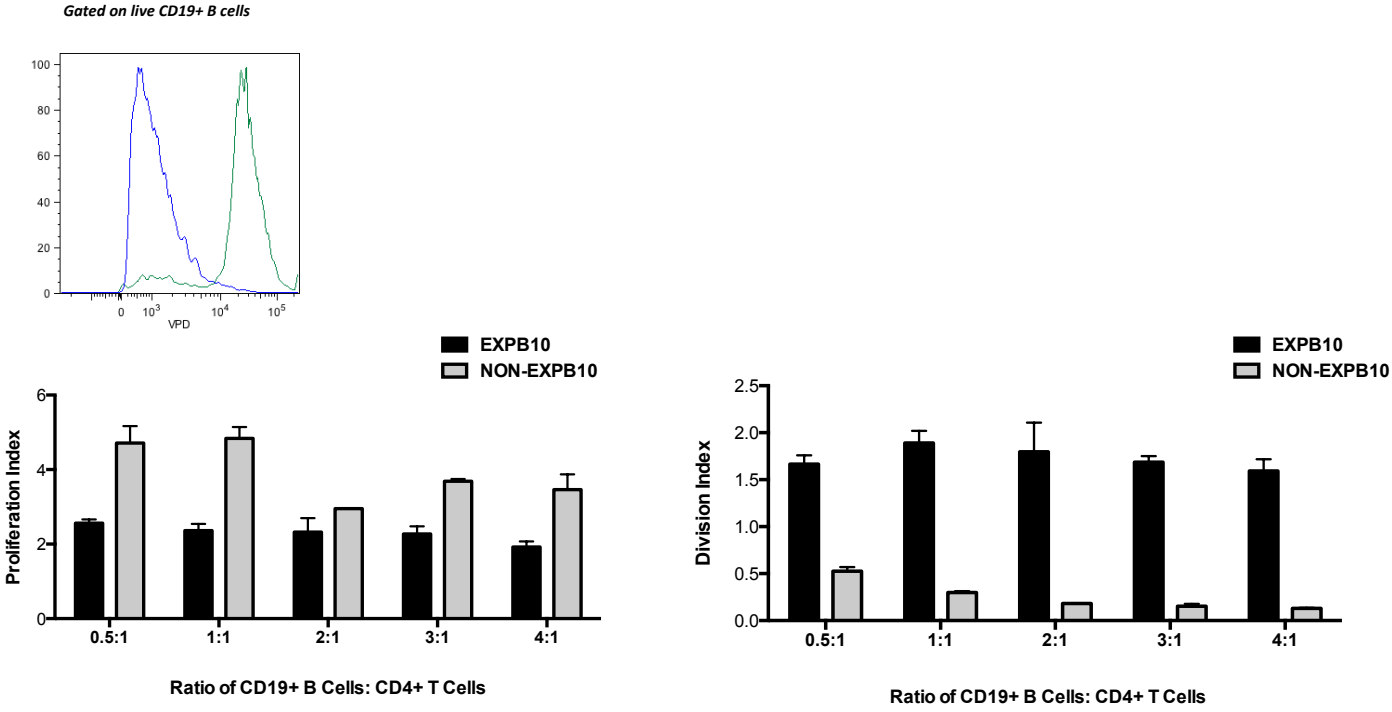

A
**
CHO Cell - CD154 MFI
20000
15000
10000
5000
0
P-3
P-6
P-9
B
10.6
10.9
CD154
FSC-A
0
50K
100K
150K
200K
250K
C
¥¥¥
% CD4+ T Cell Suppression
EXPB10 + CD154hi CHO
EXPB10 + CD154lo CHO
D
¥¥¥
¥¥¥
¥¥¥
¥¥¥
ns
% CD4+ T Cell Suppression
% Confluence of CD154+ CHO Cells
40
50
60
70
80
90
100
E
ns
¥
¥
% CD4+ T Cell Suppression
Culture Duration of CD154+ CHO Cells (days)
2
3
4
6

Fig. 3.2.3 Suppressive potency of *ex vivo* expanded $CD19^+$ B cells can vary depending on different parameters of $CD154^+$ CHO cell passage

(a) Passage of CHO cells was inversely correlated to expression levels of CD154. P-3 denotes passage-3. **p<0.007, 2-way ANOVA for repeated measurements. **(b)** $CD154^+$ CHO cells were FACS-sorted to isolate the higher and lowest 10% of $CD154^+$CHO cells, denoted as $CD154^{hi}$ and $CD154^{lo}$ CHO cells. Purities >96%. **(c)** Graph illustrating difference in suppressive potency of EXPB10 cells which were generated with $CD154^{lo}$ CHO cells or $CD154^{hi}$ CHO cells. ¥¥¥p<0.02, paired t-test. **(d)** Representative histograms of CD154 expression by live CHO cells harvested at different states of confluence. Dashed line represents mf-1 control; solid line represents CD154 staining. Graph illustrating difference in suppressive potency of EXPB10 cells when generated with $CD154^+$ CHO cells that had been cultured to varying degrees of confluence. Duration of culture and passage were kept constant. ¥¥¥p<0.02, paired t-test. **(e)** Representative histograms of CD154 expression by live CHO cells harvested after culture of different durations. Dashed line represents mf-1 control; solid line represents CD154 staining. Graph illustrating difference in suppressive potency of EXPB10 cells which are generated with $CD154^+$ CHO cells at P-5 but that had been cultured for different durations. ¥p<0.0001, 2-way ANOVA for repeated measurements. Error bars in each panel represent Mean +/- SD. Data are representative of 3 independent experiments.

Fig. 3.2.4

A


Gated on live CD19+ B cells
VPD
EXPB10
NON-EXPB10
Proliferation Index
Division Index
0.5:1
1:1
2:1
3:1
4:1
Ratio of CD19+ B Cells: CD4+ T Cells

B

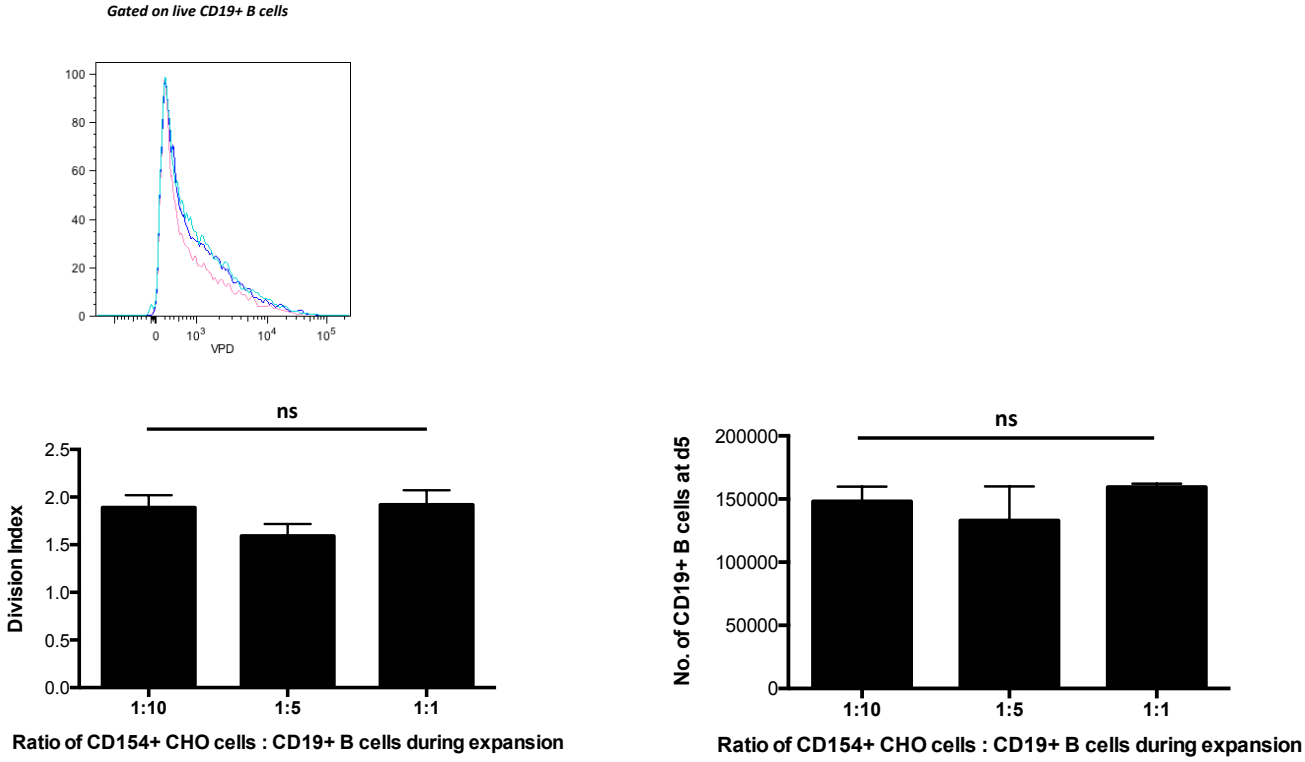

Gated on live CD19+ B cells
VPD
ns
Division Index
No. of CD19+ B cells at d5
1:10
1:5
1:1
Ratio of CD154+ CHO cells : CD19+ B cells during expansion

Fig. 3.2.4 Proliferation and survival of EXPB10 cells within the $CD4^+$ T cell suppression assay

(a) Representative histogram of live $CD19^+VPD^+$ B cells illustrating proliferation when $CD4^+$ T cells were co-cultured with EXPB10 cells or NON-EXPB10 cells at a 1:1 ratio. Green line represents NON-EXPB10 cells; blue line represents EXPB10 cells. Graphs illustrating Proliferation Index and Division Index of EXPB10 and NON-EXPB10 cells when co-cultured at decreasing ratios of $CD4^+$T cells to $CD19^+$ B cells. **(b)** Representative histogram of live $CD19^+VPD^+$ B cells illustrating proliferation when $CD4^+$ T cells were co-cultured with EXPB10 1:1, EXPB10 1:5 or EXPB10 1:10 cells. Light blue line represents EXPB10 1:1 cells; dark blue line represents EXPB10 1:5 cells; pink line represents EXPB10 1:5 cells. Graphs illustrating Division Index and total numbers of EXPB10 cells at day 5 of suppression assay when co-cultured with $CD4^+$T cells at 1:1 ratio. Paired t-test. Error bars in each panel represent Mean +/- SD. Data are representative of 3 independent experiments.

4.0 CHARACTERISTICS AND MECHANISMS OF SUPPRESSION BY EXPB10 CELLS

We have developed and optimised a strategy to generate human Breg *ex vivo* as described in *Chapter 3.0.* A large proportion of this expanded human Breg population was able to express IL-10 and have been termed 'EXPB10 cells.' This human B cell population was able to suppress an autologous or allogeneic $CD4^+$ T cell response, both in terms of proliferation and Th1 differentiation. It is important to explore the identity of this regulatory B cell population; phenotypic similarities with endogenous Breg populations may give an indication as to how this *ex vivo*-generated Breg population will function. Furthermore, in depth mechanistic study of EXPB10 cells may provide a much more detailed understanding of the role of endogenous human Breg *in vivo*. It is essential to appreciate how such regulatory cells may modulate an immune response if such populations are to be considered for clinical application and manipulation. In this chapter we investigated the following hypotheses:

- **EXPB10 cells express an endogenous human Breg phenotype**
- **EXPB10 cells can regulate a $CD4^+$ T cell response by cognate interactions and the secretion of IL-10 *in vitro***

RESULTS

4.1 Characterisation of EXPB10 cells

To characterise EXPB10 cells, it is necessary to look at cell surface markers that describe the developmental stages of human $CD19^+$ B cells, markers of known Breg subsets in both mouse and human, as well as the expression of potential immunosuppressive molecules and production of cytokines by this *ex vivo* generated Breg population.

4.1.1 Phenotype of EXPB10 cells

Human EXPB10 cells and non-expanded human $CD19^+$ B cells (NON-EXPB10) from the same human donor were stained for multiple cell markers and percentage frequency was compared between the two cell populations. Results demonstrated in fig. 4.1.1 represent combined data sets from 5 human donors. Although mean fluorescence intensity (MFI) is considered to be a more sensitive measure of change in levels of expression of cell surface markers than percentage frequency, it was not possible to reliably use this parameter due to the high auto-fluorescence of EXPB10 cells when compared to NON-EXPB10 cells. Gating was performed using mean fluorescence minus 1 controls (mf-1) for each cell surface marker and for each cell population (fig. 4.1.1a-d).

The phenotypes of EXPB10 cells expanded after 7 day co-culture at a 1:1 ratio with $CD154^+$ CHO cells in the presence of the various different cytokine combinations investigated in *Chapter 3.0* (i.e. with no cytokines, with IL-4, or with the cytokine combination IL-2, IL-4 and IL-10), were all characterized and no significant differences between these cell populations were demonstrated (data not shown). What is described below is the comparison of EXPB10 cells isolated after 7-day co-culture at a 1:1 ratio with $CD154^+$ CHO cells in the presence of the cytokine combination IL-2, IL-4 and IL-10, with NON-EXPB10 cells.

EXPB10 cells are activated, naive $CD19^+$ B cells that express high levels of co-stimulatory molecules and the high affinity receptor to IL-2

As demonstrated in fig. 4.1.1a, EXPB10 cells were highly activated directly after isolation from the expansion co-culture system and expressed significantly high levels of co-stimulatory molecules CD80 and CD86, as well as the apoptotic receptor FAS-R when compared to NON-EXPB10 cells (*$p<0.0001$).

Although NON-EXPB10 cells did express moderate levels of the IL-2 receptor subunits, CD25 and CD122, they expressed very low levels of γCR (CD132). This subunit is critical to the formation of the high affinity, trimeric IL-2 receptor, which in

turn is considered essential to enable an optimal cellular response to IL-2 [178, 179]. Therefore, although a considerable proportion of NON-EXPB10 cells found in peripheral blood appeared to be $CD122^+$ and $CD25^+$, these cells actually expressed a low affinity receptor to IL-2. EXPB10 cells however, demonstrated marked up-regulation of all three IL-2 receptor subunits CD122, CD25 and γCR (fig. 4.1.1a, **p<0.005, *p<0.0001, *p<0.0001 respectively) following expansion. Significant up-regulation of CD25 expression may also be relevant as a potential marker of B regulatory cell function [99, 100].

EXPB10 cells appeared to retain expression of IgM and IgD when compared to NON-EXPB10, demonstrating that these cells remained as functionally mature B cells. However, despite continuous stimulation through the CD154-CD40 interaction for 7 days during the expansion co-culture system, EXPB10 cells remained phenotypically naïve, and did not up-regulate the memory B cell marker CD27, nor the plasmablast marker CD138 (fig. 4.1.1b).

EXPB10 cells and NON-EXPB10 cells expressed similarly high levels of the BAFF and APRIL receptor, BAFF-R, as well as that of the low affinity BAFF receptor, BCMA. EXPB10 cells significantly up-regulated the additional BAFF and APRIL receptor, TACI, when compared to NON-EXPB10 cells, perhaps reflecting the highly proliferative nature of an activated, naïve B cell (fig. 4.1.1b, *p<0.0001).

EXPB10 cells are $CD25^+CD71^+CD73^-$ Breg which also express TIM-1

EXPB10 cells were $CD71^+$ and $CD73^-$, in marked contrast to NON-EXP10 cells (fig. 4.1.1c, *p<0.0001). Similar to $IL\text{-}10^+$ Breg identified by whole genome expression analysis [53], the majority of EXPB10 were $CD25^+CD71^+CD73^-$ (>80%, n=4 human donors). Furthermore, the majority of $CD25^+CD71^+CD73^-$ EXPB10 expressed the putative Breg marker recently described in mice, TIM-1 [35, 83-85]. At the time of writing, this marker had not yet been reported in the literature as being expressed by human Breg from healthy individuals. $CD25^-$ EXPB10 cells, whilst $CD71^+$ and $CD73^-$, expressed significantly lower levels of TIM-1 compared to $CD25^+$ EXPB10 cells (fig. 4.1.1c, *p<0.0001).

EXPB10 cells significantly down-regulated expression of cell surface markers that have been previously used to identify other known human Breg populations, such as the transitional $CD24^{hi}CD38^{hi}$ subset [20] or the memory $CD24^{hi}CD27^{+}$ subset [48], when compared to NON-EXPB10 cells (fig. 4.1.1b & 4.1.1c). The expression of other markers that have been associated with Breg phenotype in either mice or humans was either down-regulated or unchanged in EXPB10 cells when compared to NON-EXPB10 cells i.e. CD1d, CD5, CD10 and CD21 [69, 75, 78]. The expression of CD23, a marker of transitional II marginal zone precursor Breg in mice, was significantly up-regulated by EXPB10 cells in comparison to NON-EXPB10 cells; this may be reflective of the function of human CD23 as a low affinity receptor for IgE, which is often up-regulated upon human B cell activation [180, 181].

EXPB10 cells up-regulate the expression of several immunomodulatory molecules in comparison to NON-EXPB10 cells

A substantial proportion of EXPB10 cells have already been shown to express the immunosuppressive cytokine IL-10 (fig. 3.1.1f). Extracellular phenotyping demonstrated that EXPB10 cells also significantly up-regulated expression of the membrane bound precursor to TGFβ, LAP, as well as the immunosuppressive molecule B7-H4, when compared to NON-EXPB10 cells (fig 4.1.1d, *$p<0.0001$, **$p<0.005$ respectively). In addition, intracellular staining illustrated a significant up-regulation of the expression of arginase II and FAS-L by EXPB10 cells in relation to NON-EXPB10 cells (fig 4.1.1d, **$p<0.005$, *$p<0.0001$ respectively). Intracellular staining was performed to study expression of FAS-L due to the high membrane turnover of this ligand. A non-significant increase in cell surface expression of the programmed death ligands, PD-L1 and PD-L2, by EXPB10 cells was also found when compared to NON-EXPB10 cells. Expression of the immunomodulator CD22 remained similarly high on both EXPB10 and NON-EXPB10 cells. Following expansion, EXPB10 cells also retained expression of the ectonucleotidase CD39 despite having down-regulated the expression of ectonucleotidase CD73 (fig 4.1.1c & 4.1.1d). Neither Granzyme B nor TRAIL were expressed by either EXPB10 or NON-EXPB10 cells.

4.1.2 Association of suppressive potency of EXPB10 cells with phenotypic markers

The suppressive potency of EXPB10 cells could be modified as demonstrated in *Chapter 3.0*, by varying the ratio of $CD154^+$ CHO cells to $CD19^+$ B cells in the expansion co-culture system. Higher ratios correlated with increased suppressive potency of EXPB10 cells (fig 3.2.2b). In order to understand better the phenotypic identity of suppressive EXPB10 cells, the phenotype of EXPB10 cells expanded at a 1:1 ratio (EXPB10 1:1) was compared to that of EXPB10 cells expanded at a 1:10 ratio. All markers described in fig 2.1 were assessed, and only those which were found to be significantly different between EXPB10 1:1 and EXPB10 1:10 cells are now demonstrated in fig. 4.1.2. Unlike earlier data, the more sensitive measure MFI is demonstrated here, rather than percentage expression; EXPB10 1:1 and EXPB10 1:10 cells, unlike NON-EXPB10 cells, have a equivalent auto-fluorescence, thus enabling this more robust comparison. Percentage levels of expression were also measured and the same findings were established using this alternative method (*data not shown*).

More potently suppressive EXPB10 1:1 cells demonstrate higher levels of activation and of the TGFβ precursor, LAP

EXPB10 1:1 cells expressed significantly higher levels of all three components of the high affinity receptor to IL-2: γCR, CD25 and CD122. Furthermore, EXPB10 1:1 cells demonstrated significantly higher levels of the TGFβ precursor, LAP (fig. 4.1.2a, $^*p<0.0001$). These findings may be a reflection of greater suppressive function, or indeed simply of greater activation status.

More potently suppressive EXPB10 1:1 cells show greater phenotypic stability following stimulation, unlike the less potently suppressive EXPB10 1:10 cells

Following co-culture of $CD154^+$ CHO cells with $CD19^+$ B cells in the expansion system for 7 days at either ratio 1:1 or 1:10, both EXPB10 1:1 and 1:10 cells demonstrated the phenotype $CD25^+CD71^+CD73^-TIM\text{-}1^+$. However, following 5 days of co-culture with polyclonally stimulated, autologous $CD4^+$ T cells in a suppression

assay, the less potently suppressive EXPB10 1:10 cells demonstrated significantly greater down-regulation of CD71 when compared to EXPB10 1:1 cells. EXPB10 1:1 cells only slightly down-regulated both CD71 and CD25 following the 5 day co-culture (fig. 4.1.2b). MFI of CD25 remained low in EXPB10 1:10 cells at the end of the suppression assay. Both EXPB10 cell types remained $CD73^-$ (data not shown). The down-regulation of the transferrin receptor, CD71, may reflect loss of functional stability of EXPB10 1:10 cells, and thus the lower suppressive potency.

4.1.3 Cytokine production by EXPB10 cells

The investigation of the EXPB10 cell phenotype also extended to cytokine production. ELISA and cytokine bead arrays were both conducted to analyse the supernatants of EXPB10 cells which had been incubated in complete media at 37°C for 6 hours. The results were compared to those of NON-EXPB10 cells and EXPB10 1:10 cells (Fig. 4.1.3a).

EXPB10 cells express significantly higher levels of pro-inflammatory cytokines as well as the regulatory cytokine IL-10 compared to NON-EXPB10 cells

EXPB10 cells expressed pro-inflammatory cytokines IL-2, IL-4, IL-13, TNFα, IL-1β and IL-6. Perhaps unsurprisingly, NON-EXPB10 cells expressed minimal amounts of these cytokines when in a resting state. EXPB10 1:10 cells expressed similar levels of these pro-inflammatory cytokines when compared to EXPB10 1:1 cells, other than IL-1β, which was not produced by the less suppressive, EXPB10 1:10 cells. Levels of IFNγ and IL-12p70 were also measured and were found to be negligible in the supernatants of all $CD19^+$ B cell population tested (*data not shown*).

EXPB10 1:1 cells produced significantly higher levels of IL-10 than EXPB10 1:10 and NON-EXPB10 cells (**$p<0.005$, ***$p<0.02$ respectively). IL-35, another immunosuppressive cytokine reported to be expressed by some Breg populations ([40, 43], was not expressed at high levels by any $CD19^+$ B cell population tested. It has been reported that it is not absolute level of IL-10 production by human Breg which is necessarily important in controlling the immune response, but rather the ratio of IL-10

to TNFα [182]; however, there was no significant difference in this ratio when comparing EXPB10 1:1 cells to the less suppressive EXPB10 1:10 cells (fig. 4.1.3b).

4.2 Mechanisms by which EXPB10 cells suppress proliferation and differentiation of autologous $CD4^+$ T cells

Although it had been established that EXPB10 cells were able to suppress both proliferation and Th1 differentiation of autologous $CD4^+$ T cells (fig. 3.2.1c & 3.2.1f), it was not clear by what mechanisms this inhibition was achieved. Phenotypic analysis of EXPB10 cells illustrated that these particular *ex vivo* generated human Breg up-regulated or maintained the expression of several potential immunosuppressive candidates whilst existing in a highly activated state (fig 4.1.1). To establish whether one or indeed multiple molecular inhibitors are responsible for the observed suppression of autologous $CD4^+$ T cells by EXPB10 cells, potential regulatory pathways were investigated. In the majority of mechanistic experiments described, a blocking reagent or blocking mAb was used to inhibit a putative suppressive pathway. The inhibitor under investigation was added to both stimulated $CD4^+$ T cell controls and stimulated $CD4^+$ T cells co-cultured with EXPB10 cells. % Suppression was calculated as a measure of division index of $CD4^+$ T cells when co-cultured with anti-CD3/CD28 beads, EXPB10 cells and inhibitor, relative to division index of $CD4^+$ T cells when co-cultured with anti-CD3/CD28 beads and the inhibitor only; such that % suppression of the stimulated $CD4^+$ T cell control in the presence of the inhibitor is always expressed as a value of 0.

4.2.1 EXPB10 cells do not induce apoptotic cell death in autologous $CD4^+$T cells via FAS- or PD-1-dependent mechanisms

To identify whether there was an increase in apoptotic cell death of $CD4^+$ T cells when polyclonally stimulated in the presence of EXPB10 cells, autologous $CD4^+$ T cells were co-cultured with anti CD3/CD28 beads and EXPB10 cells at a 1:1 ratio of T:B cells, for 24, 48 or 72 hours. The cell co-cultures were then stained for 7-AAD and Annexin V, as detailed in *Chapter 2.0*. At all three time-points, there was no increase in Annexin V staining of stimulated $CD4^+$ T cells when co-cultured with

EXPB10 cells, as when compared to either stimulated $CD4^+$ T cells alone or to stimulated $CD4^+$ T cells co-cultured with NON-EXPB10 cells. Similarly, no increase in 7-AAD staining of stimulated $CD4^+$ T cells at these time-points was observed (fig. 4.2.1a).

In support of these findings, it was also established that blockade of the FAS and PD-1 pathways by addition of a blocking monoclonal antibody to FAS-L or PD-1 at saturating doses of 10ug/ml during the 5 day suppression assay, did not abrogate suppression of $CD4^+$ T cell proliferation mediated by EXPB10 cells (fig 4.2.1b).

4.2.2 EXPB10 cells may partially suppress proliferation of autologous $CD4^+$ T cells by means of metabolic disruption

A commonly accepted method by which immunoregulatory cells exert suppressive effects is by metabolic disruption affecting the target cell [183, 184]. EXPB10 cells maintained expression of the ectonucleotidase, CD39, whilst significantly down-regulating the expression of CD73 when compared to NON-EXPB10 cells. (fig. 4.1.1c & 4.1.1d). Recent evidence has emerged documenting the suppression of autologous human $CD4^+$ T cells by activated, $CD39^+CD73^-$ human B cells, potentially through the accumulation of products of ATP hydrolysis, 5′AMP, and adenosine [185]. Indeed, incubation of an ectonucleotidase inhibitor, 6-*N*, *N*-Diethyl-D-β,γ-dibromomethylene ATP trisodium salt (ARL 67156), in the 5 day suppression assay, did result in significant if not complete abrogation of suppression of $CD4^+$ T cell proliferation by EXPB10 cells (fig. 4.2.2a, ***$p<0.02$).

It should be noted, however, that the proliferation of $CD4^+$ T cells and beads alone in the presence of this inhibitor was significantly reduced in comparison to proliferation of $CD4^+$ T cells and beads alone in the absence of ARL 67156 (4.2.2b). Proliferation of $CD4^+$ T cells when co-cultured with beads and EXPB10 cells was unchanged in the presence or absence of ARL 67156.

EXPB10 cells do not mediate suppression of $CD4^+$ T cells by depletion of arginine

EXPB10 cells also significantly up-regulated expression of arginase II (fig 4.1.1d), an enzyme that has previously been reported to deplete supplies of the essential amino acid L-arginine upon release by regulatory cells, thereby inhibiting ongoing cellular activation and proliferation of effector cells [172]. In addition, EXPB10 cells significantly up-regulated expression of the low affinity IgE receptor, CD23 when compared to NON-EXPB10 cells (fig 4.1.1c). Ligation of CD23 in malignant human B cells has been shown to result in up-regulation of iNOS production and thereby immunosuppression of effector cells [186]. It is conceivable that competition for the substrate L-arginine by one of these processes has resulted in the observed EXPB10-mediated suppression of autologous $CD4^+$ T cells. However, blockade of neither iNOS nor arginase II, nor exogenous addition of L-arginine to suppression assays, resulted in abrogation of EXPB10 cell-mediated suppression (fig. 4.2.2c).

EXPB10 cells do not mediate suppression of $CD4^+$ T cells by depletion of IL-2

As described earlier, EXPB10 cells significantly up-regulated the high affinity IL-2 receptor, in sharp contrast to NON-EXPB10 cells, which expressed the low affinity IL-2 receptor, largely through the lack of γCR (fig. 4.1.1a). Treg are reported to suppress immune responses in part through utilisation of IL-2, thus depriving effector cells of an essential growth cytokine [187]. It was not possible to incubate a blocking mAb to human CD25 with polyclonally stimulated $CD4^+$ T cells and EXPB10 cells in a suppression assay to determine IL-2 dependency, as this blocking mAb also impeded proliferation of stimulated $CD4^+$ T cells when cultured in the absence of EXPB10 cells (*data not shown*). Thus, in order to investigate whether deprivation of IL-2 was in part responsible for EXPB10-mediated suppression of human $CD4^+$ T cells, exogenous human IL-2 was instead added to the suppression co-cultures for the 5-day duration of the assay, at increasing concentrations, ranging from 5-1000ug/ml. The hypothesis stands that if IL-2 deprivation was driving the suppression of $CD4^+$ T cell proliferation, sufficient replenishment of IL-2 should have overcome this suppression. However, the addition of IL-2 at concentrations as high as 1000 ug/ml did not result in abrogation of $CD4^+$ T cell suppression (fig. 4.2.2d). Furthermore,

potency of suppression by EXPB10 cells did not appear to increase upon addition of high doses of exogenous IL-2.

It should be noted that whilst increasing concentrations of exogenous IL-2 did not affect the suppressive function of EXPB10, apparent suppression of $CD4^+$ T cell proliferation by NON-EXPB10 cells did increase in conditions of higher IL-2 concentrations, namely at those greater and equal to 50ug/ml (fig. 4.2.2d). This increase in potential suppressive function of NON-EXPB10 cells did not correlate with an increase in absolute numbers of CD19+ B cells at day 5 of the suppression assay. Furthermore, there was no correlation with division index, suggesting that NON-EXPB10 cells were not proliferating more when exposed to more IL-2 and thereby reducing CD4+ T cell proliferation (fig. 4.2.2e).

4.2.3 Suppressive function of EXPB10 cells is not dependent on co-stimulatory molecule CD80 and CD86

CD80 and CD86 have been reported to be partly responsible for the suppressive function of human Breg subsets, possibly through an interaction with CTLA-4 expressed on activated $CD4^+$ T cells. However, blockade of CD80, CD86 or both together had no effect on suppressive potency of EXPB10 cells, either in terms of $CD4^+$ T cell proliferation or pro-inflammatory cytokine production (fig. 4.2.3a & 4.2.3b).

4.2.4 Suppressive function of EXPB10 cells is dependent on the interaction between CD40 and CD154

Several studies of mouse and human Breg have demonstrated a role for CD154 in the suppressive mechanism of this regulatory cell population [47, 69, 98]. The CD40-CD154 interaction also drove IL-10 secretion by EXPB10 cells as demonstrated in *Chapter 3.0* (fig. 3.1.1f). Co-culture of blocking mAbs to CD154, CD40, and to both CD154 and CD40 together in the suppression assay did indeed significantly reduce EXPB10-mediated suppression of $CD4^+$ T cell proliferation (fig. 4.2.4a, *$p<0.0001$, ***$p<0.002$). A modest decrease in the number of remaining EXPB10 cells at day 5

of the suppression assay was observed when the CD154 mAb was used, suggesting that survival of EXPB10 cells during blockade of CD154 and/or CD40, may in part account for reduced suppression of $CD4^+$ T cell proliferation (fig 4.2.4b). Alternatively, the observation may be an incidental affect of this particular mAb, as use of CD40 blocking mAb did not adversely affect EXPB10 cell survival.

Blockade of the CD40-CD154 interaction results in down-regulation of EXPB10 phenotypic markers and expression levels of B7-H4 and TIM-1

Phenotype of EXPB10 cells at day 5 of the suppression assay was examined in the presence of CD154 blockade. A significant decrease in both % expression and MFI of CD71 and CD25 expression by EXPB10 cells was observed when blocking mAb to CD154 had been included in the suppression co-culture; this suggests that stability of such markers may be associated with optimal suppressive potency of EXPB10 cells (fig. 4.2.4c). CD73 expression was not up-regulated (*data not shown*).

CD154 is expressed continuously by $CD4^+$ T cells throughout the 5-day suppression assay, but CD154 levels decrease in the presence of EXPB10 cells

Expression levels of CD154 by polyclonally stimulated $CD4^+$ T cells alone or in the presence of EXPB10 cells, were measured daily throughout the 5-day co-culture. CD154 was expressed by $CD4^+$ T cells throughout the culture; however, CD154 expression levels were significantly lower throughout this time-course when $CD4^+$ T cells were stimulated in the presence of EXPB10 cells (fig. 4.2.4d), up until day 5. When stimulated $CD4^+$ T cells were cultured alone for 5 days, CD154 was expressed throughout the time-course, with expression levels peaking by 48 hours and then decreasing with time to day 5 (fig. 4.2.4d). When stimulated $CD4^+$ T cells were cultured with EXPB10 cells for 5 days, CD154 expression similarly peaked at 48 hours, but then plateaued until the day 5 endpoint.

EXPB10-mediated suppression of $CD4^+$ T cells increases with time during the 5-day suppression assay

To investigate the dynamics of EXPB-mediated suppression in this *in vitro* system, proliferation profiles of $CD4^+$ T cells stimulated in the absence or presence of EXPB10 cells were examined daily on days 2, 3, 4 and 5: suppression of proliferation was calculated daily as the division index of stimulated $CD4^+$ T cells in the presence of EXPB10 cells relative to that of stimulated $CD4^+$ T cells cultured alone. It was found that EXPB10 cells were actually associated with increased $CD4^+$ T cell proliferation over the first 48 hours of the suppression co-culture; suppression of proliferation commenced by day 3 and subsequently increased in potency over the last 72 hours of the assay (fig. 4.2.4d). These findings suggest that suppression was associated with an increased exposure to CD154 expression by stimulated $CD4^+$ T cells.

CD154-CD40-dependent suppression by EXPB10 cells can be abrogated as late as day 4 of the 5-day *in vitro* suppression assay

To clarify the above findings, blocking mAb to CD154 was added to suppression co-cultures at several different time-points (fig. 4.2.4e). Suppression was significantly reduced when CD154 mAb was added at any time-point tested, even as late as day 4 of the suppression assay, supporting that view that the suppressive process is an ongoing mechanism throughout the course of the co-culture. Furthermore, given that significant suppression was occurring by day 4 of the suppression assay (fig. 4.2.4d), the observation that blockade on day 4 significantly reduced suppression (fig. 4.2.4e, $*p<0.0001$) suggests that EXPB10-mediated suppression of $CD4^+$ T cell proliferation was in fact reversible, rather than simply preventable.

Blockade of the CD40-CD154 interaction at all daily time-points results in significant down-regulation of B7-H4 and TIM-1 expressed by EXPB10 cells

Examination of EXPB10 phenotype at day 5 of the suppression assay after blocking CD154 mAb had been added at several different, daily time-points, revealed that abrogation of suppression was again associated with significant down-regulation of

CD71, CD25 (*data not shown*) as well as TIM-1 and the immunosuppressive molecule B7-H4, even as late as at day 4 of the suppression assay (fig. 4.2.4f, *p<0.0001, **p<0.005, ***p<0.02). Although these findings demonstrate only an association, it is possible that B7-H4 was a downstream mechanism by which EXPB10 cells suppressed $CD4^+$ T cells in this *in vitro* system.

4.2.5 $CD154^+$ EXPB10 cells are significantly more suppressive than $CD154^-$ EXPB10 cells

Although EXPB10 cells expressed CD40, enabling an interaction with CD154 expressed on activated $CD4^+$ T cells (fig. 4.2.4d & 4.2.5a), the percentage of $CD19^+$ B cells which expressed CD154 significantly increased from an average of 3% by NON-EXPB10 cells, to >60% by EXPB10 cells following the expansion co-culture system (fig 4.2.5a, *p<0.0001). Furthermore, there appeared to be a correlation of CD154 percentage expression and EXPB10 suppressive potency, as the percentage frequency of $CD154^+$ EXPB10 1:1 cells was significantly higher than that of EXPB10 1:10 cells (fig. 4.2.5a, *p<0.0001).

It is possible that the abrogation of suppression observed when the CD40-CD154 interaction was blocked (fig. 4.2.4a), was due to an effect of blockade of CD154 expressed on EXPB10 cells, rather than CD154 expressed on activated $CD4^+$ T cells. To investigate further, $CD154^+$ and $CD154^-$ EXPB10 cells were FACS sorted (fig. 4.2.5b, $CD154^+$ EXPB10 cells >80% purity, $CD154^-$ EXPB10 cells >95% purity). The sorted EXPB10 cells populations were then co-cultured with polyclonally stimulated autologous $CD4^+$ T cells for 5 days, to test suppressive capacity. $CD154^+$ EXPB10 cells were significantly more potent than $CD154^-$ EXPB10 cells (fig. 4.2.5c, **p<0.005).

EXPB10 cells do not gain expression of CD154 by trogocytosis involving $CD154^+$ CHO cells

Given that $CD19^+$ B cells were co-cultured with $CD154^+$ CHO cells and through this interaction, became activated and gained suppressive function that appeared to

involve CD154, it was not impossible that human B cells had gained CD154 expression through the process of trogocytosis. To investigate this, $CD154^+$ CHO cells were stained with the lipophilic membrane dye, 1,1'-Dioctadecyl-3,3,3',3' Tetramethylindocarbocyanine Perchlorate (Dil), and were co-cultured as per the expansion method with human $CD19^+$ B cells. As outlined in the trogocytosis protocol [140], Dil expression on both $CD154^+$ CHO cells and $CD19^+$ B cells was measured after 1 hour and 5 hours of co-culture. $CD19^+$ B cells did not express Dil after 1 hour of co-culture with Dil^+ $CD154^+$ CHO cells, and only expressed minimal levels (<3%) after 5 hours (fig. 4.2.5d).

4.2.6 Suppressive function of EXPB10 cells is partially dependent on immunosuppressive cytokine, IL-10, but not TGFβ

Breg have been reported to suppress autologous $CD4^+$ T cell proliferation and Th1 differentiation through the secretion of both IL-10 and TGFβ [24, 39, 188]. Blockade of TGFβ by the TGFβ type 1 receptor inhibitor, 4-[4-(1,3-benzodioxol-5-yl)-5-(2-pyridinyl)-1*H*-imidazol-2-yl]benzamide (SB 431542), did not however reduce EXPB10-mediated suppression of $CD4^+$ T cell proliferation or pro-inflammatory cytokine production (fig. 4.2.6a & 4.2.6b). However, blockade of IL-10 and IL-10R did partially restore IFNγ and TNFα expression by $CD4^+$ T cells (fig. 4.2.6c, $^{**}p<0.002$). Furthermore, blockade of IL-10 and IL-10R resulted in significant, albeit slight reduction in suppression of $CD4^+$ T cell proliferation (fig 4.2.6d, $^{*}p<0.0001$); the physiological significance of such a modest effect however, is questionable. Blockade of CD154 resulted in significantly greater reduction in suppression of $CD4^+$ T cell proliferation than blockade of IL-10 and IL-10R ($^{¥}p<0.001$), but joint blockade of CD154, IL-10 and IL-10R did not result in greater reduction in suppression than CD154 blockade alone.

In order to isolate $IL\text{-}10^+$ EXPB10 cells, an IL-10 capture kit (Miltenyi) was used to isolate both $IL\text{-}10^+$ and $IL\text{-}10^-$ EXPB10 populations. However, despite several attempts, only a mild enrichment of $IL\text{-}10^+$ EXPB10 cells was achieved (fig. 4.2.6e). Furthermore, upon co-culture of this 'enriched' $IL\text{-}10^+$ EXPB10 cell population with polyclonally stimulated autologous $CD4^+$ T cells, this EXPB10 population was unable

to suppress $CD4^+$ T cell proliferation, likely due to the finding that >95% of EXPB10 cells had died by the 5 day end-point (*data not shown*).

IL-10 secreted by EXPB10 cells supports suppressive function of EXPB10 cells by an autocrine effect

EXPB10 cells significantly up-regulated expression of IL-10R compared to NON-EXPB10 cells (fig. 4.2.6f, *$p<0.0001$). It is possible that IL-10 secreted by EXPB10 cells has exerted an autocrine effect on EXPB10 cells, rather than a solely paracrine effect on $CD4^+$ T cells. To investigate this, EXPB10 cells were generated by co-culturing $CD154^+$ CHO cells with $CD19^+$ B cells under varying conditions (fig. 4.2.6g):

i) In absence of exogenous IL-10 for 7 days (EXPB10 w/o hIL-10)
ii) In absence of exogenous IL-10 for 7 days and with the addition of blocking mAbs to IL-10 and IL-10R for the last 48 hours of the expansion co-culture, as a pre-incubation step prior to harvest (PRE-INC IL-10/R EXPB10)

An IgG isotype control mAb was added for the last 48 hours of expansion co-culture in condition i) to serve as an appropriate control for condition ii). The resulting EXPB10 cells were harvested and subsequently washed three times before being co-cultured for 5 days with polyclonally stimulated autologous $CD4^+$ T cells to determine suppressive function.

EXPB10 without hIL-10 received no exposure to exogenous IL-10 during the expansion period, but these cells were exposed to endogenous IL-10 secreted by the $CD19^+$ B cells themselves throughout the culture period. PRE-INC IL-10/R EXPB10 were not only unexposed to exogenous IL-10 for the 7 day expansion, but during the pre-incubation step, these cells were also blocked from accessing endogenous IL-10 secreted by the $CD19^+$ B cells for the last 48 hours of expansion co-culture.

There were no significant differences in MFI of either phenotypic markers such as CD71, CD73, CD25 or TIM-1, or potential mechanistic molecules such as B7-H4,

CD40 or CD154 between EXPB10 and EXPB10 w/o hIL-10 cells. However a slight but significant reduction in MFI of CD71 AND CD25 was seen in PRE-INC IL-10/R EXPB10 cells when compared to EXPB10 cells that had undergone exposure to endogenous, EXPB10-secreted IL-10 for 7 days (fig. 4.2.6h, ***$p<0.02$, ¥$p<0.001$).

EXPB10 without hIL-10, which had undergone exposure to endogenous, EXPB10-secreted IL-10, were able to suppress $CD4^+$ T cell proliferation at potencies equal to that of EXPB10 cells generated in the presence of exogenous IL-10 (i.e. normal expansion conditions). However, PRE-INC IL-10/R EXPB10, with no exposure to endogenous IL-10 for the last 48 hours of expansion or generation, were significantly less suppressive compared to EXPB10 without hIL-10, and compared to EXPB10 cells expanded in normal conditions (fig 4.2.6i, ¥¥$p<0.008$). There was no difference in survival capacity of any EXPB10 cell population tested (fig. 4.2.6j), nor was there any difference in levels of IL-10 secreted by the EXPB10 cell populations described (4.2.6k).

PRE-INC IL-10/R EXPB10 cells remained significantly less suppressive than both EXPB10 without hIL-10 and EXPB10 cells generated under normal conditions when blocking mAbs to IL-10 and IL-10R were added to suppression co-cultures (fig. 4.2.6i, **$p<0.005$). In addition, the reduced suppressive potency of PRE-INC IL-10/R EXPB10 was not further decreased by addition of blocking mAbs to IL-10 and IL-10R to suppression co-cultures. Together, these observations suggest that B cell-derived IL-10 interacts in some way with EXPB10 cells to enable optimal suppressive function of this population, and that it is the exposure of EXPB10 cells to IL-10 during the generation phase which is essential to Breg suppressive function, rather than during the actual suppression co-culture.

4.2.7 EXPB10 cells suppress $CD4^+$ T cell proliferation by both CD154-dependent cognate interactions and by a CD154-independent soluble factor

In order to corroborate the above findings of dependency by EXPB10 cells on the interaction of CD154 and CD40, suppression assays were also designed within a

transwell system. Comprising a 96-well plate much like the 96-well plates used in standard suppression assays already described, the transwell system consisted of a receiver well and an insert; such that $CD4^+$ T cells in the receiver well could be separated from the upper insert by a thin membrane and thus be prevented from directly interacting with EXPB10 cells in the insert. Suppression of proliferation of $CD4^+$ T cells in this experiment was calculated by measuring division index of $CD4^+$ T cells when co-cultured with $CD19^+$ B cells relative to that of the exact experimental conditions but in the absence of $CD19^+$ B cells +/- IgG_1 isotype mAb as required.

Cognate interactions between EXPB10 cell and stimulated $CD4^+$ T cells are required for EXPB10 cell suppressive function

When EXPB10 cells were placed in the insert and autologous $CD4^+$ T cells and anti-CD3/CD28 beads were placed in the receiver well for 5 days, suppression of $CD4^+$ T cell proliferation was completely abrogated (fig. 4.2.7a, *$p<0.0001$). Furthermore, there was no suppression of $CD4^+$ T cells when NON-EXPB10 cells were cultured either in the receiver with $CD4^+$ T cells and anti-CD3/CD28 beads or when NON-EXPB10 cells were cultured alone in the insert.

Soluble factors are released upon cognate interactions between EXPB10 cell and $CD4^+$ T cells which result in partial suppression of $CD4^+$ T cell proliferation

Although a cognate interaction was required for EXPB10-mediate suppression, this finding did not exclude the involvement of a soluble suppressive factor that could be released subsequent to the cognate interaction. To establish whether this was the case, $CD4^+$ T cells and anti-CD3/CD28 beads were also placed in the insert with EXPB10 cells. $CD4^+$ T cells and anti-CD3/CD28 beads alone in the insert, or with the addition of NON-EXPB10 cells in the insert, both served as further controls. Proliferation of the two $CD4^+$ T cell populations in the receiver well and in the insert, was individually measured by VPD dilution.

Although separation of EXPB10 cells in the insert from $CD4^+$ T cells and beads in the receiver well resulted in complete abrogation of suppression (fig. 4.2.7a & 4.2.7b), when $CD4^+$ T cells and beads were also included in the insert with the EXPB10 cells,

$CD4^+$ T cells in the receiver well were partially suppressed (fig. 4.2.7b, *p<0.0001). This observation indicates that the cognate interaction of EXPB10 cells with $CD4^+$ T cells resulted in the release of a soluble factor that partially suppressed the $CD4^+$ T cell response. $CD4^+$ T cells in the insert with EXPB10 cells were potently suppressed, significantly more so than those $CD4^+$ T cells in the receiver well (fig. 4.2.7b, *p<0.0001).

Release of the suppressive soluble factor is not dependent on the CD154-CD40 interaction

Previous experiments demonstrated that EXPB10 cells suppressed $CD4^+$ T cell proliferation through the CD154-CD40 interaction. The transwell system demonstrated that cognate interactions were required for suppression and that a soluble suppressive factor was released upon cognate interaction of EXPB10 cells and $CD4^+$ T cells, resulting in partial suppression. Therefore it is possible that the soluble factor demonstrated to partially suppress $CD4^+$ T cell responses was released upon the CD154-CD40 interaction.

To investigate this, blocking mAb to CD154 was added to the insert, along with $CD4^+$ T cells, anti-CD3/CD28 beads and EXPB10 cells. However, contrary to our hypothesis, whilst blockade with CD154 mAb significantly reduced suppression of $CD4^+$ T cells in the insert (fig. 4.2.7c, *p<0.0001) - i.e. those T cells subjected to cognate interactions with EXPB10 cells - blockade with CD154 mAb did not reduce the partial suppression of $CD4^+$ T cells in the receiver well. Furthermore, CD154 blockade did not completely abrogate suppression of $CD4^+$ T cell in the insert, unlike the complete abrogation that which occurs when cognate interactions are blocked by the transwell system; CD154 blockade can only reduce suppression of $CD4^+$ T cells in the insert to the levels of suppression seen in $CD4^+$ T cells in the receiver well. These observations suggest that although a suppressive soluble factor was produced upon cognate interactions of EXPB10 cells with $CD4^+$ T cells, and that CD154-CD40 interaction was also necessary for EXPB10 cell-mediated suppression, the production of this soluble factor was not dependent on the CD154-CD40 interaction. There may be at least two suppressive mechanisms employed by EXPB10 cells, both of which require cognate interactions.

4.2.8 TIM-1 may modulate EXPB10 cellular responses to IL-10 and affect suppressive potency of EXPB10 cells

Activation of both CD40 and IL10-R can be detected through the measurement of phosphorylated levels of the transcription factor STAT3 (pSTAT3). The response of EXPB10 cells to IL-10 can be measured through the readout of relative increase in pSTAT3 levels from baseline, following exposure of EXPB10 cells to exogenous IL-10. This readout can therefore be used to investigate whether any potential Breg markers are functionally involved in the suppressive, mechanistic pathway downstream of IL-10 secretion.

Optimisation of detecting changes in STAT3 phosphorylation levels in response to exogenous IL-10

Measurement of pSTAT3 was up-regulated in EXPB10 cells immediately after harvest from expansion co-cultures, in comparison to NON-EXPB10 cells (fig. 4.2.8a). A slight increase in levels of pSTAT3 was observed when NON-EXPB10 and EXPB10 cells were then incubated with exogenous IL-10 for 10 minutes prior to the staining process (fig. 4.2.8a), thus demonstrating that both cell types were able to respond to IL-10 via IL-10R.

When EXPB10 cells were rested in media for 1.5 hours after harvest from expansion co-cultures, and are then stained for pSTAT3, levels of phosphorylation were low (fig. 4.2.8a). This rendered subsequent increases in phosphorylation levels in response to exogenous IL-10 much more sensitive to detection (fig. 4.2.8a). Thus for subsequent experiments using pSTAT3 levels as a readout for cellular responses to IL-10, EXPB10 cells were first rested in media for 1.5 hours before undergoing the staining process.

Blockade of TIM-1 results in significantly reduced responses of EXPB10 cells to IL-10

To ask whether potential Breg markers are functionally involved in this pathway, EXPB10 cells were pre-incubated with blocking monoclonal antibodies to CD154, IL-10 and IL-10R, TIM-1, IL-2R (comprising mAbs against CD122 and CD25) or with an isotype control antibody, for the last 48 hours of the 7-day expansion system. Following harvest and three washes, the resulting EXPB10 cells were stained for levels of pSTAT3 as outlined above and in *Chapter 2.0*.

There was a significant reduction in relative increase of pSTAT3 levels in response to exogenous hIL-10 when EXPB10 cells had been pre-incubated with IL-10 and IL-10R, or with TIM-1 (fig. 4.2.8b, *$p<0.0001$, ¥$p<0.001$), when compared to that in the absence of exogenous hIL-10. This reduction was not observed when EXPB10 cells had been pre-incubated with an isotype control antibody, or antibodies blocking CD154 or CD122 and CD25. These findings demonstrate that in the presence of either IL-10 blockade or TIM-1 blockade by antagonistic mAbs, the intracellular response of EXPB10 cells to IL-10 was significantly reduced. The absolute % levels of pSTAT3 in absence and in presence of IL-10 as expressed by both EXPB10 cells and EXPB10 cells pre-incubated with TIM-1 mAb, are illustrated as FACS histograms (fig. 4.2.8b): baseline levels of pSTAT3 were equivalent in both Breg populations but levels of pSTAT3 in response to IL-10 were substantially decreased in EXPB10 cells pre-incubated with TIM-1, in comparison to EXPB10 cells generated under normal conditions.

Blockade of TIM-1 or CD154 results in significantly reduced expression of CD71, increased expression of CD73 but no change in expression of IL-10R or CD25

The reduced responsiveness of EXPB10 cells when TIM-1 was blocked may be accounted for by a reduction in levels of IL-10R expression in those EXPB10 cells pre-incubated with TIM-1 mAb. However, FACS-staining revealed a no significant difference in MFI of IL-10R in this EXB10 cell population when compared to EXPB10 cells generated without a pre-incubation step or compared to EXPB10 pre-

incubated with CD154 mAb (fig. 4.2.8c). EXB10 cells pre-incubated with IL-2R mAbs demonstrated a significant up-regulation of IL-10R expression (fig. 4.2.8c, **p<0.005). EXPB10 cells pre-incubated with IL-10 and IL-10R mAbs for 48 hours did express lower levels of IL-10R, in keeping with the blockade, as well as reduced levels of CD25 expression (fig. 4.2.8c, **p<0.005). PRE-INC IL-10/R EXPB10 cells however maintained CD71 expression.

EXPB10 cells pre-incubated with either CD154 or TIM-1 mAbs demonstrated a significant reduction in MFI of CD71, but not CD25. Both of these EXPB10 cell populations also slightly but significantly up-regulated CD73 expression (fig. 4.2.8c, **p<0.005, ***p<0.02). No EXPB10 cell population demonstrates any changes in TIM-1 or B7-H4 expression. PRE-INC CD154 EXPB10 cells significantly up-regulated CD40, unlike any other EXPB10 cell population (fig. 4.2.8c, *p<0.0001); other than those EXPB10 cells pre-incubated with CD154, no other EXPB10 cell population shows altered expression of CD154.

Blockade of TIM-1 results in significantly reduced suppressive potency of EXPB10 cells

Regulatory function of EXPB10 cell populations that had undergone the pre-incubation step outlined above was tested in the 5-day suppression assay with anti-CD3/CD28 bead-driven $CD4^+$ T cells. EXPB10 cells which had been pre-incubated with CD154, IL-10 and IL-10R, or TIM-1 antagonistic mAbs, had significantly reduced suppressive potency compared to EXPB10 cells generated under normal conditions, or indeed compared to EXPB10 cells pre-incubated with IL-2R blocking mAbs (fig. 4.2.8d, **p<0.005, ***p<0.02, ¥¥p<0.008). This did not appear to be due to reduced survival capacity of PRE-INC TIM-1 EXPB10 or PRE-INC IL-10/R EXPB10 cells, as there was no difference in the number of $CD19^+$ B cells at day 5 of the suppression assay between these cell subsets and EXPB10 cells pre-incubated with IL-2R mAbs or an isotype control (fig. 4.2.8e). Similar to findings when blocking mAb to CD154 was added to suppression co-cultures, PRE-INC CD154 EXPB10 cell numbers were significantly reduced at day 5 of the suppression assay (***p<0.02).

DISCUSSION

Although IL-10 expression has been the principle characteristic used to define populations of suppressive B cells in mice and humans, several surface markers that have been used to identify Breg subsets are up- or down-regulated during immune activation; there are many different stimuli which can instigate IL-10 secretion by B cells, which inevitably results in different variations of cell surface marker expression patterns [39, 69, 71, 76, 78, 98]. This is turn leads to inherent problems in the definition of different Breg cell subsets among different experimental settings, and may help account for the huge heterogeneity found across the many different Breg subsets already identified.

The term, 'B10 cell' was initially coined by Tedder et al. in 2011 [77, 188], and was defined as a B cell that could express IL-10 following 5 hours of PMA and ionomycin stimulation. This definition was extended to include the B10 progenitor cells, or 'B10pro cell,' as those B cells which could express IL-10 following 48 hours of CD40 stimulation and 5 hours of subsequent LPS, PMA and ionomycin stimulation [48]. EXPB10 cells were originally defined at the commencement of this project as *ex vivo* expanded $CD19^+$ B cells with regulatory function that could express IL-10. Although EXPB10-secreted IL-10 could be readily detected immediately after harvest from expansion co-cultures by using ELISA or cytokine bead arrays (fig. 4.1.3), additional stimulation with PMA and ionomycin for 5 hours was required in order to visualise and quantify $IL\text{-}10^+$ B cells using flow cytometry (fig. 3.1.1f). However, it does not necessarily follow that it is this $IL\text{-}10^+$ B cell population which is the suppressive subset qualified in the *in vitro* suppression assay used here. Such potent, non-specific stimulation by PMA and ionomycin may have induced an alternate or additional IL-10-secreting Breg population to that obtained immediately after harvest from the expansion co-cultures. Thus phenotyping the $IL\text{-}10^+$ population after PMA and ionomycin stimulation may prevent a precise description of the Breg population as it existed when suppressive function was actually tested within the $CD4^+$ T cell suppression assay. It is therefore more accurate to assess EXPB10 phenotype immediately after isolation from the expansion co-culture system, rather than after additional stimulation with PMA and ionomycin.

EXPB10 cells, similar to the transitional, human $CD24^{hi}CD38^{hi}$ and mouse T2-MZP B cell subsets previously identified [20, 73], were $CD27^-$ and expressed high levels of IgM and IgD, demonstrating that EXPB10 cells remained a naïve B cell population despite 7 days of constant stimulation with membrane-bound CD154 and the cytokines IL-2, IL-4 and IL-10 (fig. 4.1.1b & 4.1.1c). This was reinforced by the absence of expression of the plasma cell marker, CD138 (fig. 4.1.1b). Unlike human $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^+$ B cells, as well as mouse T2-MZP and B10 cells, EXPB10 cells did not express elevated levels of other markers such as CD1d, CD5, CD10 and CD21; indeed, expression of these surface molecules was generally down-regulated by EXPB10 cells (fig. 4.1.1c). Furthermore, expression of both CD24 and CD38 was significantly reduced following expansion (fig. 4.1.1c). Cell surface markers CD71 and CD25 were significantly up-regulated and CD73 down-regulated in EXPB10 cells when compared to freshly isolated $CD19^+$ B cells (NON-EXPB10), much like the $IL\text{-}10^+$ Breg population identified through whole genome analysis by Van de Veen et al. [53]. However unlike EXPB10 cells, $CD25^+CD71^+CD73^-$ B cells identified by gene arrays comprised both naïve $CD27^-$ and memory $CD27^+$ B cells.

To our knowledge, this is the first documentation of TIM-1 expression by *ex vivo* generated human Breg subset. EXPB10 cells up-regulated expression of TIM-1 following the expansion protocol, and it may be the $TIM\text{-}1^+$ subset of $CD25^+CD71^+CD73^-$ EXPB10 cells (fig. 4.1.1c) that is the functional Breg within this population. Furthermore, there were a significantly higher percentage of $TIM\text{-}1^+$ EXPB10 cells in $CD25^+$ EXPB10 cells compared to $CD25^-$ EXPB10 cells (fig. 4.1.1c, $*p<0.0001$). It is not clear from this data whether the EXPB10 cell phenotype $CD25^+CD71^+CD73^-TIM\text{-}1^+$ represented all or part of a unifying phenotype encompassing all activated, human $IL\text{-}10^+$ Breg cells, whether this phenotype simply represented that of a highly activated $CD19^+$ B cell, or whether this phenotype represented just one of many developmentally discrete Breg populations. The functional significance of TIM-1 expression is discussed later in this chapter.

Similar to mouse *ex vivo*-generated B10 cells [98], human *ex-vivo* EXPB10 cells up-regulated expression of IL-21R (fig. 4.1.1c). Exogenous IL-21 was not added to expansion co-cultures during the generation of human EXPB10 cells, and IL-21 was

not expressed by EXPB10 cells, thus reducing the likelihood of autocrine stimulation by EXPB10-produced IL-21during expansion (*data not shown*). It may be that IL-21 is not required for the function or expansion of human EXPB10 cells, unlike their mouse counterpart. Alternatively, it may be that addition of exogenous IL-21 could drive huge expansion of EXPB10 cells. Given that IL-21 is readily produced by activated $CD4^+$ T cells [129, 189, 190] and is a known potent inducer of IL-10 in Tr1 cells [191, 192], it is tempting to speculate that polyclonally activated $CD4^+$ T cells which are co-cultured with EXPB10 cells in the suppression assay, are a source of IL-21 in this *in vitro* system, thus enabling or improving EXPB10 regulatory function. This possibility would help account for the finding that when $CD4^+$ T cells were not activated in the presence of EXPB10 cells, slight proliferation of T cells was revealed, whereas when $CD4^+$ T cells were activated, T cell proliferation was significantly suppressed (fig 3.2.1g). There are several other possible explanations of this phenomenon, which will be discussed later in this chapter; measurement of IL-21 as well as blockade of IL-21 during in the *in vitro* suppression assay would help determine this particular hypothesis.

EXPB10 cells maintained high expression levels of BAFF receptor BAFF-R and the BAFF and APRIL receptor BCMA after co-culture with $CD154^+$ CHO cells. The additional BAFF and APRIL receptor, TACI, was significantly up-regulated in EXPB10 cells when compared to NON-EXPB10 cells (fig. 4.1.1b). These molecules and their receptors have an important role in murine and human B-cell differentiation, maturation, and survival. BAFF-R, TACI and BCMA are mainly expressed on B cells, although their expression depends on the stage of B cell maturation [193]. BAFF-R, found across the B cell lineage, mediates the survival and proliferation of early and mature B cell subsets [194-196] and participates in class-switch recombination [196, 197]. BCMA is thought to be generally involved in the final steps of differentiation from B cells to plasma cells. TACI is mainly expressed on marginal zone and memory B cells, although it can also be found on activated B and T cells, and intervenes in class-switch recombination in T-cell dependent and independent humoral immune responses [197-201].

The up-regulation of TACI by EXPB10 cells may simply be a consequence of prolonged activation and may offer no significant functional role. A study by Ferrer et

al. looked at the expression and function of BAFF and APRIL receptors on both normal and leukaemic B cells [202] when activated. Stimulation of B cells from peripheral blood of healthy individuals by CD40 + IL-4R ligation over 48 hours resulted in significant up-regulation of TACI and moderate up-regulation of BCMA, similar to that seen in EXPB10 cells. Stimulation of B cells via the BCR had no such effect. The measurement of p52, the proteolysed form of NFkB2 p100/p52, can be used as a read-out of activation of the NFκB pathway, which is usually triggered by BAFF and APRIL stimulation. The authors demonstrated that through the increase in TACI expression, B cells activated by CD40 + IL-4R ligation were more sensitive to both BAFF and APRIL, and had increased cell viability when compared to B cells stimulated via the BCR. It may be that these signals, together with the increased expression of TACI, have had an additive effect in the differentiation and survival of EXPB10 cells *in vitro* or indeed, *in vivo*. It would be interesting to include exogenous BAFF in expansion co-cultures, similar to the *in vitro* expansion protocols with mouse B10 cells used by Yoshizaki et al. [98], to investigate whether either expansion rate or suppressive potency of EXPB10 cells could be significantly improved.

EXPB10 cells were highly activated, as demonstrated by up-regulation of co-stimulatory molecules such as CD80 and CD86, activation markers CD71 and FAS-R, as well as of the high affinity receptor to IL-2 (fig. 4.1.1a & 4.1.1b). EXPB10 cells, in keeping with activation, also secreted pro-inflammatory cytokines such as IL-2, IL-4, TNFα, IL-6 and IL-1β (fig. 4.1.3a). Activation status of EXPB10 cells appeared to be closely linked to suppressive function: EXPB10 1:1 cells were significantly more suppressive than EXPB10 1:10 cells (fig. 3.2.2b), and this was mirrored by significant (albeit slight) differences in expression levels of activation markers including the high affinity receptor to IL-2 (fig. 4.1.2a). It is difficult to separate the two parameters of activation and suppression; by definition, EXPB10 1:10 cells would be likely to be less activated than EXPB10 1:1 cells as they were subjected to lower levels of $CD154^+$ CHO cell-mediated stimulation. It is entirely possible that activation status and suppressive potency were independent from each other and were two separate measures of function that happened to arise from the same processes during the expansion co-culture. However the finding that the $CD25^+CD71^+$ phenotype appeared to be less stable in less suppressive and less activated EXPB10 cells at the end of the suppression assay (fig. 4.1.2b), does suggest that the two states of

activation and suppressive function were inextricably linked. It may be that EXPB10 1:10 cells were intrinsically less stably activated and thus less stably suppressive than EXPB10 1:1 cells; or that the more activated EXPB10 1:1 cells were able to respond to an exogenous stimulus such as a polyclonally stimulated $CD4^+$ T cell in such a way as to better maintain regulatory function. Indeed, *in vivo* experiments examining mouse Breg function have illustrated that Breg did need to be activated in some way, either by antigenic or innate signals in order to suppress an effector response [35, 82].

There is evidence to support the view that both activation status and CD25 expression are associated with human $CD19^+$ B cell regulatory function [48, 99, 100]. Tretter et al. activated human $CD19^+$ B cells through the BCR using *S aureus* Cowan I antigen (SAC) and in the presence of IL-2 for 72 hours; they identified that large $CD25^+$ B cells were able to suppress proliferation of autologous $CD4^+$ T cells, unlike small $CD25^-$ B cells [99]. The authors found that various other means of B cell stimulation such as CpG-ODN (TLR9 ligation associated with innate immune responses) and anti IgG/IgM (BCR crosslinking associated with adaptive responses) also resulted in suppressive, large $CD25^+$ B cells. Freshly isolated $CD25^+$ B cells from peripheral blood were unable to suppress a $CD4^+$ T cell response, although this may be because unactivated $CD19^+$ B cells have low levels of γCR and thus a low affinity receptor to IL-2 (fig. 4.1.1a). In contrast, Kessel et al. isolated $CD25^{hi}$ human B cells from peripheral blood and found that not only were they larger than $CD25^-$ human B cells, but also that they had mild regulatory function [100]. These findings reinforce the idea that activation of the B cell is required for regulatory function as well as CD25 expression, possibly to enable a functional IL-2R. Indeed, EXPB10 cells were large, activated cells (fig. 3.2.1b & 4.1.1a) which significantly up-regulated high affinity IL-2R (fig 4.1.1a) and were potently suppressive (fig. 3.2.1c). $CD25^+$ EXPB10 cells also expressed higher levels of TIM-1 compared to $CD25^-$ EXPB10 cells (fig. 4.1.1c); this differential expression may have contributed to Breg function as discussed later in this chapter.

In line with this work, Tedder et al. found that that freshly isolated $CD24^{hi}CD27^+$ B cells from healthy individuals were unable to suppress a $CD4^+$ T cell response without further manipulation [48]. Following 24 hours of stimulation with CD40L and CpG however, $CD24^{hi}CD27^+$ B cells were enriched for IL-10 and were able to suppress

TNFα secretion by $CD4^+$ T cells stimulated by CD3 mAb. It is worth noting that under these activation conditions, $CD24^{lo}CD27^-$ B cells (which express x10 fold less IL-10 if stimulated for 5 hours with PMA and ionomycin) could also suppress $CD4^+$ T cell responses to the same extent. Furthermore, both $IL\text{-}10^+$ and $IL\text{-}10^-$ human B cells in this study expressed high levels of CD25 following stimulation, reflecting activation status and possibly suppressive activity.

Thus different methods of human B cell stimulation result in activated, $CD25^+$ B cells with regulatory function, regardless of whether whole $CD19^+$ B cells or more specific subsets are stimulated. IL-10 production was induced in EXPB10 cells through prolonged CD154-CD40 stimulation usually associated with an adaptive immune response [203, 204], whereas the IL-10 secretion which led to identification of the $CD25^+CD71^+CD73^-$ phenotype by Van de Veen et al. [53], was induced through CpG-ODN, a TLR9 ligand, often associated with innate mechanisms [205]. It is of course possible that these different modes of activation of different B cell populations result in multiple, heterogeneous Breg subsets; indeed, not all of these activated $CD25^+$ Breg populations appear to be solely dependent on IL-10 [48, 99]. However it is equally plausible that once progenitor Breg within any B cell lineage reach a certain 'threshold' of activation whilst in the presence of an optimal combination of cytokines, regardless of whether this activation is mediated through antigen-dependent mechanisms, regulatory function usurps effector function. The downstream suppressive mechanisms may be multiple, with IL-10 being one of several depending on the microenvironment. In essence, the human B cell regulates an otherwise overactive immune response. This view is supported by the findings of Rosser et al., that mouse Breg increase in number and restrain excessive inflammation in response to inflammatory signals such as IL-6 and IL-1β, which in turn are induced by both gut flora and arthritis [71].

Given that EXPB10 cells secreted IL-2 (fig. 4.1.3a), they were expanded in the presence of IL-2 (fig. 3.1.1a) and they significantly up-regulated the high affinity IL-2R (fig. 4.1.1a), a potential role of IL-2 in the generation of regulatory function in human B cells should be considered. This possibility is supported by the finding that adding high concentrations of IL-2 (>50μg/ml) to suppression assays appeared to induce mild regulatory function in NON-EXPB10 cells (fig. 4.2.2d). Similar findings

were reported by Tretter et al. with regards to small $CD25^-$ B cells (at 1000U/ml) [99]. It may be that the combination of high levels of IL-2 over a prolonged period (5 days) and the exposure to CD40 activation (via the polyclonally stimulated $CD4^+$ T cells) provided sufficient conditions to elevate B cell stimulation to a threshold permissive for Breg (or EXPB10) generation: thus suppression of the on-going T cell response by NON-EXPB10 cells in the presence of high dose IL-2. It should be noted that pre-incubation of blocking mAbs to CD25 and CD122 with EXPB10 cells for the last 48 hours of the expansion protocol, did not affect suppressive function of EXPB10 cells (fig. 4.2.8d). IL-2 may have already promoted EXPB10 cell differentiation to a point where this cytokine is no longer required at high levels, or this finding may reflect CD25 expression as a phenotypic marker of EXPB10 cells, rather than as a functional component.

Tretter et al. found that large $CD25^+$ B cells were only potently suppressive when in the presence of moderate concentrations of IL-2 (>50U/ml) within the suppression assay. Given that EXPB10 cells were very strongly stimulated via CD154 and in the presence of a similarly high concentration of IL-2 (50U/ml) for a sustained 7-day period prior to co-culture with $CD4^+$ T cells, it may be that EXPB10 cells were more activated than the large $CD25^+$ B cells described by Tretter et al. and so were 'closer' to the activation threshold for regulation; thus EXPB10 cells did not require exogenous IL-2 during the suppression assay, whilst less activated $CD25^+$ B cells did require exogenous IL-2 [99].

A commonly cited mechanism of regulation by suppressive cell populations is that of metabolic disruption [183, 184]. One of the many processes by which $CD4^+CD25^+$ Treg are thought to control an immune response is by the deprivation of IL-2 [187]. However the addition of exogenous IL-2 even at high doses did not abrogate or reduce EXPB10 regulatory function (fig. 4.2.2d). The finding that EXPB10 1:1 cells were functionally stable in the presence of extremely high doses of IL-2, suggests that this Breg population was able to retain a functional identity even in microenvironments of severe inflammation (fig. 4.2.2d). It should be noted that suppressive potency of EXPB10 cells was not increased either; perhaps this reflects the role of IL-2 in the generation of EXPB10, rather than in a downstream suppressive mechanism. Furthermore, although NON-EXPB10 cells did acquire what appeared to

be regulatory function at high IL-2 concentrations, this did not correlate with increased rates of B cell proliferation (fig. 4.2.2e), suggesting that deprivation of growth factors was not the primary inhibitory mechanism in this *in vitro* system.

There are of course, other ways in which cell metabolism can be disrupted. EXPB10 cells maintained expression of CD39, an ectonucleoside triphosphate diphosphohydrolase-1, whilst down-regulating expression of CD73, an ecto-5′-nucleotidase. Both nTreg and iTreg have been reported to employ these enzymes to hydrolyse exogenous adenosine triphosphate (ATP) to adenosine 5′-monophosphate (AMP) and finally to adenosine (ADO), both of which are known to be immunosuppressive [206, 207]. With regards to EXPB10 cells, Saze et al. similarly found that co-culture of human $CD19^+$ B cells with CD40L and IL-4 for 5 days resulted in highly activated $CD39^+CD73^-$ B cells which inhibited the proliferation of autologous $CD4^+$ T cells [185]. Indeed, inhibition of endonucleatidase function did significantly reduce, but did not wholly abrogate suppression of $CD4^+$ T cell proliferation by EXPB10 cells, suggesting that accumulation of immunosuppressive cAMP may have been partly responsible for EXPB10-mediated regulation in this *in vitro* system (fig. 4.2.2a, ***$p<0.02$).

It is important to note that proliferation of $CD4^+$ T cells in the presence of such an inhibitor was also reduced relative to that of $CD4^+$ T cells in the absence of such inhibition (fig. 4.2.2b, ***$p<0.02$). It may be that the breakdown of ATP was critical to intrinsic $CD4^+$ T cell function in this context, rather than the actual suppressive function of EXPB10 cells. Alternatively, it may be that the inhibition of ATPase inhibited activation of the $CD4^+$ T cell in such a way that this T cell was then unable to stimulate suppressive mechanisms within the EXPB10 cell, and so the highly activated EXPB10 cell provided co-stimulatory support to the $CD4^+$ T cell rather than suppression. Therefore, the indicative role for endonucleatidases in the suppressive function of EXPB10 cells should be interpreted cautiously. It may be that the use of another ecto-ATPase inhibitor and blocking mAb to CD39 may help to clarify this result.

Much like analysis of NON-EXPB10 cells here (fig. 3.2.1c, 4.1.1c & 4.1.1d), Saze et al. confirmed that despite expressing functionally active CD39 and CD73,

unactivated, freshly isolated $CD19^+$ B cells could not regulate an immune response [185]. Mass spectrometry confirmed that activated $CD19^+$ B cells produced more AMP than unactivated $CD19^+$ B cells; the group speculated that the state of B cell activation is critical to regulatory function, and that only activated $CD19^+$ B cells were able to utilise endonucleotidases effectively to suppress an immune response. The authors hypothesised that the accumulation of cAMP, rather than ADO, resulted in $CD4^+$ T cell suppression. To establish whether the role of ectonucleotidases in EXPB10 suppressive function is a true finding, it would be useful to measure levels of AMP & ADO in this *in vitro* system, as well as the expression of ADO receptors on NON-EXPB10, EXPB10 and activated CD4+ T cells.

A well-characterised immunosuppressive mechanism disrupts metabolic processes through alterations in L-arginine catabolism and the production of reactive nitric species (fig. 4.2.2c) [208]. $CD25^+CD71^+CD73^-$ EXPB10 cells up-regulated intracellular expression of arginase II when compared to NON-EXPB10 cells (fig. 4.1.1d). At least two isoforms of mammalian arginase exists (types I and II) which differ in their tissue distribution, subcellular localisation, cross-reactivity and physiological function [209]. The type II isoform is located in the mitochondria and is generally expressed in extra-hepatic tissues such as the kidney [210]. Indeed, both acute myeloid leukaemic B cells and neonatal $CD71^+$ erythroid cells have been described to potently suppress immune responses through the secretion of arginase II [172]. However, despite both addition of exogenous L-arginine and the inhibition of human arginase II by use of the blocking reagent S-(2-boronoethyl)-L-cysteine (BEC), EXPB10-mediated inhibition of $CD4^+$ T cell proliferation was not abrogated *in vitro* (fig. 4.2.2c). It may be that although EXPB10 cells up-regulated translation of arginase II, this enzyme was not actively secreted by *ex vivo*-generated human Breg into the microenvironment.

Another regulatory candidate, iNOS, reported to be induced upon ligation of CD23 on malignant human B cells, as well as on endothelial cells and macrophages in the context of infection [186, 211, 212], did not appear to drive immunosuppression by EXPB10 cells; blockade of iNOS by 2-Amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine hydrochloride (AMT HCl) had no effect on suppressive potency by EXPB10 cells, >90% of which had up-regulated CD23 (fig. 4.1.1c & 4.2.2c). Disrupted metabolism

of L-arginine and the ornithine cycle therefore did not appear to play a principle role in EXPB10-mediated immunosuppression. Other immunoregulatory mechanisms which are able to disrupt cellular metabolism were not tested, such as reactive oxygen species (ROS) or indoleamine 2,3-dioxygenase (IDO) [213]; future experiments may warrant investigation of these more innate-associated processes.

Many regulatory cell populations are able to suppress an immune response through the triggering of apoptotic cell death [213, 214]. Indeed this is a common mechanism by which malignant B cells evade the immune response [215]. EXPB10 cells significantly up-regulated expression of FAS-L, and also demonstrated a trend towards increased expression of the PD ligands 1 and 2 (fig. 4.1.1d). In addition, only when T cells were activated in this suppression assay were they susceptible to EXPB10-mediated regulation (fig. 3.2.1g). Due to the known correlation between T-cell activation and susceptibility to apoptosis, it is possible that activation-induced cell death (AICD) played a role in this *in vitro* system. However staining for apoptosis and *in vitro* blockade of FAS-L or the PD-1 pathway suggests that these molecules were not involved in EXPB10-mediated regulation (fig 4.2.1a & 4.2.1b). Annexin V and 7-AAD staining of $CD4^+$ T cells after co-culture with anti CD3/CD28 beads and EXPB10 cells for 24, 48 or 72 hours revealed no increase in apoptosis or cell death when compared to $CD4^+$ T cells co-cultured with anti CD3/CD28 beads and NON-EXPB10 cells. Blockade of either FAS-L or PD-1 did not reduce or abrogate suppression.

Given that EXPB10 cells expressed high levels of both FAS-L and FAS-R (fig. 4.1.1a & 4.1.1d), it is surprising that a degree of fratricide was not seen [216] in either the expansion co-culture, or the suppression assay. One might also have expected that activated $CD4^+$ T cells would have induced apoptosis in EXPB10 cells, given that CD40L-activated B cells have been reported as highly sensitive to the killing action of Th1 cells [217]. It has been reported that incubation of CD40L-activated B cells with IL-4-producing Th2 cells induced B cell growth and differentiation instead of inducing B cell apoptosis [218]. The presence of IL-4, either exogenous or secreted by Th2 cells, may in fact have protected CD40L-activated B cells that express Fas-R, from apoptosis [147]. The addition of exogenous IL-4 to the expansion co-cultures, the secretion of IL-4 by EXPB10 cells (fig. 4.1.3a), together with possible IL-4

production by polyclonally stimulated $CD4^+$ T cells in the suppression assay, may have conferred this protection to EXPB10 cell survival. The caveat remains, as with other mechanistic inhibitors described in this chapter, that there was no positive control available, i.e. FAS-L or PD-L expressing cell lines, which could be used to verify the blockade achieved by the antagonistic mAbs used.

Cognate interactions have been widely reported to be crucial to the suppressive function of both mouse and human Breg [20, 21, 47, 98-100, 219], both through the combined action of CD80 and CD86 [20, 47], as well as through the CD40-CD154 interaction [24, 47, 69, 98]. Indeed, transwell experiments confirmed that EXPB10 cells do require a cognate interaction with $CD4^+$ T cells (fig. 4.2.7a). It has been postulated that the interaction with CD80/CD86 expressed on Breg, with the inhibitory receptor CTLA-4 expressed on activated $CD4^+$ T cells, can trigger a regulatory cascade within the T cell, resulting in suppression [47]. However, although blockade of CD80 and CD86 has been demonstrated to slightly reduce $CD24^{hi}CD38^{hi}$ Breg-mediated suppression of pro-inflammatory cytokine production by $CD4^+$ T cells [47], those findings could not be replicated with EXPB10 cells (fig. 4.2.3a & 4.2.3b); blockade of CD80 and/or CD86 at both 10ug/ml and 50ug/ml could not reduce suppression of pro-inflammatory cytokine production or proliferation of $CD4^+$ T cells. This may have been due to inefficacy of the blocking antibodies (again there was no positive control to confirm the action of antagonist antibodies to CD80 or CD86); to the lack of relevance of this particular mechanism within EXPB10 cells; or to the overriding action of anti-CD28 beads forming a complex with CD28 on $CD4^+$ T cells during polyclonal activation within the *in vitro* suppression assay, thus preventing CD80 and CD86 from initiating complex-formation with CTLA-4. These experiments do not therefore exclude the possibility that EXPB10 cells can harness CTLA-4 in suppression of an immune response, but the findings do suggest that this pathway is not essential for EXPB10-mediated suppression of $CD4^+$ T cells in this *in vitro* system. An MLR may help clarify this issue.

Blockade of the CD154-CD40 interaction, did however significantly reduce EXPB10-mediated suppression of $CD4^+$ T cell proliferation (fig. 4.2.4a). This finding supports other human and mouse studies, which have identified a role for the CD154-CD40 interaction in both the generation and function of Breg populations [47, 69, 98].

However it is worth noting that suppression of $CD4^+$ T cell proliferation, even with combined blockade to both CD40 and CD154, was not completely abrogated; additional mechanisms may have been at work. It may be that insufficient blockade of the CD154-CD40 interaction was occurring; however this is unlikely given that even at mAb concentrations as high as 50ug/ml, no further reduction in suppression was seen (*data not shown*). Greater reduction in suppression was seen after CD154 blockade than with CD40 blockade. This is reflected in the moderate reduction in total number of CD19+ B cells remaining at day 5 when CD154 is blocked (fig. 4.2.4b), suggesting that survival capacity of EXPB10 cells was diminished in the presence of the CD154 mAb. It may be that this particular CD154 mAb was much more effective at blocking the CD154-CD40 interaction; it may be that this particular CD154 mAb had an adverse effect on EXPB10 cell survival by virtue of artefact, but this is unlikely given that this antibody had no affect on NON-EXPB10 cell number (*data not shown*); it may be that this mAb has depleted $CD154^+$ EXPB10 cells. What is apparent from these data is that blockade of the CD154-CD40 interaction significantly reduced suppression possibly due to inhibition of a downstream suppressive mechanism, as well as through the lack of T cell help and reduced EXPB10 cell survival.

Given that CD154 was up-regulated on activated $CD4^+$ T cells (fig. 4.2.4d), these observations also suggest that EXPB10 cells required ongoing signals from activated $CD4^+$ T cells in order to suppress the proliferation of these same T cells. This could potentially explain why inhibition of proliferation was seen when $CD4^+$ T cells were polyclonally stimulated in the presence of EXPB10 cells, whereas $CD4^+$ T cell proliferation was mildly increased when not stimulated by anti-CD3/CD28 beads in the presence of EXPB10 cells (fig. 3.2.1g). Proliferation of $CD4^+$ T cells may have been seen under these conditions due to high expression of co-stimulatory molecules such as CD80 and CD86 on EXPB10 cells, which in turn were unable to exert suppressive mechanisms due to the lack of CD154 expression on unstimulated $CD4^+$ T cells. Indeed, EXPB10 cells appeared to promote $CD4^+$ T cell proliferation during the first 48 hours of co-culture with anti-CD3/CD28 beads (fig. 4.2.4d). Suppression of proliferation occurred by day 3 and then gradually increased in potency until day 5. CD154 expression by activated $CD4^+$ T cells under these conditions peaked at 48 hours and then plateaued for the remainder of the co-culture. It may that EXPB10

cells required an optimal level of engagement by CD154 i.e. at 48 hours in this *in vitro* system, before being able to activate downstream suppressive signals. The continued exposure to high intensity CD154 signals may have thus maintained the suppressive process until the day 5 endpoint of the assay.

To investigate the dynamics of suppression of $CD4^+$ T cells suppression within the 5-day co-culture, blocking CD154 mAb was added at either day 1, 2, 3 or 4 of the co-culture (fig. 4.2.4e). Blocking the CD154-CD40 interaction on any of the first 4 days of the 5-day suppression assay resulted in potent abrogation of EXPB10 cell-mediated suppression. This would support the view that such suppression was a continuous process, whereby $CD4^+$ T cell proliferation - in other words $CD4^+$ T cell division - was constantly being held back in the presence of EXPB10 cells. Indeed, suppression of $CD4^+$ T cell proliferation could be significantly reduced by CD154 blockade as late as day 4 of the assay (fig. 4.2.4e), even though significant suppression had already occurred by this point (fig. 4.2.4d). These findings suggest that EXPB10-mediated suppression was not only preventable, but may also have been reversible in the *in vitro* setting.

It is not clear why $CD4^+$ T cells expressed lower levels of CD154 when activated in the presence of EXPB10 cells (fig. 4.2.4d). This may be a reflection of reduced T cell activation mediated by the suppressive EXPB10 cells. The apparent dependency on CD154 expression by $CD4^+$ T cells for EXPB10 cell suppressive function may offer an insight into an antigen-specific mode of action. In the *in vivo* setting, only allospecific $CD4^+$ T cells should be activated in response to allograft, and thus only allospecific $CD19^+$ B cells will be able to interact with the responding T cell population and receive the prolonged CD154-CD40 engagement required to recruit suppressive function. However, the promotion of $CD4^+$ T cell proliferation at early stages of the suppression assay, and potential reversibility of suppression when CD154 is blocked, does raise questions about the stability of suppressive function within this Breg population. Furthermore, increased CD154 expression by $CD4^+$ T cells and prolonged $CD19^+$ B cell activation have been reported in patients suffering from autoimmune diseases such as SLE and RA [220, 221]; it is this state of hyperactivation that is believed to promote humoral responses which subsequently

drive pathology. However, it is also thought that IL-10$^+$ Breg are defective in these patients due to being refractory to T cell help [20].

It will be important to clarify how these B cells behave in the absence of CD4$^+$ T cell stimulation *in vivo*, as well as the nature of their function once the inflammatory stimulus has subsided. In addition, naïve and memory T cells differentially respond to activation [222]. Following antigenic challenge, both T-lymphocyte subpopulations express high levels of CD154 within hours, but only memory T lymphocytes have preformed CD154 molecules, which can be targeted to the cell membrane within 5–15 min after cellular activation [223, 224]. It will be interesting to see whether EXPB10-mediated suppression of naïve vs. memory CD4$^+$ T cells is affected by this differential expression.

It was demonstrated earlier in this chapter that less suppressive and less activated EXPB10 1:10 cells down-regulated phenotypic markers CD71 when co-cultured with CD4$^+$ T cells during the suppression assay (fig. 4.1.2b). The same phenomenon was seen when the CD154-CD40 interaction was blocked (fig. 4.2.4c): CD71 expression was significantly down-regulated by EXPB10, and EXPB10-mediated suppression was almost completely abrogated. The changes in expression of B cell activation markers and potential EXPB10 phenotypic markers supports the theory that suppression by EXPB10 cells requires on-going signaling from activated CD4$^+$ T cells. CD154 could be considered as 'signal 1.'

CD154 expression was up-regulated on EXPB10 cells, and levels correlated with suppressive potency of EXPB10 1:1 cells vs. EXPB10 1:10 cells (fig. 4.2.5a). Sorting of both CD154$^+$ and CD154$^-$ Breg from whole EXPB10 1:1 cells, further reinforced that high levels of CD154 expression are associated with suppressive potency (fig. 4.2.5c). Trogocytosis assays confirmed that EXPB10 cells had not gained CD154 through membrane transfer and had instead intrinsically up-regulated CD154 expression through activation (fig. 4.2.5e). It is possible that blockade of CD154 or CD40 by antagonistic antibodies was actually impeding the action of CD154$^+$ EXPB10 cells in the suppression assay. CD154 in this context could be a marker of B cell activation, a marker of suppression or a functional molecule auto-stimulating EXPB10 cells by binding to CD40 also expressed on EXPB10 cells and thereby

triggering downstream suppressive mechanisms. Indeed, this mechanism of auto-stimulation has been reported in $CD40^+$ T cells in a model of autoimmunity [225]. A series of elegant experiments using CD40 agonistic mAb have also demonstrated that B cell-expressed CD40 ligand play an important role in facilitating responses of activated B cells [226].

Although it has been long established that CD40 on $CD19^+$ B cells is ligated by CD154 expressed on activated $CD4^+$ T cells in order to engage T cell help [227], $CD154^+$ B cells have been reported to be increased in children suffering acute rejection following intestinal transplantation as well as in patients suffering from SLE (although these patients did not have active disease) [228, 229]. Furthermore, IL-10 produced by human B cells both when resting and after stimulation with Cowan I Strain *Staphylococcus* (CSS), was confined solely to $CD154^+$ B cells [229]. It has been postulated that CD154 expression on B cells is associated with autoantibody production and represents a hyperactive cellular state, thus the link with allograft rejection and patients with SLE. However in light of the recently identified Breg population, it is also possible that $CD154^+$IL-10^+ B cells represent a regulatory subset in both cohorts of patients. It may be that $CD154^+$ EXPB10 cells produce more IL-10 than $CD154^-$ EXPB10 cells.

The down-regulation of both TIM-1 and immunomodulatory molecule B7-H4 upon CD154 blockade at any time-point suggests a potential role in suppression of CD4+ T cells in this system (fig. 4.2.4f). B7-H4, a member of the B7 family (which also includes CD80 and CD86), was able to inhibit $CD4^+$ and $CD8^+$ T-cell proliferation, cytokine production, as well as the generation of alloreactive cytotoxic lymphocytes when in the form of plate-bound B7-H4 immunoglobulin, by arresting the cell cycle at the G_0-G_1 stage [230-233]. Human studies have identified that antigen presenting cells such as monocytes, monocyte-derived dendritic cells (MDDCs) and myeloid dendritic cells (MDCs) were able up-regulate both B7-H4 mRNA and protein surface expression partly in response to autocrine IL-10 [234, 235]; these APCs subsequently suppressed T effector cell proliferation *in vitro*. The expression of IL-10 by APCs was initially stimulated through co-culture with Treg. Both IL-10 and IL-6 have been found at high concentrations within the tumour microenvironment and have also been found to induce B7-H4 in tumour associated macrophages (TAMs) [236]. Given that

EXPB10 cells were exposed to exogenous IL-10 added during the expansion protocol (fig. 3.1.1a), and that EXPB10 cells themselves secreted IL-10 and IL-6 upon CD40 ligation (fig. 4.1.3a), it is possible that EXPB10 cells have up-regulated B7-H4 expression in response to IL-10 and IL-6 present in the microenvironment, and thus suppress $CD4^+$ T cell responses through a cognate interaction.

mRNA expression of B7-H4 is widely distributed in both mouse and human peripheral tissues, but protein expression appears to be limited to malignancies such as ovarian, lung, uterine, breast and renal cancer tissue and has been associated with a poor prognosis [237-241]. In addition to tumour cells and TAMs, endothelial cells of small blood vessels in the cancer microenvironment have also been found to constitutively express B7-H4 and may contribute to tumour progression [242]. Blockade of B7-H4 translation using blocking anti-sense oligonucleotides resulted in significant abrogation of immunoregulation, both *in vitro* and in a humanised mouse model of ovarian cancer [236]. B7-H4 knockout mice demonstrate variable augmentation of Th1 responses in models of infection and cancer depending on the mouse background [243]. This inconsistency in effect has been suggested to represent a 'fine-tuning' role for B7-H4 in controlling the adaptive immune response. Unfortunately, blocking mAbs to human B7-H4 are not commercially available to readily test whether EXPB10 cells employ this mechanism of immunosuppression. Sorting $B7\text{-}H4^+$ cells was attempted but a consistent, accurate population could not be isolated due to the rapidly shuttling nature of the protein from membrane to cytoplasm (*data not shown*) [244, 245]. It would be valuable in future experiments to attempt translational blocking of B7-H4 through the use of anti-sense oligonucleotides previously designed by Kryczek et al. [236], to further explore EXPB10 cell-mediated suppression of $CD4^+$ T cells.

Whilst a cognate interaction through the CD154-CD40 interaction has been established as necessary to achieve EXPB10-mediated suppression, this by no means excludes the need for further soluble reagents. Recent work identifying IL-35 as an immunosuppressive cytokine produced by Breg documented the secretion of IL-35 by regulatory plasma cells [43]. Analysis of EXPB10 supernatants using ELISA excluded the presence of IL-35 (fig. 4.1.3a), in keeping with a naïve, $CD19^+$ EXPB10 phenotype and the lack of CD138 expression (fig 4.1.1b). However, EXPB10 cells

also significantly up-regulated LAP, the membrane precursor to TGFβ (fig 4.1.1d). LAP is a pro-peptide that is non-covalently associated to the amino terminal domain of TGF-β, forming a latent TGF-β complex. Serum proteinases catalyse the proteolytic cleavage of the complex, resulting in the release of active TGFβ which can then regulate immune responses through initiation of apoptosis, cell cycle arrest or induction of Treg [246]. Furthermore, LAP^+ Breg have been reported to regulate the immune system through the action of TGFβ [39, 247]. However, blockade of TGFβ using SB 431542 did not abrogate or reduce suppression of $CD4^+$ T cell responses by EXPB10 cells (fig.4.2.6a & 4.2.6b). Similar to the blockade of CD80 and CD86, these experiments do not exclude the potential action of TGFβ in an *in vivo* setting: firstly there was no positive control to confirm adequate blockade using SB 431542; secondly, proteases capable of cleaving LAP and which may be present in cellular matrices of solid tissue, may not be present in these *in vitro* cultures. Therefore whilst TGFβ does not appear to be necessary for *in vitro* suppression by EXPB10 cells, its *in vivo* action is not necessarily precluded.

IL-10 is the immunosuppressive cytokine by which the majority of endogenous natural mouse and human Breg reported in the literature [20, 48, 69, 77] as well as *ex vivo*-generated EXPB10 cells were originally defined. Blockade of both IL-10 and IL-10R (IL-10/R) was able to partially restore pro-inflammatory cytokine production by $CD4^+$ T cells (fig. 4.2.6c), although suppression of $CD4^+$ T cell proliferation was only slightly reduced even when high concentrations of IL-10/R mAbs were used (fig. 4.2.6d). Thus IL-10 appeared to regulate Th1 differentiation but did not appear to be primarily responsible for EXPB10-mediated suppression of proliferation of $CD4^+$ T cells. The combined blockade of CD154, CD40, IL-10 and IL-10R, did not increase the reduction of suppression seen when CD154 and CD40 were blocked (fig. 4.2.6d); thus supporting previous evidence that IL-10 secretion by EXPB10 cells is a consequence of CD154 stimulation, and acts along the same suppressive pathway as CD154 in this system. These findings correspond to other human studies of Breg that have also identified a clear role for CD154-stimulated, IL-10-dependency of Breg-mediated suppression of pro-inflammatory cytokine production, but not of $CD4^+$ T cell proliferation [20, 47, 169].

Use of a transwell system enabled a more detailed study of mechanisms of suppressive function as mediated by EXPB10 cells. Separation of EXPB10 cells from stimulated $CD4^+$ T cells resulted in complete abrogation of suppression (fig. 4.2.7a), confirming that cognate interactions between these two cell populations were essential to suppressive function. The addition of $CD4^+$ T cells and anti-CD3/CD28 beads to EXPB10 cells within the insert demonstrated that although cognate interactions were required, a soluble suppressive factor appeared to be produced upon this interaction that in turn partially mediated suppression of $CD4^+$ T cell proliferation (fig. 4.2.7b).

Given that earlier experiments have demonstrated a requirement for CD154-CD40 engagement, as well as the finding that this engagement resulted in the release of IL-10 which also partially mediated suppression, it would be logical to assume that the cognate interaction and subsequent release of a suppressive soluble factor, are both CD154-CD40 engagement and IL-10 respectively. However, in an unexpected twist, addition of blocking CD154 mAb to the insert along with $CD4^+$ T cells, anti-CD3/CD28 beads and EXPB10 cells, did not abrogate or indeed affect at all the suppression of $CD4^+$ T cells in the receiver well (fig. 4.2.4c). Instead, only suppression of $CD4^+$ T cells in the insert was reduced, and that too only to the level of suppression as exerted on the $CD4^+$ T cells in the receiver well. These findings illustrate that at least two different suppressive pathways have taken place in this *in vitro* culture, both of which were dependent on cognate interactions. Only one of these pathways was dependent on CD154-CD40 engagement and presumably therefore, IL-10. The second, hitherto unidentified pathway, involved both a cognate interaction other than CD154-CD40 engagement, which then stimulated the production of an unknown suppressive soluble factor. This soluble factor is unlikely to be IL-10, as combined blockade of CD154-CD40 and IL-10 did not fully abrogate $CD4^+$ T cell response, nor did this blockade increase suppression compared to blockade of the CD154-CD40 interaction alone (fig. 4.2.6d). However, an additional experiment looking at joint blockade of IL-10 and CD154 is required before one can reach any definitive conclusion.

It is not clear what the identity of either this additional cognate interaction or soluble suppressive factor could be from these experiments. In addition to a physical barrier,

the transwell insert also provides a physical distance between cell populations that is not physiological. It may be that the perceived cognate interaction in this second pathway does not represent an actual physical engagement between EXPB10 cells and $CD4^+$ T cells, but instead a reaction or process which requires close proximity of cells to each other. The generation of cAMP as catalysed by CD39 expressed on EXPB10 cells could be a possible candidate for this reaction, given that earlier data has demonstrated a potential role for endonucleotidase action in EXPB10 suppressive function (4.2.2a). ATP may be generated by activated $CD4^+$ T cells to provide substrate for this reaction. To establish whether this is a valid possibility, it would be first prudent to block both endonucleotidase activity and CD154-CD40 interactions in the transwell system. One would expect suppression of both $CD4^+$ T cells in the insert and those in the receiver well to be completely abrogated. There are reports that elevated cAMP levels may also result in up-regulation of CD154 expression and prolonged CD154-CD40 interactions [248, 249]; if cAMP metabolism is important in EXPB10-mediated suppression, the pathway may not therefore be necessarily CD154-independent.

Given that both the definition and function of EXPB10 cells do require B cell-derived IL-10, isolation of the IL-10^+ subset within the EXPB10 cell population would ideally enable further study of what is assumed to be the Breg cell type (only about 30% of EXPB10 cells express IL-10 after PMA and ionomycin stimulation, as demonstrated in fig. 3.1.1f). An IL-10 capture kit supplied by Miltenyi, which was used by Van de Veen et al. [53] to identify the previously described IL-10-enriched $CD25^+CD71^+CD73^-$ Breg population, employs the use of an IL-10 'capture' antibody which is able to bind to IL-10 as it is secreted from the target cell. The use of microbeads and magnetic columns enable subsequent isolation of the bead-bound IL-10^+ cells. Unfortunately, despite trying multiple permutations within the kit protocol, I was unable to successfully isolate a pure population of IL-10^+ EXPB10 cells, although a very slight enrichment was achieved (fig. 4.2.6e). Furthermore, suppression assays using this very mildly IL-10-enriched population of EXPB10 cells also failed, largely attributable to large-scale death of EXPB10 cells by day 5 of the suppression assay (>95% death, *data not shown*).

The unsuccessful use of this assay may be due to technical failure as the protocol involves multiple, complicated steps. In addition, it may have been prudent to stimulate the EXPB10 cells with PMA and ionomycin prior to subjecting the cells to the IL-10 capture kit. This additional stimulation step had been avoided, so to enable capture of the actual EXPB10 population that was secreting IL-10 when added to suppression co-cultures. However in hindsight, stimulation with PMA and ionomycin would have boosted levels of IL-10 secretion by EXPB10 cells (fig. 3.1.1f), and thus increased the likelihood of IL-10 detection and cell capture. Death during the suppression assay of those EXPB10 cells and NON-EXPB10 cells isolated using magnetic columns and microbeads, may have occurred due to further stimulation triggered by the microbeads themselves.

It remains unclear from the literature as to whether IL-10 secreted by Breg mediated a direct immunoregulatory effect on the target effector cells, or whether IL-10 effected an autocrine action on the Breg subset, perpetuating a downstream suppressive mechanism. Seminal experiments using IL-$10^{-/-}$ mice demonstrated the need for both CD40 stimulation and IL-10 secretion by B cells for successful Breg function [24] and this has since been confirmed in other models [78, 98]. IL-10 has been widely documented to directly inhibit antigen presentation [250], pro-inflammatory cytokine secretion by APCs [251, 252], Th1 and/or Th17 responses [94, 95], and to promote differentiation of Tr1 cells and $Foxp3^{+}$ Treg cells [253, 254]. However an autocrine role for B cell-derived IL-10 has also been identified which has been associated with IgG and IgM secretion [255] as well as the expansion and function of mouse $CD40^{hi}CD5^{+}$ Breg [118]. B cell-derived IL-10 is also known to prevent apoptosis of B cells, to enhance B cell proliferation and differentiation, MHC II expression as well as to promote B cell survival [116, 117, 256, 257].

Given that EXPB10 cells also expressed increased levels of IL-10R compared to NON-EXPB10 cells (fig. 4.2.6f, *$p<0.0001$), we asked whether IL-10 had a paracrine or autocrine effect in this system (fig. 4.2.6g). EXPB10 cells which were generated in the absence of exogenous IL-10 (condition i) EXPB10 w/o hL-10) showed no difference in suppressive potency when compared to EXPB10 which had been exposed to exogenous IL-10 as per the standard protocol optimised in *Chapter 3.0.* However EXPB10 cells which had been generated in the absence of exogenous IL-10

and which had also been blocked from endogenous IL-10 exposure for the last 48 hours of culture (condition ii) PRE-INC IL-10/R EXPB10), had significantly reduced suppressive potency when compared to the first two conditions in which EXPB10 cells had access to endogenous IL-10 (fig. 4.2.6i, ¥p<0.007). Furthermore, when IL-10 and IL-10R were both blocked by the addition of antagonistic mAbs to the suppression assay co-cultures of EXPB10 cells with polyclonally stimulated $CD4^+$ T cells, no further reduction in suppressive potency was observed in EXPB10 cells generated under any condition (fig. 4.2.6i). These findings suggest that firstly IL-10 secreted by EXPB10 cells exerted an important autocrine effect on EXPB10 suppressive function and that secondly, this effect appeared to be more critical to EXPB10 suppressive function than a potentially paracrine, suppressive effect of EXPB10-secreted IL-10 directed at $CD4^+$ T cells in this *in vitro* system.

Phenotypic analysis revealed that PRE-INC IL-10/R EXPB10 expressed slightly lower levels of both CD71 and CD25, but otherwise appear to be unchanged when compared to EXPB10 cells generated under standard conditions or without exogenous IL-10 (fig. 4.2.6h). Despite the importance of the CD154-CD40 interaction to EXPB10 suppressive function, there was no difference in expression of either of these molecules across any group. It was the exposure of EXPB10 cells to IL-10 during the Breg generation phase that was essential for optimal EXPB10 suppressive function. Up-regulation of IL-10R and the secretion of IL-10 could be termed 'signal 2.'

One could argue that given IL-10 is an important factor in proliferation and differentiation of $CD19^+$ B cells, that this accounts for the functional change seen in EXPB10 cells when underexposed to IL-10: a simple case of growth factor deprivation. However, there was no difference in expansion factor between any group (*data not shown*), and furthermore there appeared to be no reduction in B cell survival capacity when EXPB10 were generated under condition ii), as there was no difference in the number of $CD19^+$ B cells at day 5 of the suppression assay between any group (fig 4.2.6j). These observations would suggest that intrinsic, baseline cell function was not compromised when EXPB10 cells were underexposed to IL-10 for a short period of time.

IL-10 is a known positive and negative regulator of its own secretion, depending on the different transcription factors targeted [258]; it is possible that EXPB10 cells which were underexposed to endogenous IL-10 were less suppressive because these cells were producing lower levels of IL-10 in response to the lack of IL-10 in the microenvironment. However, analysis of supernatants from EXPB10 cells pre-incubated with either an isotype control or with blocking mAb to IL-10 and IL-10R (which had subsequently been incubated in media at 37°C for 6 hours), demonstrated that there was no difference in levels of IL-10 production between these EXPB10 cell populations (fig 4.2.6k). The underexposure of EXPB10 cells to endogenous IL-10 did not result in decreased IL-10 production by this Breg subset, but did result in reduced suppressive potency (fig. 4.2.6i, 4.2.8d), thus indicating that IL-10 expressed by EXPB10 cells has a predominantly autocrine role in the suppressive mechanism of this regulatory cell subset. In order to conclusively demonstrate autocrine action of IL-10 on EXPB10 cells, it would be ideal to co-culture EXPB10 cells in a suppression assay with IL-10R$^{-/-}$ CD4^{+} T cells. One would expect suppressive function of EXPB10 cells to be minimally effected.

To summarise the findings of these mechanistic experiments so far: it appears that cognate interactions between CD40 expressed on EXPB10 cells and CD154 expressed on activated CD4^{+} T cells were essential to EXPB10-mediated suppression and to the continued high level expression of CD71 by EXPB10 cells. CD154-mediated stimulation of EXPB10 cells resulted in IL-10 secretion by EXPB10 cells, and this B cell-derived IL-10 then acted in an autocrine manner to propagate the suppressive function of EXPB10 cells. Transwell experiments suggested that there was an additional mechanism of suppression at work that was CD154-independent, but required cognate or close interactions of EXPB10 cells with CD4^{+} T cells, as well as an unidentified soluble factor. Endonucleatidase action and accumulation of cAMP may form part of this mechanism.

To investigate what was occurring downstream of IL-10R ligation within the EXPB10 cells, the STAT3 pathway was utilised as a read-out of the cellular response to IL-10 [20, 258]. Importantly, this signaling pathway has been previously shown to be critical in the regulatory mechanisms of human IL-10^{+} Breg in the context of transplantation, GVHD as well as autoimmune disease [20, 52, 63]. Given that CD40

ligation also employs the STAT3 signaling pathway [259], it was necessary to rest EXPB10 cells for 1.5 hours before testing cellular responses to IL-10 (fig. 4.2.8a). As expected, EXPB10 cells were able to respond to exogenous IL-10, but this response was effectively abolished when EXPB10 cells had been pre-incubated with a blocking mAb to TIM-1 for the last 48 hours of expansion (PRE-INC TIM-1 EXPB10). Pre-incubation with IL-10 and IL-10R blocking mAbs also reduced the cellular response to IL-10, but to a lesser extent than for PRE-INC TIM-1 EXPB10 cells (fig. 4.2.8b). The finding that EXPB10 cells pre-incubated with an isotype control antibody or blocking mAbs to the high affinity IL-2R or to CD154, were able to respond adequately to exogenous IL-10, indicated that these observations were not attributable to incidental effects of antibody binding. Furthermore, the correlation of reduced suppressive potency of PRE-INC TIM-1 EXPB10 cells and PRE-INC IL-10/R EXPB10 cells with the inability to respond to IL-10 supported both the autocrine role of IL-10 in the suppressive mechanism of EXPB10 cells, and the functional significance of TIM-1 expression in EXPB10-mediated regulation (fig. 4.2.8b & 4.2.8d).

Given that PRE-INC TIM-1 EXPB10 cells expressed similar levels of IL-10R as compared to EXPB10 cells generated by the standard protocol (fig. 4.2.8c), it is improbable that PRE-INC TIM-1 EXPB10 cells were unable to respond to IL-10 due to lack of receptor expression. Similar observations regarding expression of CD40 and CD154 also suggest that this interaction was not impeded and so could not account for the reduced potency of suppression (fig. 4.2.8c). PRE-INC TIM-1 EXPB10, together with PRE-INC CD154 EXPB10, both demonstrated significant down-regulation of the transferrin receptor and activation marker, CD71. PRE-INC IL-10/R EXPB10 cells demonstrated significant down-regulation in expression of CD25 (fig. 4.2.6h & 4.2.8c). Ding et al. have illustrated that mouse TIM-1^+ Breg need to be activated *in vivo*, through immunisation either by OVA or allograft, in order to suppress an immune response and induce tolerance [35]. Our findings here have demonstrated that activation status of the EXPB10 cell is important for suppressive potency of this *ex vivo*-generated human TIM-1^+ Breg population and that TIM-1 is important in modulating EXPB10 cell responses to IL-10 as well as in EXPB10 suppressive potency (fig. 4.2.8d). The up-regulation and action of TIM-1 could be considered as 'signal 3.'

It may be that TIM-1, having been up-regulated on EXPB10 cells and following cellular activation, was able to induce a conformational change in IL-10R; this conformational change in turn could have resulted in a higher affinity IL-10R for IL-10. Alternatively, TIM-1 could have manipulated downstream intracellular pathways to enhance the response to IL-10 when this cytokine bound to its receptor. Furthermore, the down-regulation of CD71 expression upon blockade of either CD154 or TIM-1 indicates that TIM-1 may have also been important in maintaining high levels of activation within the cell; high levels of activation in turn correlated with suppressive potency (fig. 4.1.2b, 4.2.4c, 4.2.6h, 4.2.8c, 4.2.8d). Given that blockade of IL-10R on EXPB10 cells resulted in only a slight down-regulation of CD71, it is possible that this particular function of TIM-1 was IL-10-independent. One must however interpret such results with caution: the function of TIM-1 may not be restricted to Breg-mediated suppression, but may actually be more widespread, affecting multiple cell functions. In addition, it may be that the particular TIM-1 mAb used is simply stereotactically blocking the IL-10R from binding to IL-10. Gene knockouts of TIM-1 in human EXPB10 cells as well as affinity binding studies would be essential in future experiments to investigate this further.

Different forms of CD40 activation result in different downstream signaling pathways, in particular those involving multiple TNF receptor associated factors (TRAF) and phosphoinositide 3-kinases (PI3K) [127]. TIM-1 also requires activation of PI3K in order to function in the context of T cell activation [260]. It may be that in Breg, this particular downstream signaling pathway, when triggered by the form of CD154 expressed on CHO cells, enables phosphorylation of the cytoplasmic domain of TIM-1, and thus results in increased cellular ability of Breg to respond to IL-10 and subsequent triggering of a currently unidentified suppressive mechanism. The functional epitope of CD154 expressed on CHO cells should be characterised, as well as signaling pathways (other than STAT3) that are activated upon CD40 ligation of EXPB10 cells, to help ascertain the effect of this stimulation on TIM-1 expression and function.

Using both a *TIM-1*$^{-/-}$ mouse as well as a *TIM-1*$^{\Delta mucin}$ mouse model in which the mucin domain of the TIM-1 molecule is deleted, Kuchroo's group has demonstrated that

TIM-1 expression and signaling is essential for maintenance and promotion of IL-10 production in mouse B cells [83-85]. Deletion of TIM-1 specific to B cells resulted in progressive loss of IL-10 production by B cells with age, and this was associated with severe multi-organ inflammation. IL-10 production by B cells after stimulation by anti CD40 mAb, IgM crosslinking or IL-21 treatment, was significantly reduced in both *TIM-1*$^{-/-}$ and *TIM-1*$^{\Delta mucin}$ B cells. In addition, *TIM-1*$^{-/-}$ and *TIM-1*$^{\Delta mucin}$ B cells not only expressed lower levels of IL-10, but they also expressed higher levels of pro-inflammatory cytokines including IL-6, IL-12 and IL-1b [84]. It would be interesting to examine whether pre-incubation of EXPB10 cells with blocking mAbs to TIM-1 would result in changes in cytokine expression of this human Breg population.

A potential alteration in cytokine profile in the presence of TIM-1 blockade, could suggest that TIM-1 plays a part in directing differentiation of a potential Breg towards either a regulatory or pro-inflammatory phenotype. Studies examining the effects of high affinity vs. low affinity agonistic TIM-1 antibody have demonstrated a dual action of TIM-1. Ligation with a high-affinity mAb, 3B3, promoted expansion of antigen-specific T cells expressing Th1 and Th17 cytokines while inhibiting Treg [261-263]. Concordantly, 3B3 treatment exacerbated EAE [262] and prevented allograft tolerance mediated by anti-CD154 [263]. In contrast, a lower-affinity anti–TIM-1 mAb, RMT1-10, inhibited EAE [262] and, when combined with rapamycin, induced long-term allograft acceptance in mice [264]. Prolonged engraftment by RMT1-10 was dependent on Th2-cytokine skewing, Breg induction and Treg activity. The identity of the ligand for TIM-1 in these *in vitro* EXPB10 co-cultures was not explored, although both TIM-4 and apoptotic cells have been reported to ligate TIM-1 in the literature [35, 265]. TIM-1 may act as a functional gateway for potential human Breg, modulating the balance between pro-inflammatory and regulatory differentiation through cellular activation and responses to IL-10. The potential involvement of Treg in EXPB10-mediated suppression is further explored in *Chapter 5.0*.

SUMMARY

Looking collectively at these data, the first steps of a potential mechanism by which EXPB10 cells regulate $CD4^+$ T cells are beginning to emerge (fig 4.2.9). Results described above and in *Chapter 3.0* have demonstrated that through stimulation by CD154 expressed on CHO cells, $CD19^+$ B cells and subsequently EXPB10 cells, expressed IL-10 as well as up-regulated expression of IL-10R, CD71, CD25, TIM-1 and CD154. EXPB10 cells were also capable of significantly suppressing an autologous, polyclonally-driven $CD4^+$ T cell response. This suppression appeared to be dependent on the CD40-CD154 interaction and ensuing IL-10 secretion. Endogenous IL-10 secreted by EXPB10 cells exerted an autocrine effect on EXPB10 cells, and this appeared to be associated with suppressive function. TIM-1 was up-regulated on EXPB10 cells and modulated the response of EXPB10 cells to IL-10. Furthermore, functional TIM-1 appeared to be necessary for optimal suppression of $CD4^+$ T cells by EXPB10 cells, possibly by modulating the Breg response to autocrine IL-10. Furthermore, CD154 expressed on EXPB10 cells may have also served to auto-stimulate EXPB10 cells by directly engaging with CD40, to maintain activation status and propagate the pSTAT3-signaling pathway. It may be that these responses of EXPB10 cells to CD154-driven cellular activation and subsequent IL-10 secretion, resulted in an up-regulation of B7-H4 that could represent the downstream mechanism by which EXPB10 cells suppress $CD4^+$ T cells (fig. 4.2.9a).

Purinergic signaling may also play a role in EXPB10 cell-mediated suppression of $CD4^+$ T cells (fig. 4.2.9b). Engagement of CD154 and CD40 resulted in down-regulation of CD73 whilst expression of CD39 was maintained. The loss of CD73 in the face of persistent CD39 expression may have led to the accumulation of cAMP in the cellular microenvironment as a direct result of the extracellular breakdown of ATP. cAMP has been shown in other models to both up-regulate CD154 expression and prolong the CD154-CD40 interaction and so may propagate the CD154-TIM-1-IL10-pSTAT3 pathway described above to result in eventual suppression of $CD4^+$ T cells. Alternatively, cAMP may act directly within the $CD4^+$ T cell to inhibit proliferative responses. This may account for the CD154-independent, mechanistic

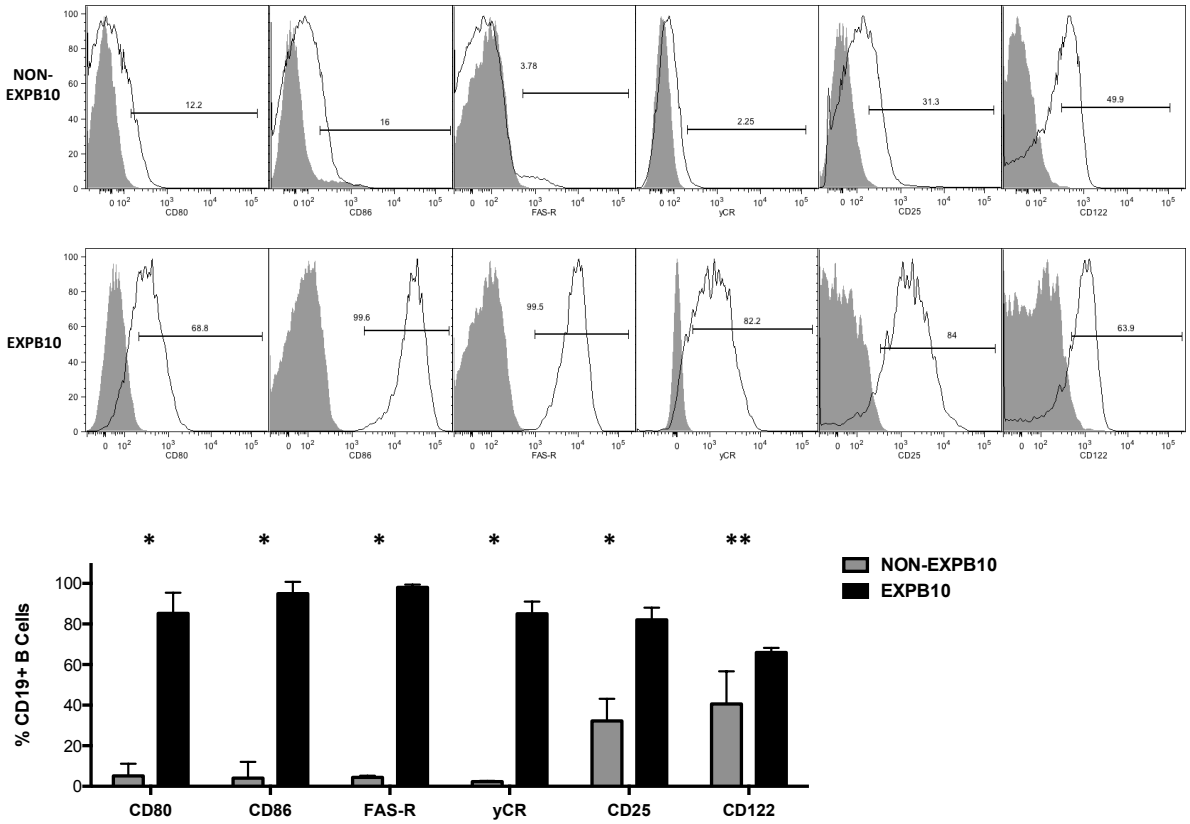
pathway revealed by transwell experiments. An in depth study of this pathway is required before any conclusions can be made.

Ultimately, it is essential to identify the precise mechanism by which Breg function, in order to accurately identify those B cells which can regulate an immune response. Given the finding that it is not necessarily the direct, paracrine effect of EXPB10-secreted IL-10 on $CD4^+$ T cells, but instead it is the autocrine effect of EXPB10-secreted IL-10 on EXPB10 cells which drives the suppression of $CD4^+$ T cells in this *in vitro* system, perhaps it would be more informative to focus on EXPB10 cells which express IL-10R, rather than IL-10 itself. The prospect of TIM-1 as a gatekeeper to regulatory function is a tempting one. A 'switch' of potential or progenitor Breg to a regulatory function, depending on the way in which TIM-1 is stimulated, opens up the possibility of fine-tuning an already highly controlled system, in order to manipulate biological systems in both health and disease.

In the next chapter, the function of EXPB10 cells is interrogated in a much more complex system, in the form of an *in vivo* humanised mouse model of skin transplantation. Although EXPB10 cells appear to suppress $CD4^+$ T cell responses *in vitro*, this function does not necessarily translate to a much more dynamic and hostile *in vivo* system. EXPB10 cells have so far been investigated in the absence of antigen. It is vital to understand how such cells behave in live environments in order to truly understand their biological roles both as an endogenous regulatory population and as a potential cellular therapy.

Fig. 4.1.1

A


NON-EXPB10
12.2
16
3.78
2.25
31.3
49.9
CD80
CD86
FAS-R
yCR
CD25
CD122
EXPB10
68.8
99.6
99.5
82.2
84
63.9
CD80
CD86
FAS-R
yCR
CD25
CD122
% CD19+ B Cells
CD80
CD86
FAS-R
yCR
CD25
CD122
NON-EXPB10
EXPB10

B

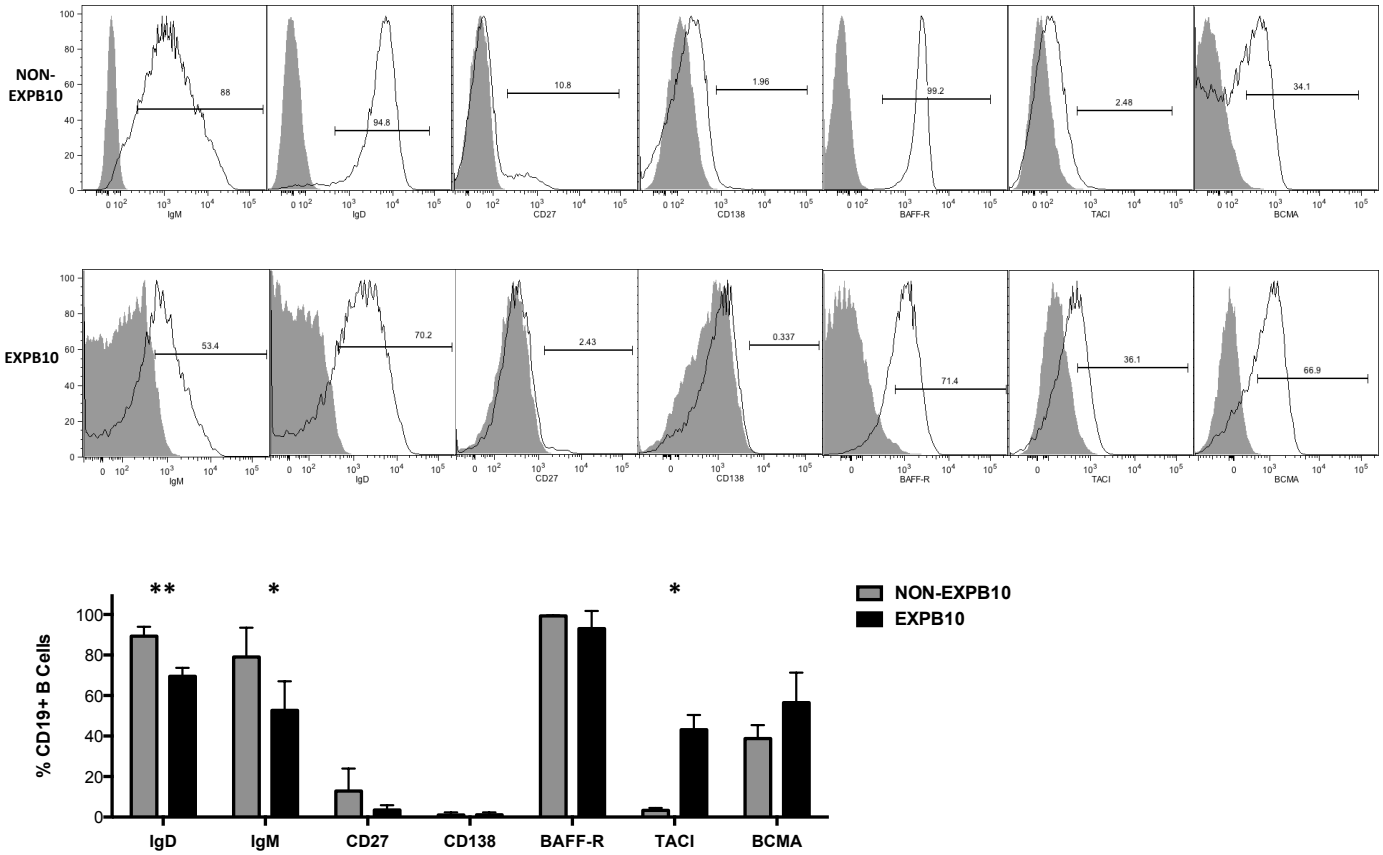
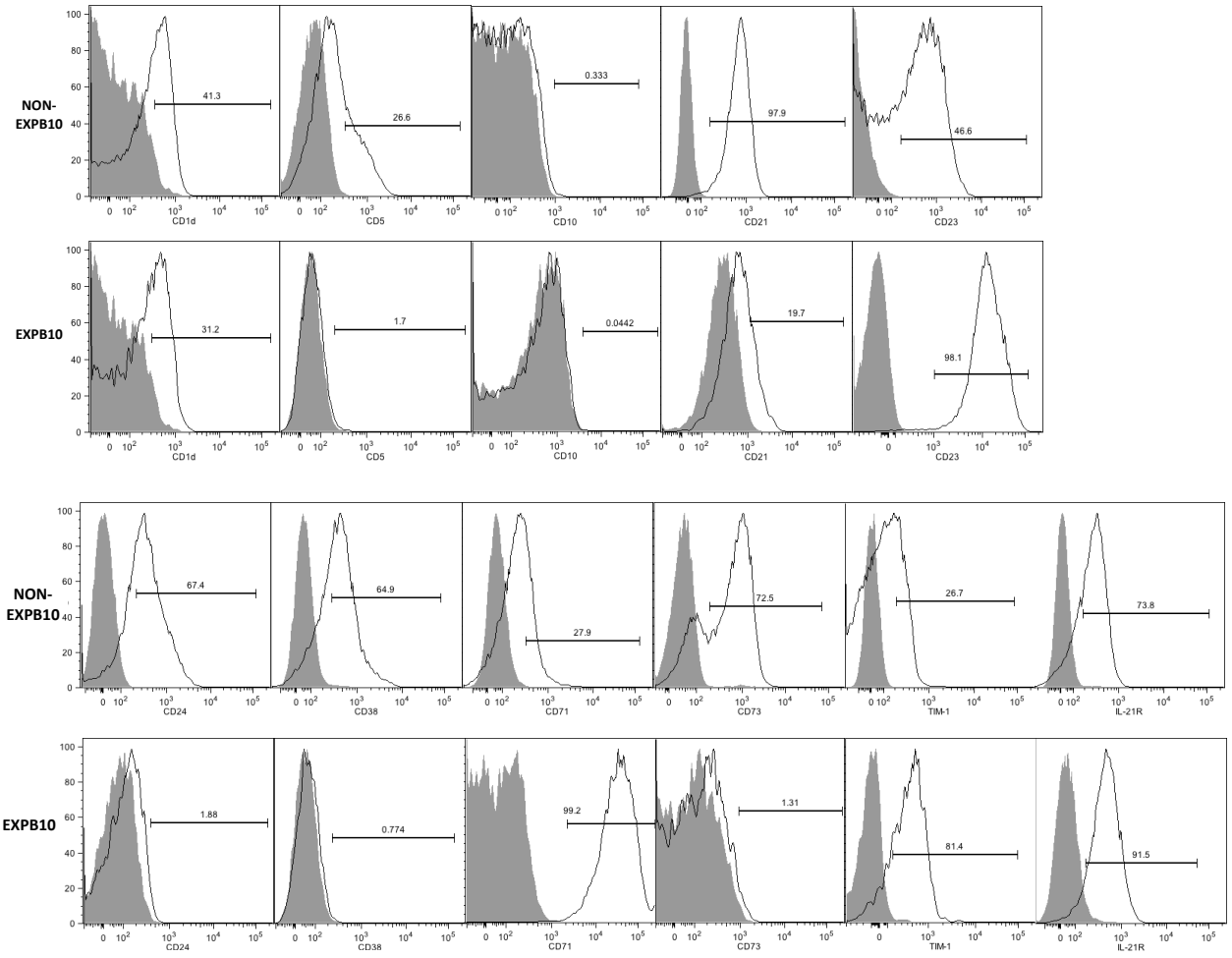
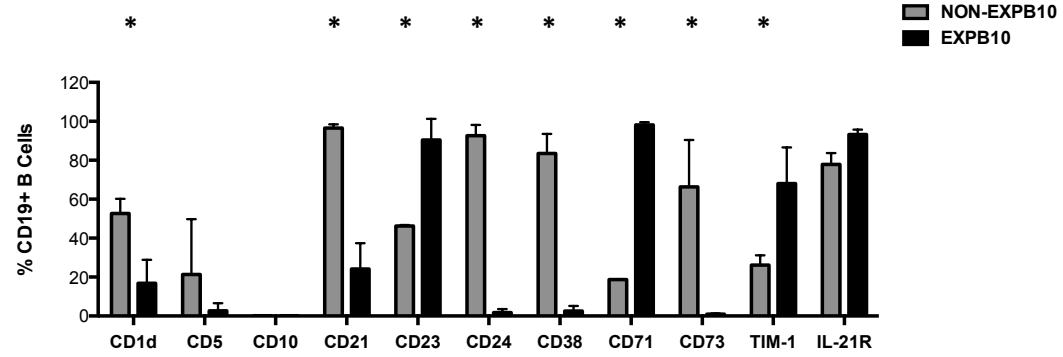

NON-EXPB10
88
94.8
10.8
1.96
99.2
2.48
34.1
IgM
IgD
CD27
CD138
BAFF-R
TACI
BCMA
EXPB10
53.4
70.2
2.43
0.337
71.4
36.1
66.9
IgM
IgD
CD27
CD138
BAFF-R
TACI
BCMA
% CD19+ B Cells
IgD
IgM
CD27
CD138
BAFF-R
TACI
BCMA
NON-EXPB10
EXPB10

Fig. 4.1.1 (cont.)

C





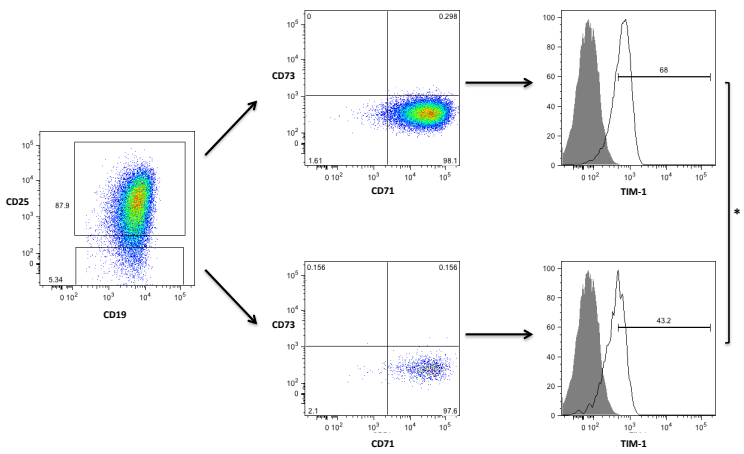
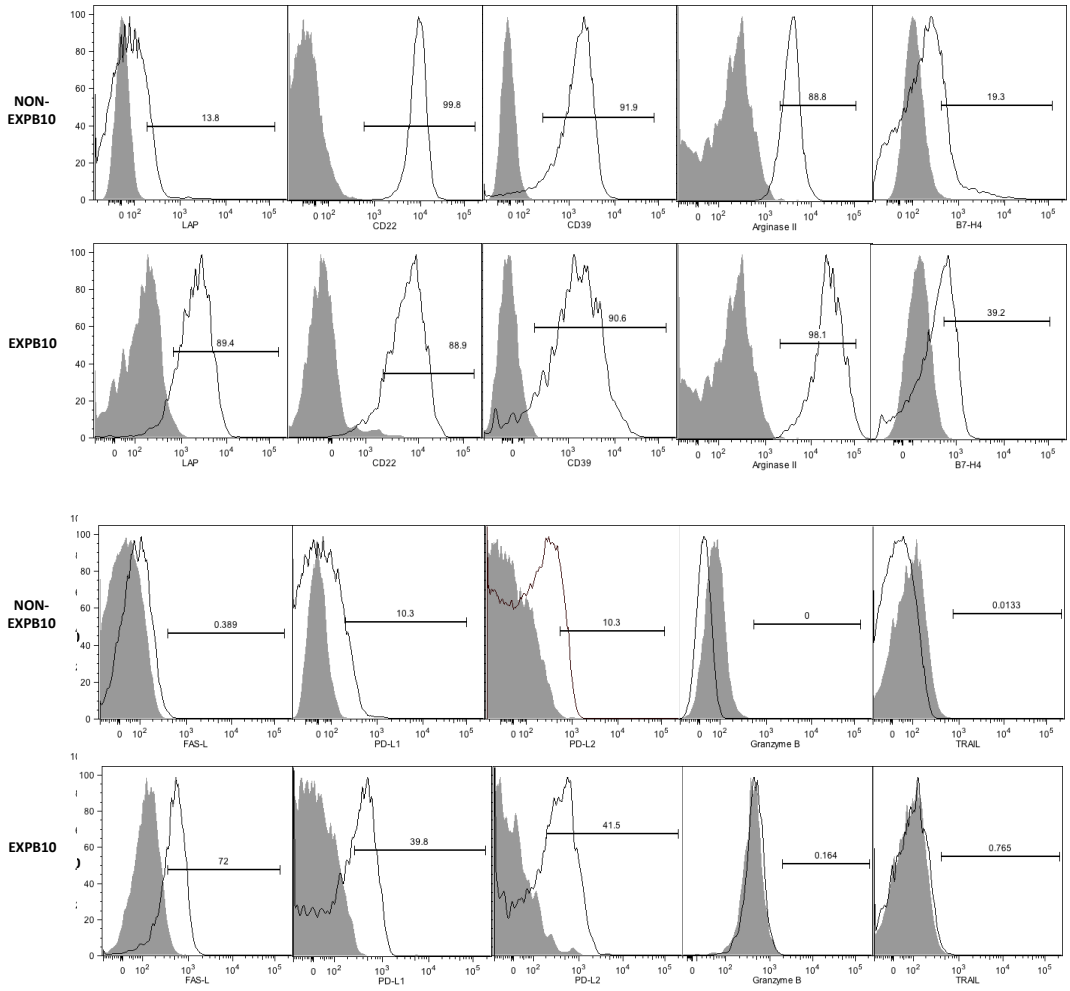


Fig. 4.1.1 (cont.)

D



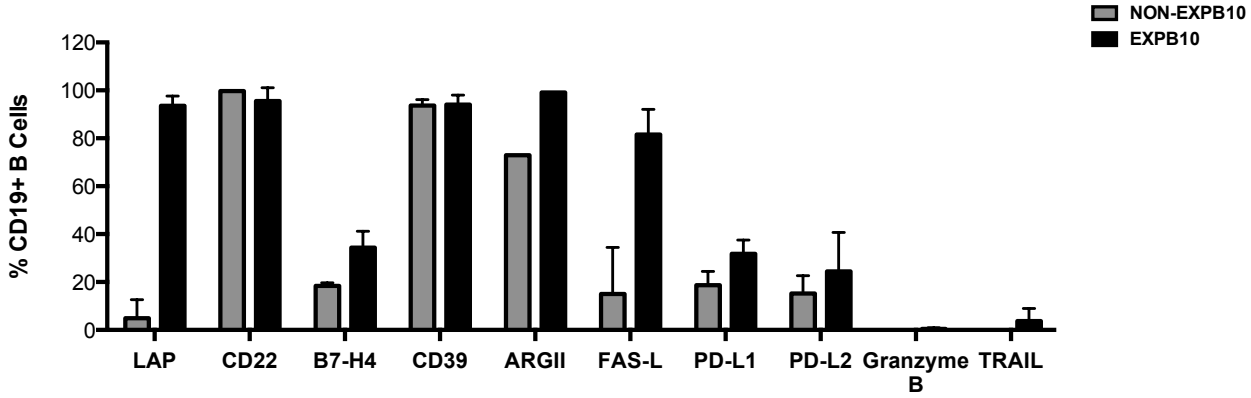


Fig. 4.1.1 Phenotype of EXPB10 cells

(a) - (d) Representative histograms gated on live $CD19^+$ cells. Filled histogram represents the mf-1 control; solid line represents stained $CD19^+$ B cells. Error bars in each panel represent Mean +/- SD. **(c)** Representative FACS plots illustrating significant differences in TIM-1 expression in EXPB10 cells. *$p<0.0001$, **$p<0.005$, paired t-test. Data are representative of 5 human donors.

Fig. 4. 1.2

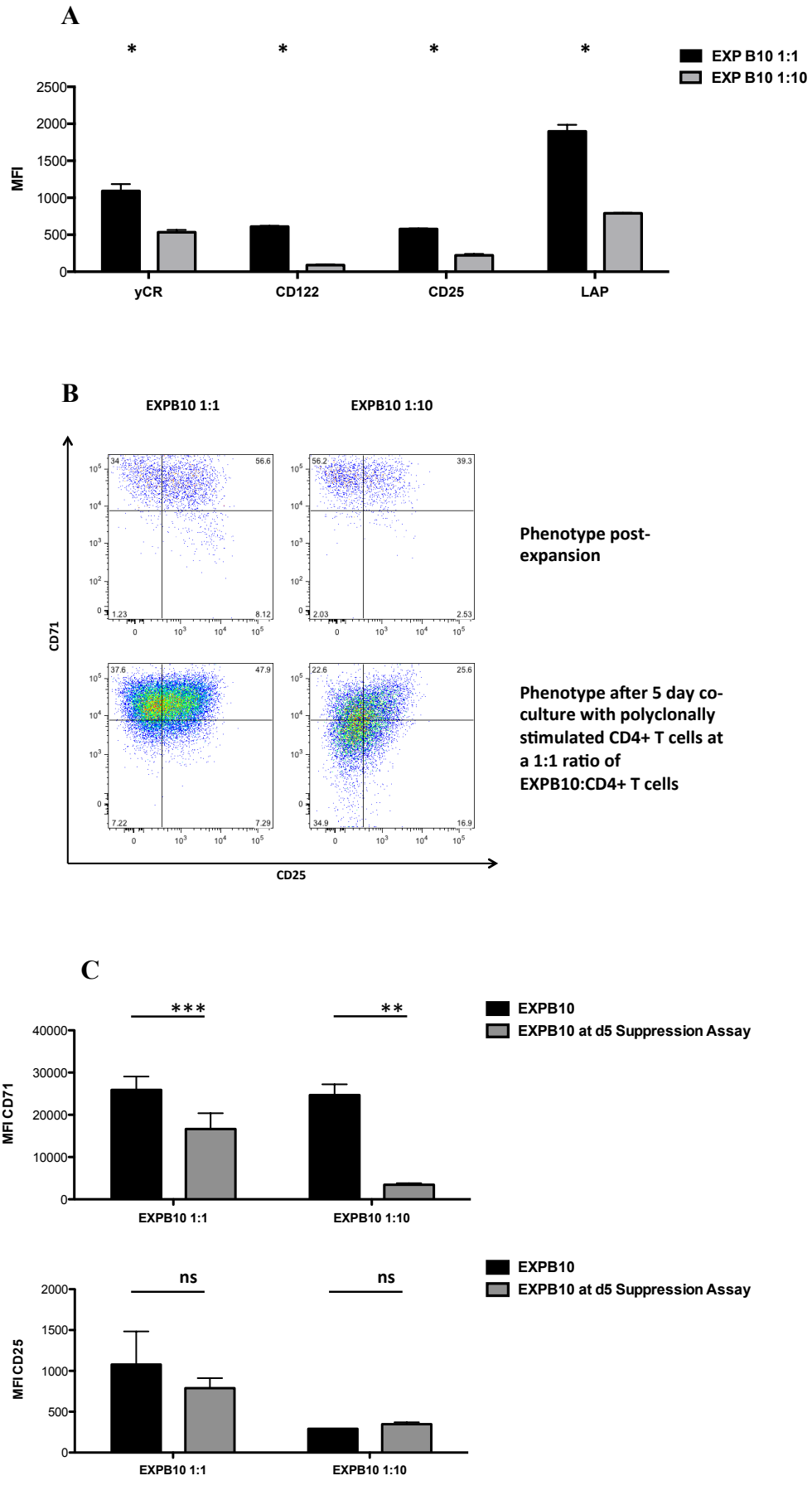
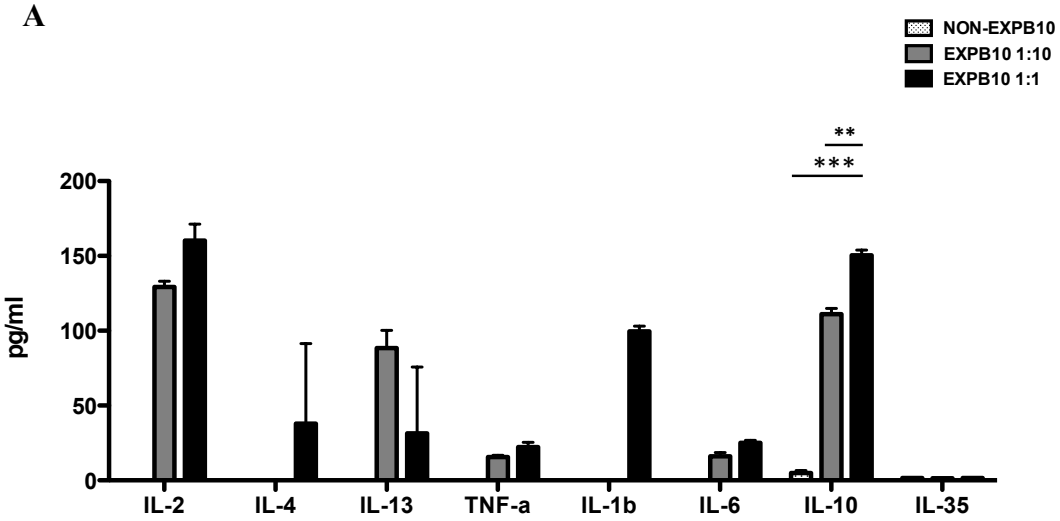

A
*
*
*
*
EXP B10 1:1
EXP B10 1:10
MFI
2500
2000
1500
1000
500
0
yCR
CD122
CD25
LAP
B
EXPB10 1:1
EXPB10 1:10
CD71
CD25
34
56.6
1.23
8.12
56.2
39.3
2.03
2.53
37.6
47.9
7.22
7.29
22.6
25.6
34.9
16.9
Phenotype post-expansion
Phenotype after 5 day co-culture with polyclonally stimulated CD4+ T cells at a 1:1 ratio of EXPB10:CD4+ T cells
C
***
**
EXPB10
EXPB10 at d5 Suppression Assay
MFI CD71
40000
30000
20000
10000
0
EXPB10 1:1
EXPB10 1:10
ns
ns
EXPB10
EXPB10 at d5 Suppression Assay
MFI CD25
2000
1500
1000
500
0
EXPB10 1:1
EXPB10 1:10

Fig. 4.1.2 Association of suppressive potency of EXPB10 cells with phenotypic markers

(a) Mean fluorescence intensity (MFI) of EXPB10 1:1 and EXPB10 1:10 cells where a significant difference in expression of phenotypic markers was found. *$p<0.0001$, paired t-test. **(b)** Representative FACS plots of EXPB10 1:1 and EXPB10 1:10 cells after expansion and after 5-day suppression assay co-culture with autologous $CD4^+$ T cells and anti-CD3/CD28 beads. Graphs illustrate differences in MFI of CD71 and CD25 expressed by EXPB10 1:1 and EXPB10 1:10 cells before and after suppression assay. ***$p<0.02$, **$p<0.005$, paired t-test. Error bars in each panel represent Mean +/- SD. Data are representative of 3 independent experiments.

Fig. 4.1.3

A


NON-EXPB10
EXPB10 1:10
EXPB10 1:1
**
***
pg/ml
200
150
100
50
0
IL-2
IL-4
IL-13
TNF-a
IL-1b
IL-6
IL-10
IL-35

B

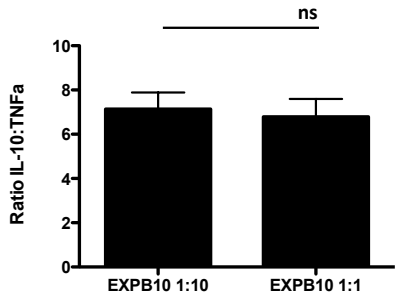

ns
Ratio IL-10:TNFa
10
8
6
4
2
0
EXPB10 1:10
EXPB10 1:1

Fig. 4.1.3 Cytokine production by EXPB10 cells

(a) Cytokine bead array of supernatants from NON-EXPB10, EXPB10 1:10 and EXPB10 1:1 cells after 6 hours incubation in complete media (c.RPMI). **$p<0.005$, ***$p<0.02$, paired t-test. **(b)** Ratio of IL-10:TNFα produced by EXPB10 1:1 and EXPB10 1:10 cells. Error bars in each panel represent Mean +/- SD. Data are representative of 3 independent experiments.

Fig. 4.2.1

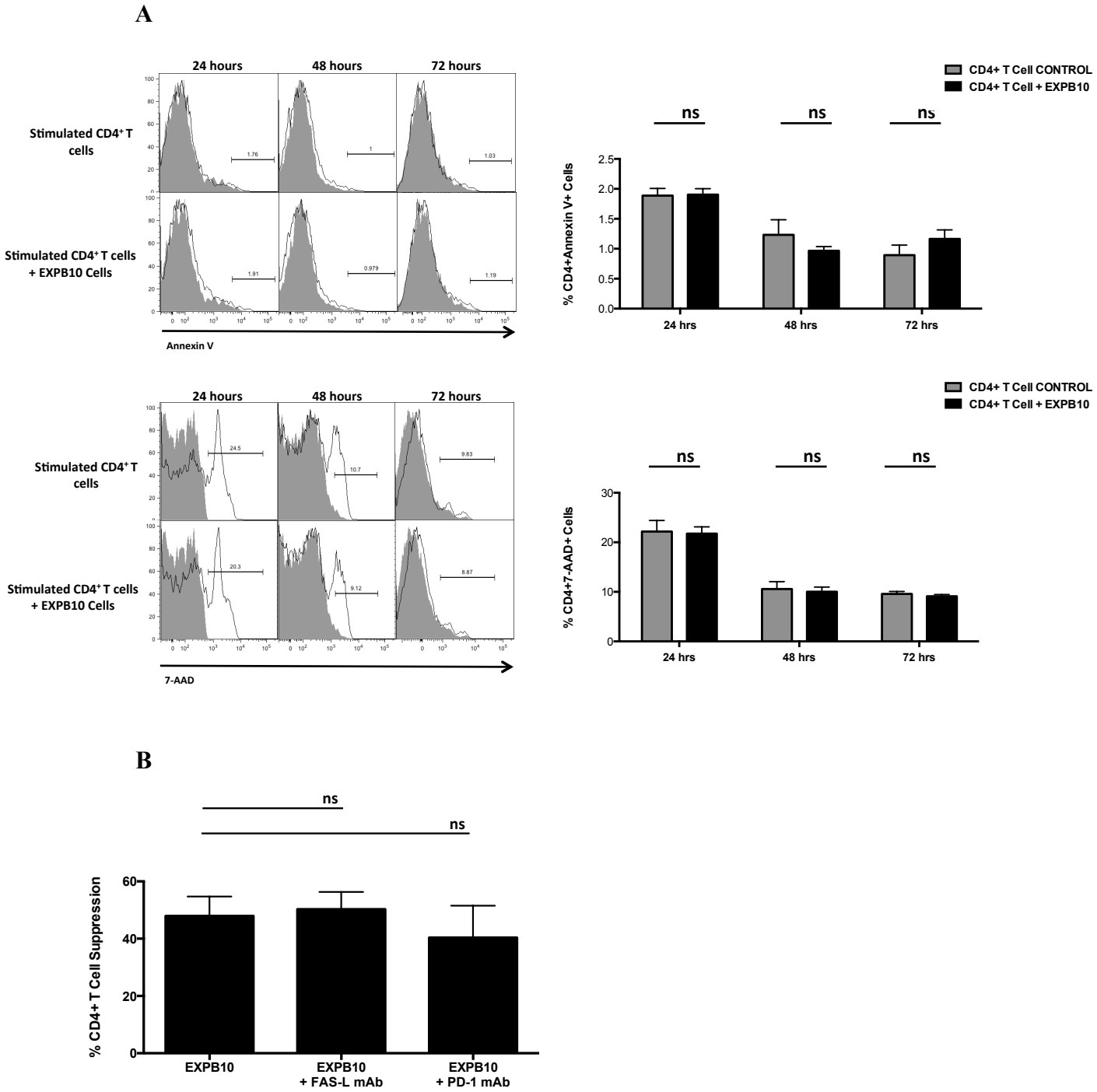

A
24 hours
48 hours
72 hours
Stimulated CD4+ T cells
Stimulated CD4+ T cells + EXPB10 Cells
Annexin V
CD4+ T Cell CONTROL
CD4+ T Cell + EXPB10
ns
% CD4+Annexin V+ Cells
24 hrs
48 hrs
72 hrs
7-AAD
% CD4+7-AAD+ Cells
B
% CD4+ T Cell Suppression
EXPB10
EXPB10 + FAS-L mAb
EXPB10 + PD-1 mAb

Fig. 4.2.1 EXPB10 cells do not induce apoptotic cell death in autologous $CD4^+$T cells via FAS- or PD-1-dependent mechanisms

(a) Representative FACS plots and demonstration of Annexin-V and 7-AAD staining of $CD4^+$ T cells when autologous $CD4^+$ T cells were co-cultured with anti CD3/CD28 beads and EXPB10 cells at a 1:1 ratio of T:B cells, for 24, 48 or 72 hours. **(b)** % $CD4^+$ T cell suppression mediated by EXPB10 cells in presence of blocking mAb to FAS-L 10ug/ml or PD-1 mAbs 10ug/ml. Paired t-test. Error bars in each panel represent Mean +/- SD. Data are representative of 3 independent experiments.

Fig. 4.2.2

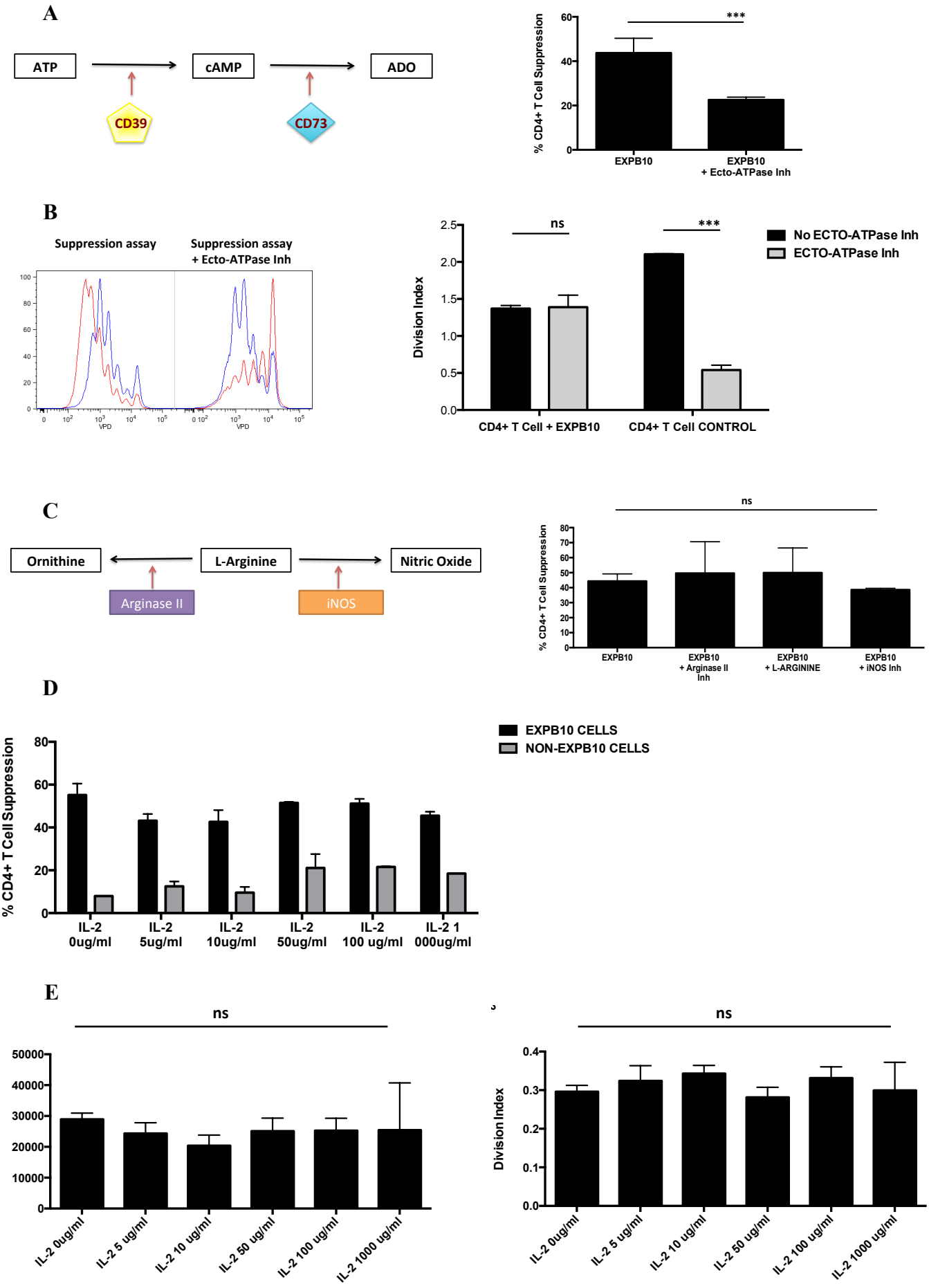
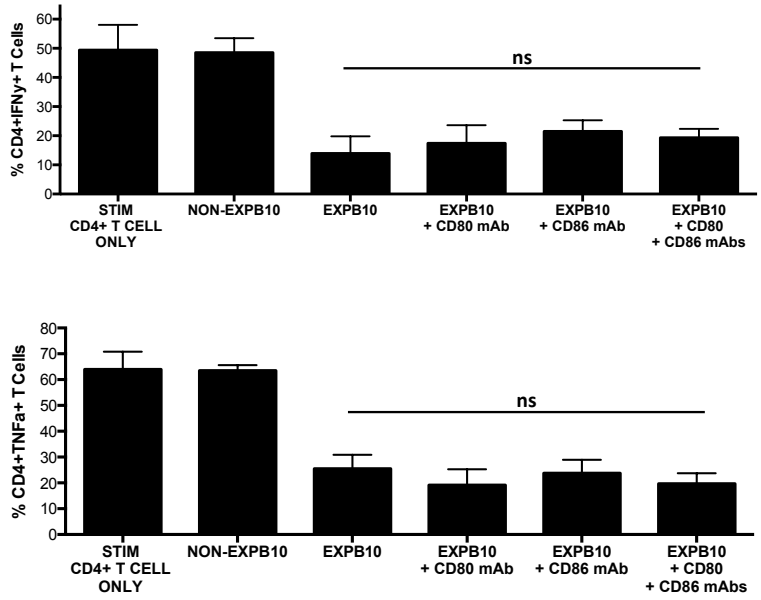

A
ATP
cAMP
ADO
CD39
CD73
% CD4+ T Cell Suppression
***
EXPB10
EXPB10 + Ecto-ATPase Inh
B
Suppression assay
Suppression assay + Ecto-ATPase Inh
VPD
Division Index
ns
***
No ECTO-ATPase Inh
ECTO-ATPase Inh
CD4+ T Cell + EXPB10
CD4+ T Cell CONTROL
C
Ornithine
L-Arginine
Nitric Oxide
Arginase II
iNOS
ns
% CD4+ T Cell Suppression
EXPB10
EXPB10 + Arginase II Inh
EXPB10 + L-ARGININE
EXPB10 + iNOS Inh
D
EXPB10 CELLS
NON-EXPB10 CELLS
% CD4+ T Cell Suppression
IL-2 0ug/ml
IL-2 5ug/ml
IL-2 10ug/ml
IL-2 50ug/ml
IL-2 100 ug/ml
IL-2 1 000ug/ml
E
ns
No. of CD19+ Bcells at day 5
IL-2 0ug/ml
IL-2 5 ug/ml
IL-2 10 ug/ml
IL-2 50 ug/ml
IL-2 100 ug/ml
IL-2 1000 ug/ml
ns
Division Index
IL-2 0ug/ml
IL-2 5 ug/ml
IL-2 10 ug/ml
IL-2 50 ug/ml
IL-2 100 ug/ml
IL-2 1000 ug/ml

Fig. 4.2.2 EXPB10 cells may partially suppress proliferation of autologous $CD4^+$ T cells by means of metabolic disruption

(a) Simplified diagram outlining the pathway of ATP breakdown catalysed by endonucelotidases CD39 and CD73. Graph demonstrates significant reduction in % $CD4^+$ T cell suppression mediated by EXPB10 cells when in the presence of an ecto-ATPase inhibitor (Inh) ARL 67156 at 0.1 mM/L. ***$p<0.02$, paired t-test. **(b)** Representative histograms of live $CD4^+VPD^+$ T cells at day 5 of the suppression assay in both the absence and presence of ARL 67156. Red line represents $CD4^+$ T cells when cultured with beads alone ($CD4^+$ T cell CONTROL); blue line represents $CD4^+$ T cells when cultured with beads and EXPB10 cells ($CD4^+$ T Cell + EXPB10). Graph illustrates that division index of $CD4^+$ T cells was significantly different in the absence and presence of ARL 67156, when co-cultured with beads alone. There was no difference between division index of $CD4^+$ T cells when co-cultured with beads and EXPB10 cells in the absence or presence of ARL 67156. ***$p<0.02$, paired t-test. **(c)** Simplified diagram outlining metabolism of L-arginine catalysed by either arginase II or inducible nitric oxide synthase (iNOS). Graph demonstrates no difference in % $CD4^+$ T cell suppression mediated by EXPB10 cells when in the presence of inhibitors of arginase II **(**S-(2-boronoethyl)-L-cysteine (BEC)**)**, iNOS **(**AMT HCl at 0.002 mM/L**)** or upon the addition of exogenous L-arginine (4mM/L). **(d)** Graph demonstrating % $CD4^+$ T cell suppression by EXPB10 cells when co-cultured in the presence of exogenous IL-2 in increasing concentrations. (e) Number and division index of $CD19^+$ B cells at day 5 of suppression assay when NON-EXPB10 cells were co-cultured with autologous $CD4^+$ T cells, anti-CD3/CD28 beads, and exogenous IL-2 is added at increasing concentrations. Error bars in each panel represent Mean +/- SD. Data is representative of 4 independent experiments.

Fig. 4.2.3

A


% CD4+IFNγ+ T Cells
60
50
40
30
20
10
0
ns
STIM CD4+ T CELL ONLY
NON-EXPB10
EXPB10
EXPB10 + CD80 mAb
EXPB10 + CD86 mAb
EXPB10 + CD80 + CD86 mAbs
% CD4+TNFa+ T Cells
80
70
60
50
40
30
20
10
0
ns
STIM CD4+ T CELL ONLY
NON-EXPB10
EXPB10
EXPB10 + CD80 mAb
EXPB10 + CD86 mAb
EXPB10 + CD80 + CD86 mAbs

B

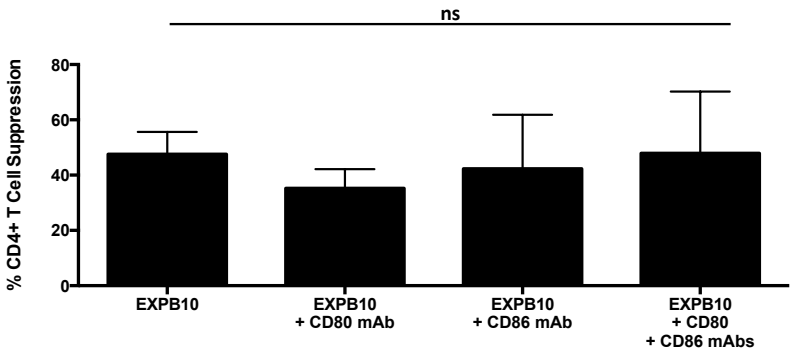

ns
% CD4+ T Cell Suppression
80
60
40
20
0
EXPB10
EXPB10 + CD80 mAb
EXPB10 + CD86 mAb
EXPB10 + CD80 + CD86 mAbs

Fig. 4.2.3 Suppressive function of EXPB10 cells is not dependent on co-stimulatory molecule CD80 and CD86

(a) EXPB10 cell-mediated suppression of % IFNγ and TNFα expression by $CD4^+$ T cells was not reduced by blockade of CD80, CD86 or CD80 + CD86 when each mAb was added at 10ug/ml. Paired t-test. **(b)** EXPB10 cell-mediated suppression of $CD4^+$ T cell proliferation was not reduced by blockade of CD80, CD86 or CD80 + CD86 when each mAb was added at 10ug/ml. Paired t-test. Error bars in each panel represent Mean +/- SD. Data are representative of 3 independent experiments.

Fig. 4.2.4

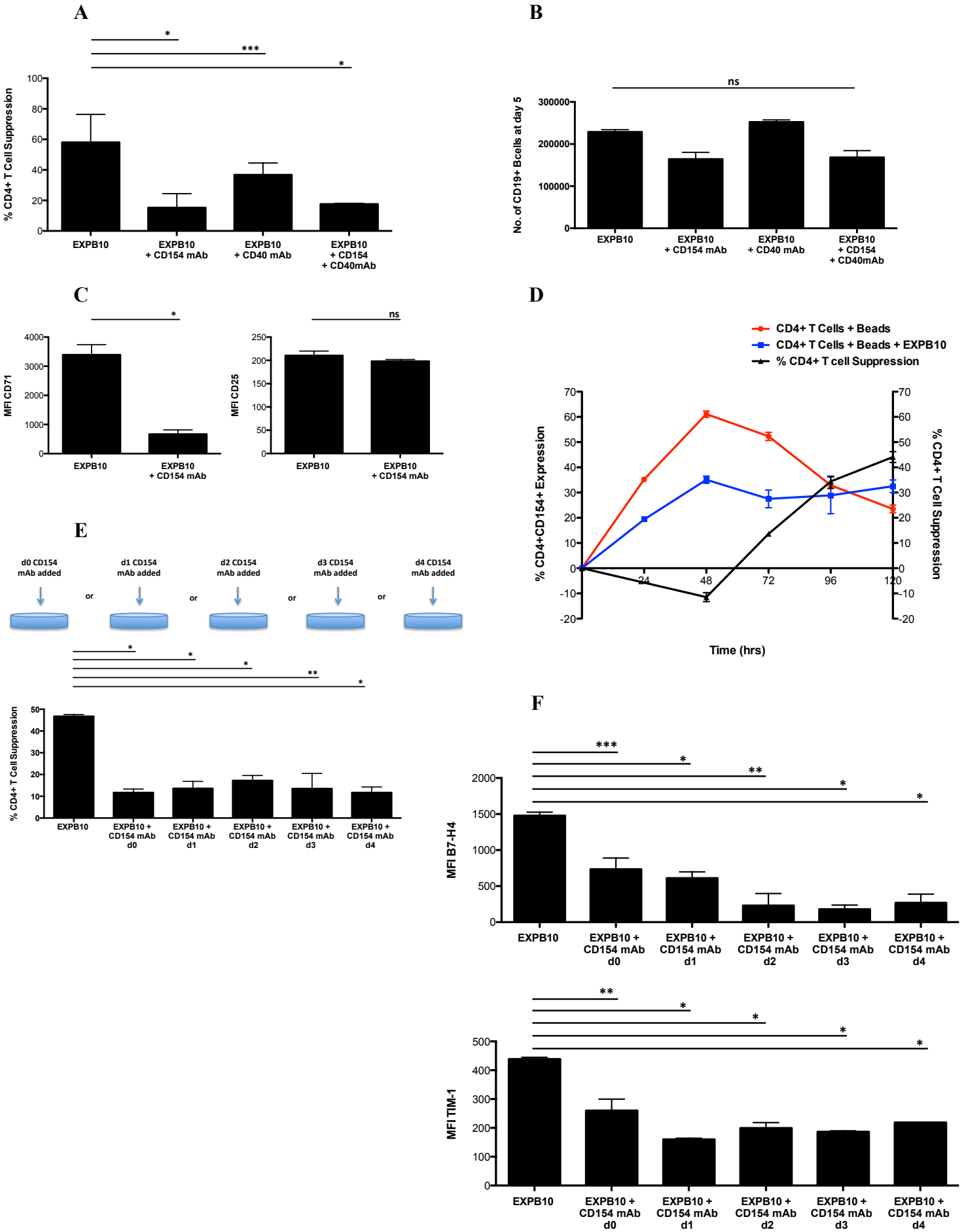

A
% CD4+ T Cell Suppression
EXPB10
EXPB10 + CD154 mAb
EXPB10 + CD40 mAb
EXPB10 + CD154 + CD40mAb
B
No. of CD19+ Bcells at day 5
ns
C
MFI CD71
MFI CD25
D
CD4+ T Cells + Beads
CD4+ T Cells + Beads + EXPB10
% CD4+ T cell Suppression
% CD4+CD154+ Expression
Time (hrs)
E
d0 CD154 mAb added
d1 CD154 mAb added
d2 CD154 mAb added
d3 CD154 mAb added
d4 CD154 mAb added
or
F
MFI B7-H4
MFI TIM-1

Fig. 4.2.4 Suppressive function of EXPB10 cells is dependent on the interaction between CD40 and CD154

(a) % $CD4^+$ T cell suppression by EXPB10 cells was significantly reduced by blockade of CD154, CD40 or CD154 & CD40 where all mAbs are added at 10ug/ml. *p<0.0001, ***p<0.02, paired t-test. **(b)** There was no significant decrease in number of EXPB10 cells at day 5 of suppression assay in presence of blocking mAbs to CD154 or CD40. **(c)** MFI of CD71 was significantly reduced at day 5 of suppression co-culture when CD154 was blocked. MFI of CD25 was not affected. *p<0.0001, paired t-test. **(d)** Graph demonstrating expression of CD154 by stimulated $CD4^+$ T cells over 5 days culture +/- EXPB10 cells at 1:1 ratio. Graph also displaying % suppression of $CD4^+$ T cell proliferation by EXPB10 cells that was calculated daily at 48, 72, 96 and 120 hours of suppression assay. **(e)** When blocking mAb to CD154 at 10ug/ml was added either on day (d) 1, d2, d3 or d4, % suppression of $CD4^+$ T cells by EXPB10 cells was significantly reduced compared to % suppression in the absence of CD154 blockade. *p<0.0001, **p<0.005 paired t-test. **(f)** Significant decrease in MFI of B7-H4 and TIM-1 when CD154 mAb at 10ug/ml was added either on d1, d2, d3 or d4. *p<0.0001, **p<0.005, ***p<0.02, paired t-test. Error bars in each panel represent Mean +/- SD. Data are representative of 3 independent experiments.

Fig. 4.2.5

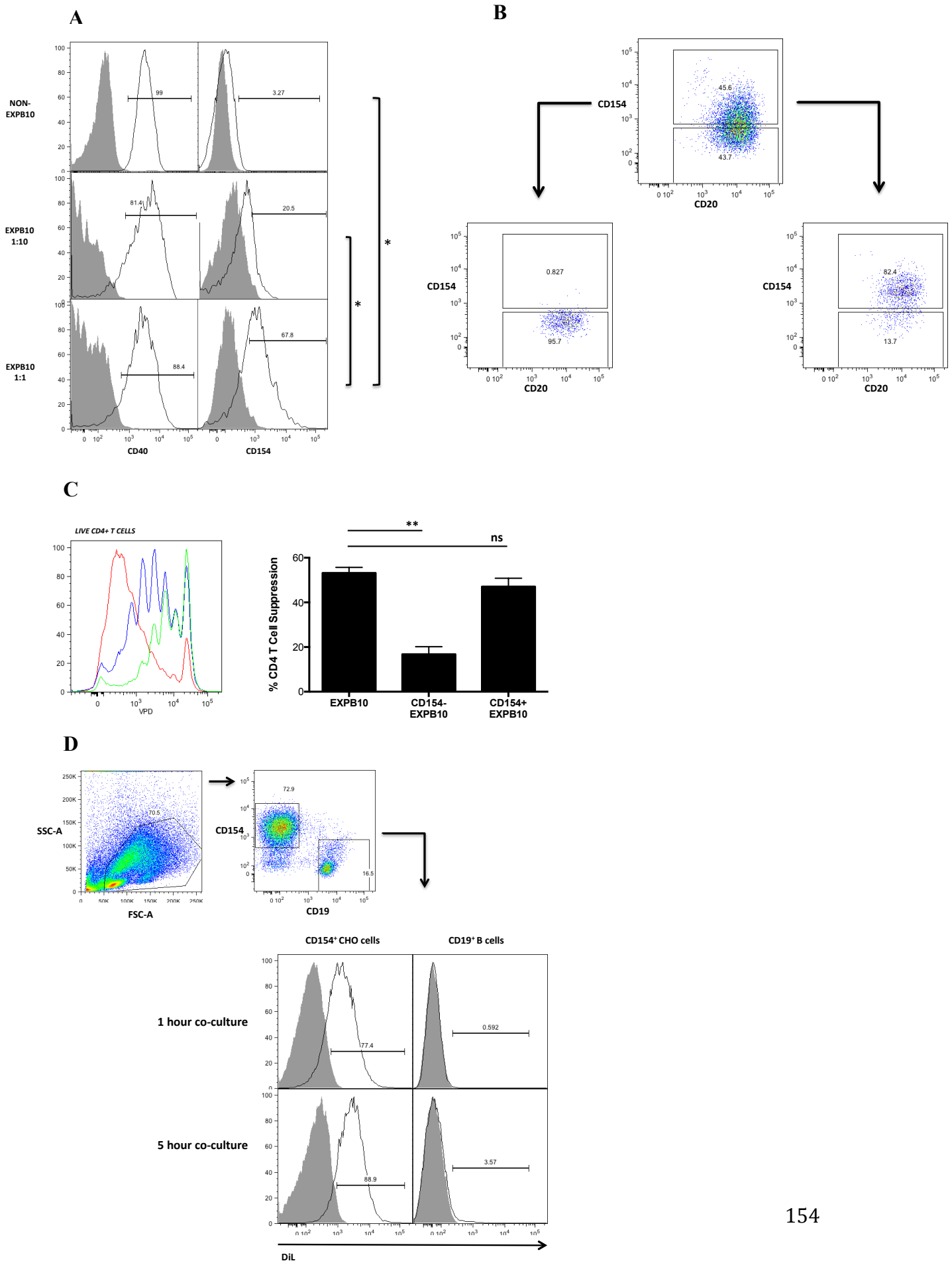

A
NON-EXPB10
EXPB10 1:10
EXPB10 1:1
99
3.27
81.4
20.5
88.4
67.8
CD40
CD154
*
*
*
B
CD154
CD20
45.6
43.7
0.827
95.7
82.4
13.7
C
LIVE CD4+ T CELLS
VPD
**
ns
% CD4 T Cell Suppression
EXPB10
CD154- EXPB10
CD154+ EXPB10
D
SSC-A
FSC-A
70.5
CD154
CD19
72.9
16.5
CD154+ CHO cells
CD19+ B cells
1 hour co-culture
5 hour co-culture
77.4
0.592
88.9
3.57
DiL

Fig. 4.2.5 $CD154^+$ EXPB10 cells are significantly more suppressive than $CD154^-$ EXPB10 cells

(a) Representative histograms of live NON-EXPB10, EXPB10 1:10 and EXPB10 1:1 cells when stained for CD40 or CD154. Filled histogram represents mf-1 control; solid line represents antibody staining. *$p<0.0001$, paired t-test. **(b)** FACS-sort of $CD154^+$ and $CD154^-$ EXPB10 cells. Purities >82%. **(c)** Representative histograms of live $CD4^+CFSE^+$ T cells when cultured for 5 days with anti-CD3/CD28 beads alone or with $CD154^+$ or $CD154^-$ EXPB10 cells. Red line represents stimulated $CD4^+$ T cell control; blue line represents stimulated $CD4^+$ T cells co-cultured with $CD154^-$ EXPB10 cells; green line represents stimulated $CD4^+$ T cells co-cultured with $CD154^+$ EXPB10 cells. Graph demonstrating significant reduction in $CD4^+$ T cell suppression by $CD154^-$ EXPB10 cells when compared to $CD154^+$ EXPB10 cells and whole EXPB10 cells. **$p<0.005$, paired t-test. **(d)** EXPB10 cells did not gain CD154 expression through trogocytosis from $CD154^+$ CHO cells. $CD154^+$ CHO cells stained with lipophilic membrane dye Dil or unstained $CD154^+$ CHO cells were co-cultured with $CD19^+$ B cells at a 1:1 ratio in presence of cytokines IL-2, IL-4 and IL-10. Representative FACS plots illustrate that $CD154^+$ CHO cells retained Dil staining at 1 hour and 5 hours of incubation; $CD19^+$ B cells did not acquire Dil expression after 1 hour or 5 hours of incubation. Filled histograms represent unstained $CD154^+$ CHO cells and $CD19^+$ B cells which have been co-cultured with unstained $CD154^+$ CHO cells; unfilled histograms represent Dil-stained $CD154^+$ CHO cells and $CD19^+$ B cells which have been co-cultured with Dil-stained $CD154^+$ CHO cells. Error bars in each panel represent Mean +/- SD. Data are representative of 3 independent experiments.

Fig. 4.2.6

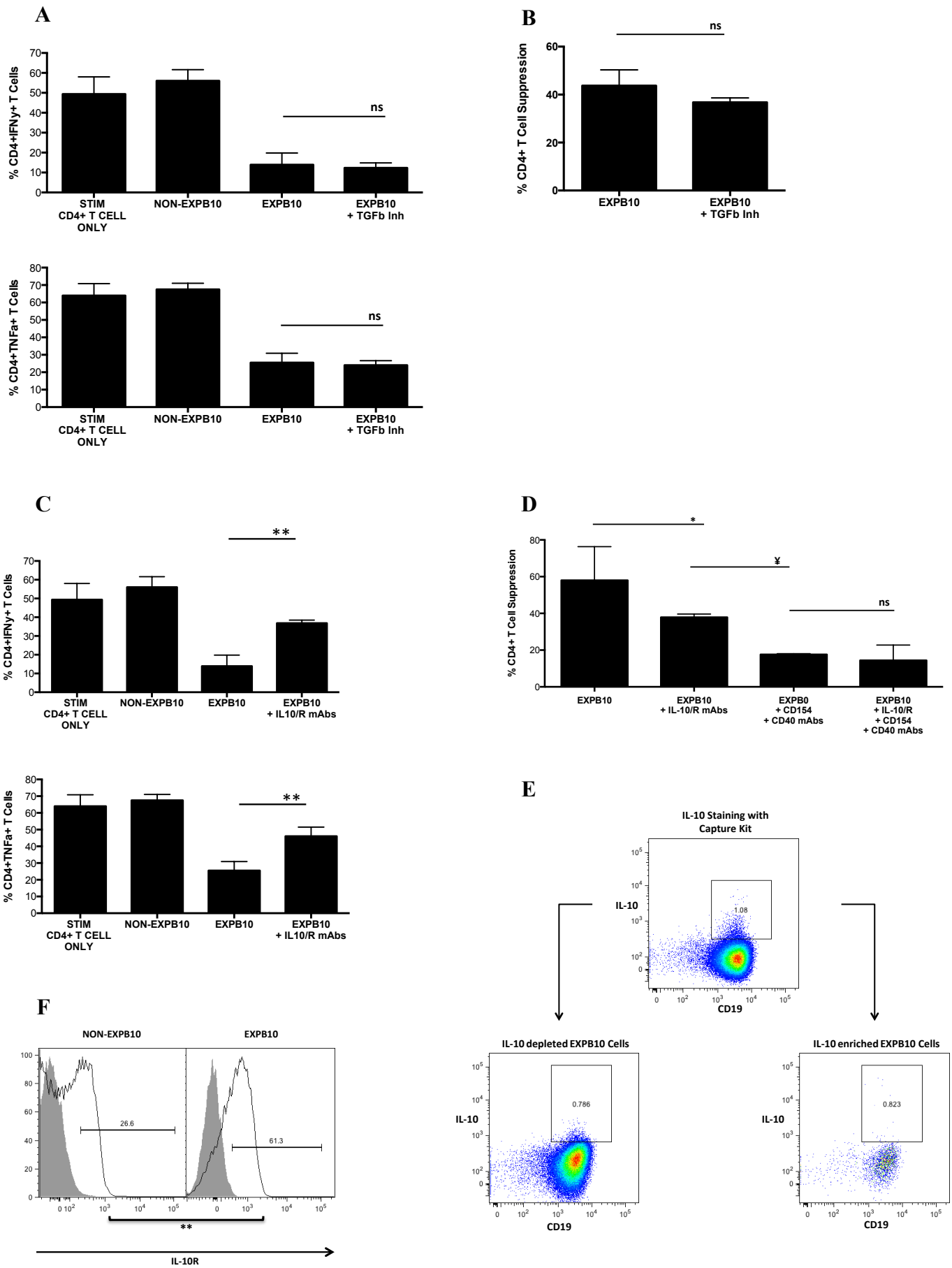
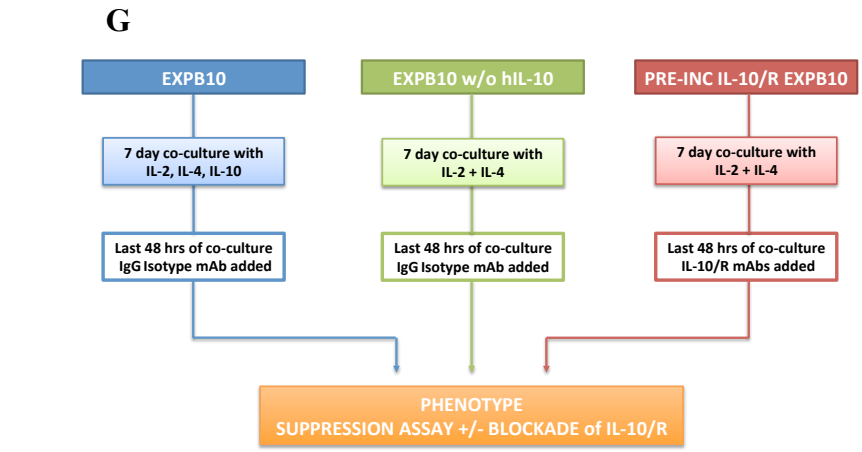
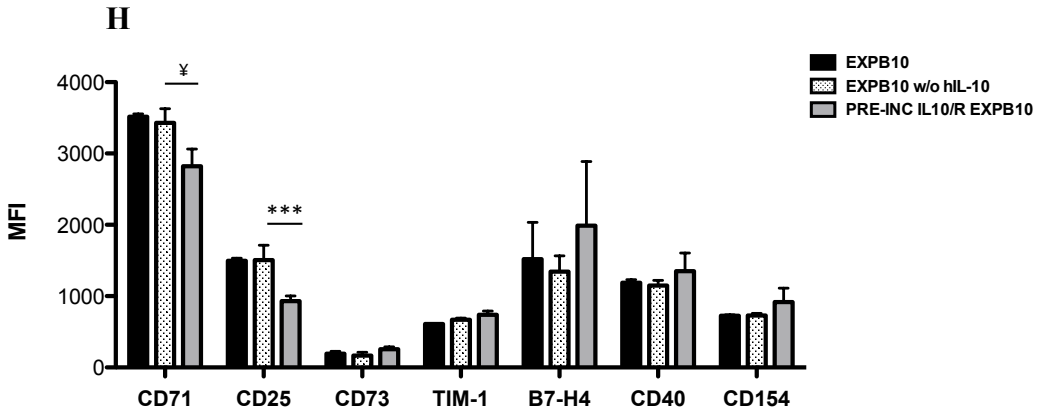
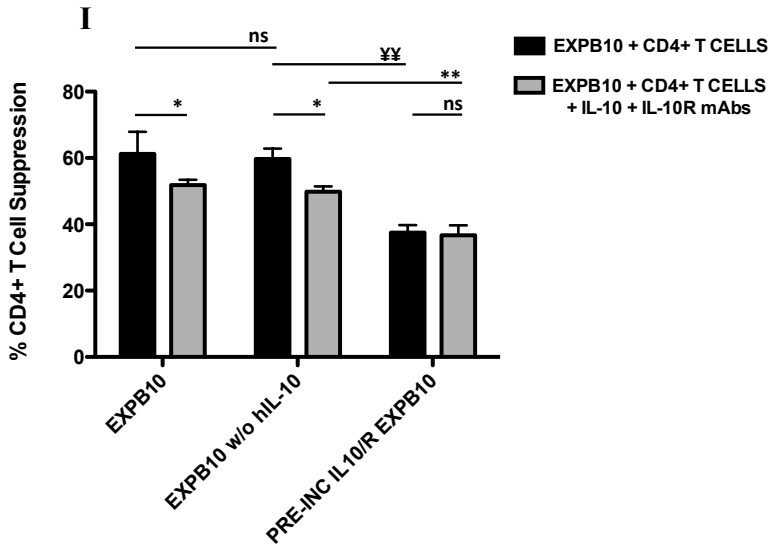
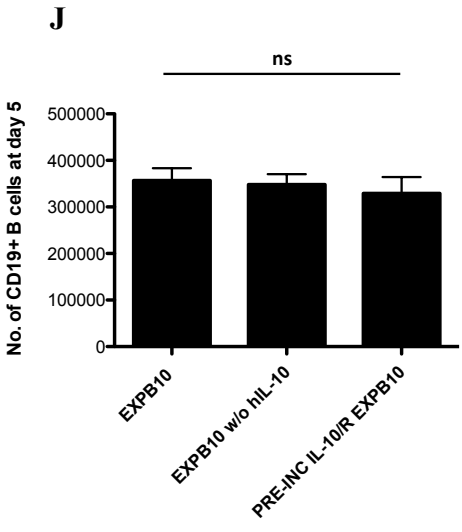

A
% CD4+IFNγ+ T Cells
ns
STIM CD4+ T CELL ONLY
NON-EXPB10
EXPB10
EXPB10 + TGFb Inh
% CD4+TNFa+ T Cells
B
% CD4+ T Cell Suppression
C
**
EXPB10 + IL10/R mAbs
D
*
¥
EXPB10 + IL-10/R mAbs
EXPB0 + CD154 + CD40 mAbs
EXPB10 + IL-10/R + CD154 + CD40 mAbs
E
IL-10 Staining with Capture Kit
IL-10
CD19
1.08
IL-10 depleted EXPB10 Cells
0.786
IL-10 enriched EXPB10 Cells
0.823
F
NON-EXPB10
EXPB10
26.6
61.3
IL-10R

Fig. 4.2.6 (cont.)


G
EXPB10
EXPB10 w/o hIL-10
PRE-INC IL-10/R EXPB10
7 day co-culture with IL-2, IL-4, IL-10
7 day co-culture with IL-2 + IL-4
7 day co-culture with IL-2 + IL-4
Last 48 hrs of co-culture IgG Isotype mAb added
Last 48 hrs of co-culture IgG Isotype mAb added
Last 48 hrs of co-culture IL-10/R mAbs added
PHENOTYPE SUPPRESSION ASSAY +/- BLOCKADE of IL-10/R


H
EXPB10
EXPB10 w/o hIL-10
PRE-INC IL10/R EXPB10
MFI
4000
3000
2000
1000
0
¥
***
CD71
CD25
CD73
TIM-1
B7-H4
CD40
CD154


I
EXPB10 + CD4+ T CELLS
EXPB10 + CD4+ T CELLS + IL-10 + IL-10R mAbs
% CD4+ T Cell Suppression
80
60
40
20
0
ns
¥¥
**
*
*
ns
EXPB10
EXPB10 w/o hIL-10
PRE-INC IL10/R EXPB10


J
No. of CD19+ B cells at day 5
500000
400000
300000
200000
100000
0
ns
EXPB10
EXPB10 w/o hIL-10
PRE-INC IL-10/R EXPB10

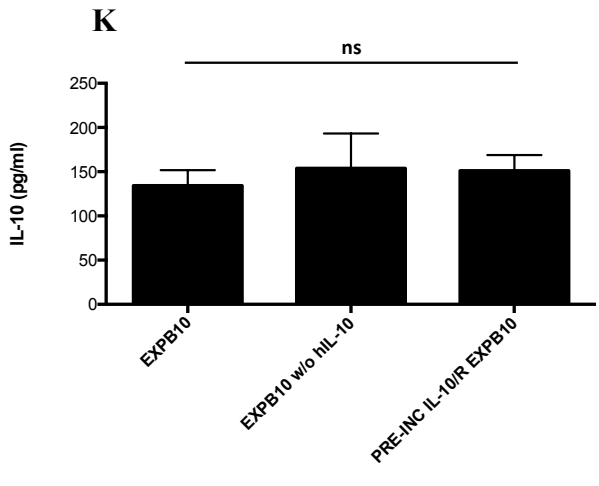
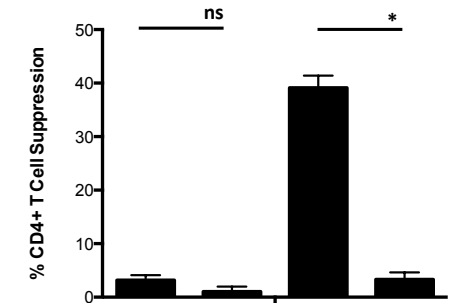

K
IL-10 (pg/ml)
250
200
150
100
50
0
ns
EXPB10
EXPB10 w/o hIL-10
PRE-INC IL-10/R EXPB10

Fig. 4.2.6 Suppressive function of EXPB10 cells is partially dependent on immunosuppressive cytokine, IL-10, but not TGFβ

(a) Suppression of % expression of pro-inflammatory cytokines by $CD4^+$ T cells was not reduced by inhibition of TGFβ by addition of SB 431542 at 10uM/L (TGFb Inh) during co-culture. Paired t-test. **(b)** % Suppression of $CD4^+$ T cell proliferation was not reduced by inhibition of TGFβ. Paired t-test. **(c)** Blockade of IL-10 and IL10R by addition of blocking mAbs at 10ug/ml partially restored IFNy and TNFa expression by $CD4^+$ T cells *in vitro*. **p<0.005, paired t-test. **(d)** Blockade of IL-10 and IL10R by addition of blocking mAbs at 10ug/ml partially reduced EXPB10 cell-mediated suppression of $CD4^+$ T cell proliferation. CD154 blockade by addition of CD154 mAb at 10ug/ml resulted in significantly greater reduction in suppression of $CD4^+$ T cell proliferation than IL-10/R blockade. Joint blockade of IL-10/R and CD154 did not result in greater reduction in suppression of $CD4^+$ T cell proliferation than blockade with CD154 alone. *p<0.0001, $^{¥}$p<0.001, paired t-test. **(e)** Poor enrichment of IL-10^+ EXPB10 cells through use of an IL-10 capture kit. **(f)** Representative histograms of IL-10R expression by EXPB10 and NON-EXPB10 cells. Filled histogram represents the mf-1 control; unfilled histogram represents $CD19^+$ B cells. EXPB10 cells significantly up-regulated expression of IL-10R compared to NON-EXPB10 cells. **p<0.005, paired t-test. **(g)** EXPB10 cells were generated under different conditions. EXPB10 standard protocol is in blue: co-culture with $CD154^+$ CHO cells for 7 days with cytokines IL-2, IL-4, IL-10; during the last 48hrs of co-culture, IgG isotype mAb control was added. EXPB10 w/o hIL-10 protocol is in green: co-culture with $CD154^+$ CHO cells for 7 days with cytokines IL-2 and IL-4; during the last 48hrs of co-culture, IgG isotype mAb control was added. PRE-INC IL-10/R EXPB10 protocol is in red: co-culture with $CD154^+$ CHO cells for 7 days with cytokines IL-2 and IL-4; during the last 48hrs of co-culture, blocking mAbs to IL-10/R at 10ug/ml were added. EXPB10 cells were harvested on day 7. Phenotype was analysed by FACS. Suppressive potency was analysed by co-culture with autologous $CD4^+$ T cells at 1:1 ratio and with anti-CD3/CD28 beads. In some suppression assays, blockade of IL-10 was achieved by addition of IL-10/R blocking mAbs at 10ug/ml to the 5-day suppression co-culture **(h)** MFI of cell markers expressed by EXPB10, EXPB10 w/o hIL-10 and PRE-INC IL-10/R EXPB10 cells are demonstrated. **p<0.005, $^{¥}$p<0.001. **(i)** % Suppression of $CD4^+$T cell proliferation by EXPB10, EXPB10 w/o hIL-10 and PRE-INC IL-10/R EXPB10 cells, when $CD4^+$ T cells were co-cultured with EXPB10 cells a 1:1 ratio and with anti-CD3/CD28 beads for 5 days, either in the absence or presence of IL-10/R blocking mAbs at 10ug/ml. PRE-INC IL-10/R EXPB10 cells were significantly less suppressive than EXPB10 w/o hIL-10. There was no difference in suppressive potency between EXPB10 cells and EXPB10 w/o hIL-10. IL-10/R blockade during the suppression assay resulted in significantly reduced suppressive potency of both EXPB10 and EXPB10 w/o hIL-10 cells, but had no effect on suppressive potency of PRE-INC IL-10/R EXPB10 cells. *p<0.0001, **p<0.005, $^{¥¥}$p<0.008, paired t-test. **(j)** Total no. of EXPB10, EXPB10 w/o hIL-10 or PRE-INC IL-10/R EXPB10 cells at day 5 of suppression assay demonstrated no differences in $CD19^+$ B cell survival. Paired t-test. (**k)** Level of IL-10 produced by EXPB10, EXPB10 w/o hIL-10 and PRE-INC IL-10/R EXPB10 cells, after 6-hour incubation period in cRPMI. ***p<0.02, paired t-test**.** Error bars in each panel represent Mean +/- SD. Data are representative of 4 independent experiments.

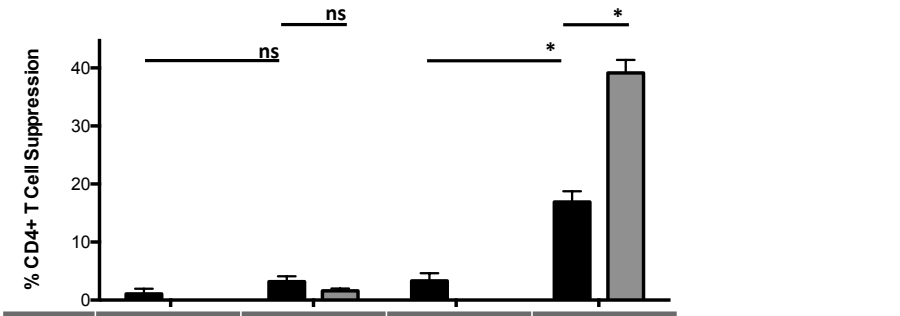
Fig. 4.2.7

A



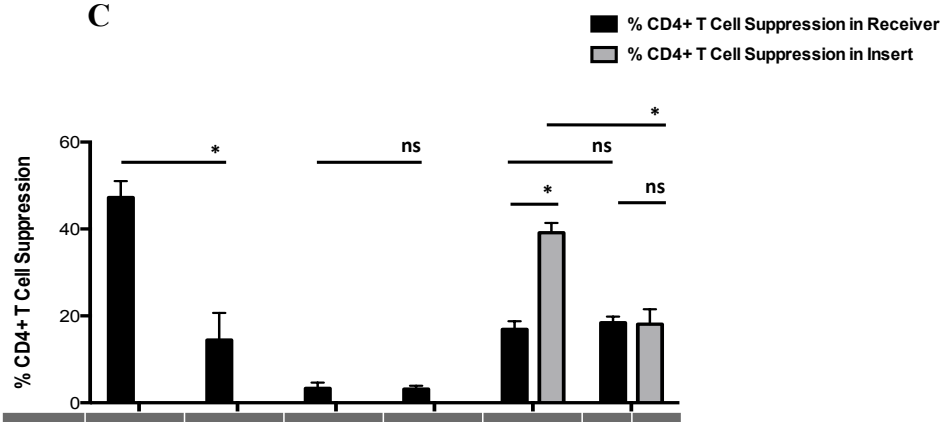
Receiver				
CD4+ T CELLS + BEADS	+	+	+	+
NON-EXPB10 CELLS	+	-	-	-
EXPB10 CELLS	-	-	+	-
Insert				
NON-EXPB10 CELLS	-	+	-	-
EXPB10 CELLS	-	-	-	+

B



Receiver				
CD4+ T CELLS + BEADS	+	+	+	+
Insert				
CD4+ T CELLS + BEADS	-	+	-	+
NON-EXPB10 CELLS	+	+	-	-
EXPB10 CELLS	-	-	+	+

C



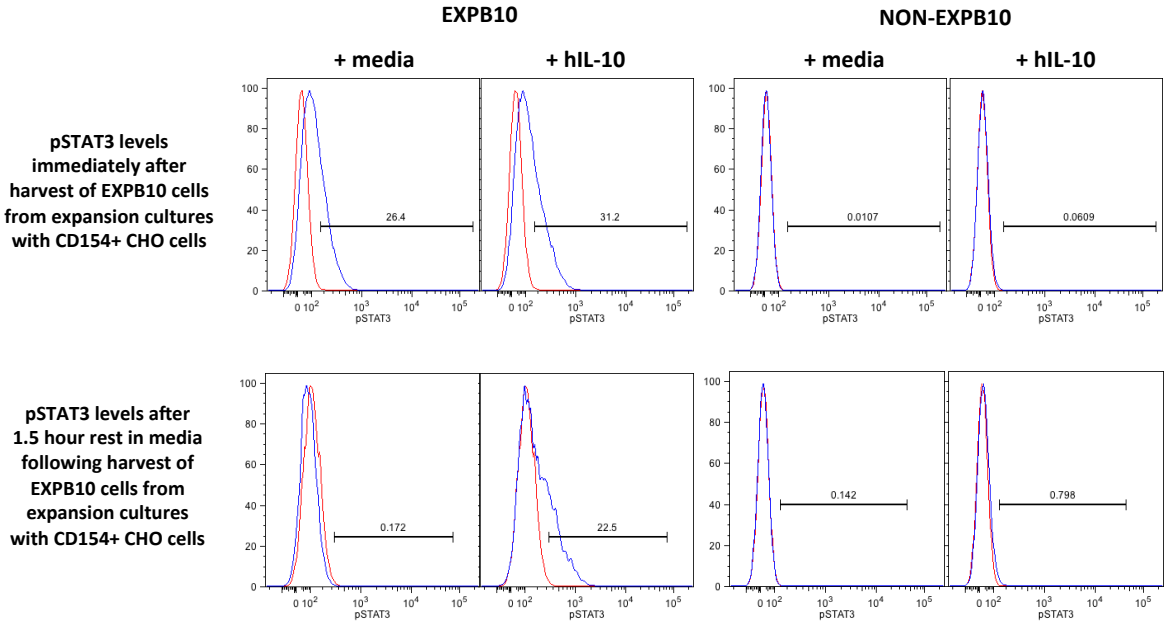
Receiver						
CD4+ T CELLS + BEADS	+	+	+	+	+	+
EXPB10 CELLS	+	+	-	-	-	-
CD154 mAb	-	+	-	-	-	-
Insert						
CD4+ T CELLS + BEADS	-	-	-	-	+	+
EXPB10 CELLS	-	-	+	+	+	+
CD154 mAb	-	-	-	+	-	+

Fig. 4.2.7 EXPB10 cells suppress $CD4^+$ T cell proliferation by both CD154-dependent cognate interactions and by a CD154-independent soluble factor

(a) Use of transwell plates demonstrated that cognate interaction between $CD4^+$ T cells and EXPB10 cells was required for EXPB10 cell-mediated suppression of $CD4^+$ T cells. Conditions in each experiment are described in table. $CD4^+$ T cells were cultured in receiver plate with anti-CD3/CD28 beads. NON-EXPB10 cells and EXPB10 cells were cultured either in the receiver plate or the insert. NON-EXPB10 cells did not significantly suppress $CD4^+$ T cell proliferation when co-cultured in the receiver plate or the insert. EXPB10 cells suppressed $CD4^+$ T cells when cultured in the receiver plate. $CD4^+$ T cell suppression was completely abrogated when EXPB10 cells were cultured in the insert. *$p<0.0001$, paired t-test. **(b)** EXPB10 cells suppressed $CD4^+$ T cell proliferation by both cognate interactions and a soluble factor released following that cognate interaction. $CD4^+$ T cells and anti-CD3/CD28 beads were cultured either in the receiver only or in the receiver and in the insert as indicated in table. NON-EXPB10 cells and EXPB10 cells were cultured in the insert only. Black bar represents suppression of $CD4^+$ T cell proliferation in the receiver; grey bar represents suppression of $CD4^+$ T cell proliferation in the insert. EXPB10 cells were unable to suppress $CD4^+$ T cell proliferation in the receiver when EXPB10 cells only were cultured in the insert and cognate interaction was abrogated. Suppression of $CD4^+$ T cell proliferation in the receiver was partially restored when EXPB10 cells were co-cultured with $CD4^+$ T cells and anti-CD3/CD28 beads in the insert. $CD4^+$ T cell proliferation in the insert was significantly more suppressed by EXPB10 cells in the insert when compared to $CD4^+$ T cell proliferation in the receiver. NON-EXPB10 cells were unable to suppress $CD4^+$ T cell proliferation either in the insert or receiver. *$p<0.0001$, paired t-test. **(c)** EXPB10 cells suppressed proliferation of $CD4^+$ T cells by cognate interaction mediated by CD154-CD40 interaction and also by another unidentified cognate mechanism. $CD4^+$ T cells and anti-CD3/CD28 beads were cultured either in the receiver only or in the receiver and in the insert as indicated in table. EXPB10 cells were cultured in the insert or in the receiver +/- blocking mAb to CD154. When EXPB10 cells and $CD4^+$ T cells were cultured with anti-CD3/CD28 beads in the receiver, proliferation of $CD4^+$ T cells was significantly suppressed. When blocking CD154 mAb was also added to receiver, suppression of $CD4^+$ T cell proliferation was significantly reduced but not abrogated. When EXPB10 cells were cultured in the insert and $CD4^+$ T cells and anti-CD3/CD28 beads were cultured in the receiver, cognate interaction was blocked and suppression was completely abrogated. Addition of CD154 mAb to the insert with EXPB10 cells hd no effect on this abrogation of suppression. When EXPB10 cells were cultured with $CD4^+$ T cells and anti-CD3/CD28 beads in the insert and $CD4^+$ T cells and anti-CD3/CD28 beads were also cultured in the receiver, proliferation of $CD4^+$ T cells in the receiver was partially suppressed but significantly less so than proliferation of $CD4^+$ T cells in the insert. When CD154 mAb was added to the insert under these conditions, suppression of $CD4^+$ T cells in the receiver was unaffected and suppression of $CD4^+$ T cells in the insert was partially reduced but only to the level of suppression of $CD4^+$ T cells in the receiver. *$p<0.0001$, paired t-test. Error bars in each panel represent Mean +/- SD. Data is representative of 3 independent experiments.

Fig. 2.4.8

A


EXPB10
NON-EXPB10
+ media
+ hIL-10
+ media
+ hIL-10
pSTAT3 levels immediately after harvest of EXPB10 cells from expansion cultures with CD154+ CHO cells
26.4
31.2
0.0107
0.0609
pSTAT3 levels after 1.5 hour rest in media following harvest of EXPB10 cells from expansion cultures with CD154+ CHO cells
0.172
22.5
0.142
0.798
pSTAT3

B

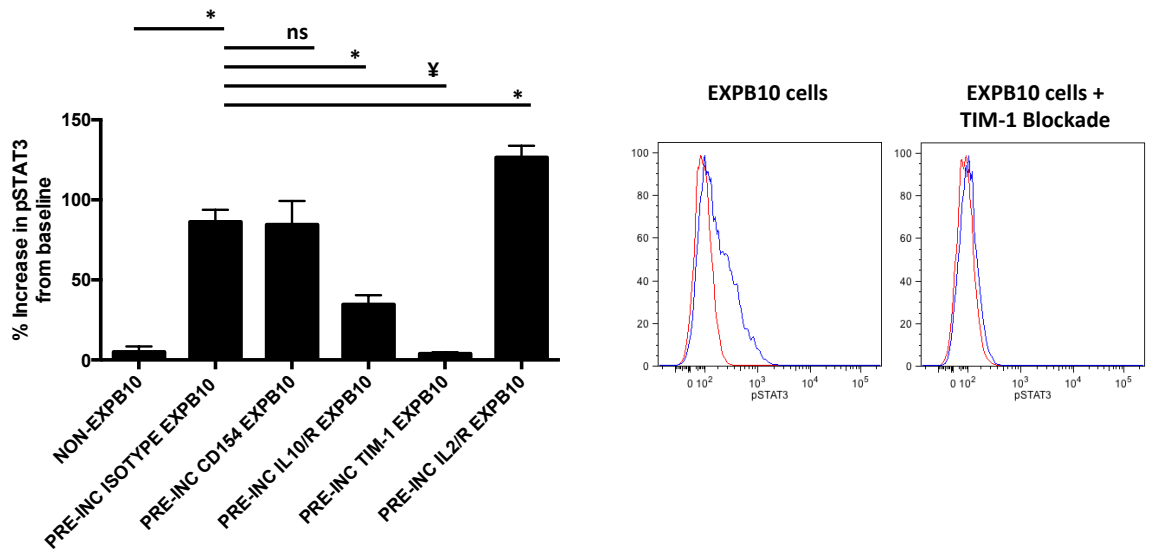

*
ns
*
¥
*
% Increase in pSTAT3 from baseline
150
100
50
0
NON-EXPB10
PRE-INC ISOTYPE EXPB10
PRE-INC CD154 EXPB10
PRE-INC IL10/R EXPB10
PRE-INC TIM-1 EXPB10
PRE-INC IL2/R EXPB10
EXPB10 cells
EXPB10 cells + TIM-1 Blockade
pSTAT3

Fig. 2.4.8 (cont.)

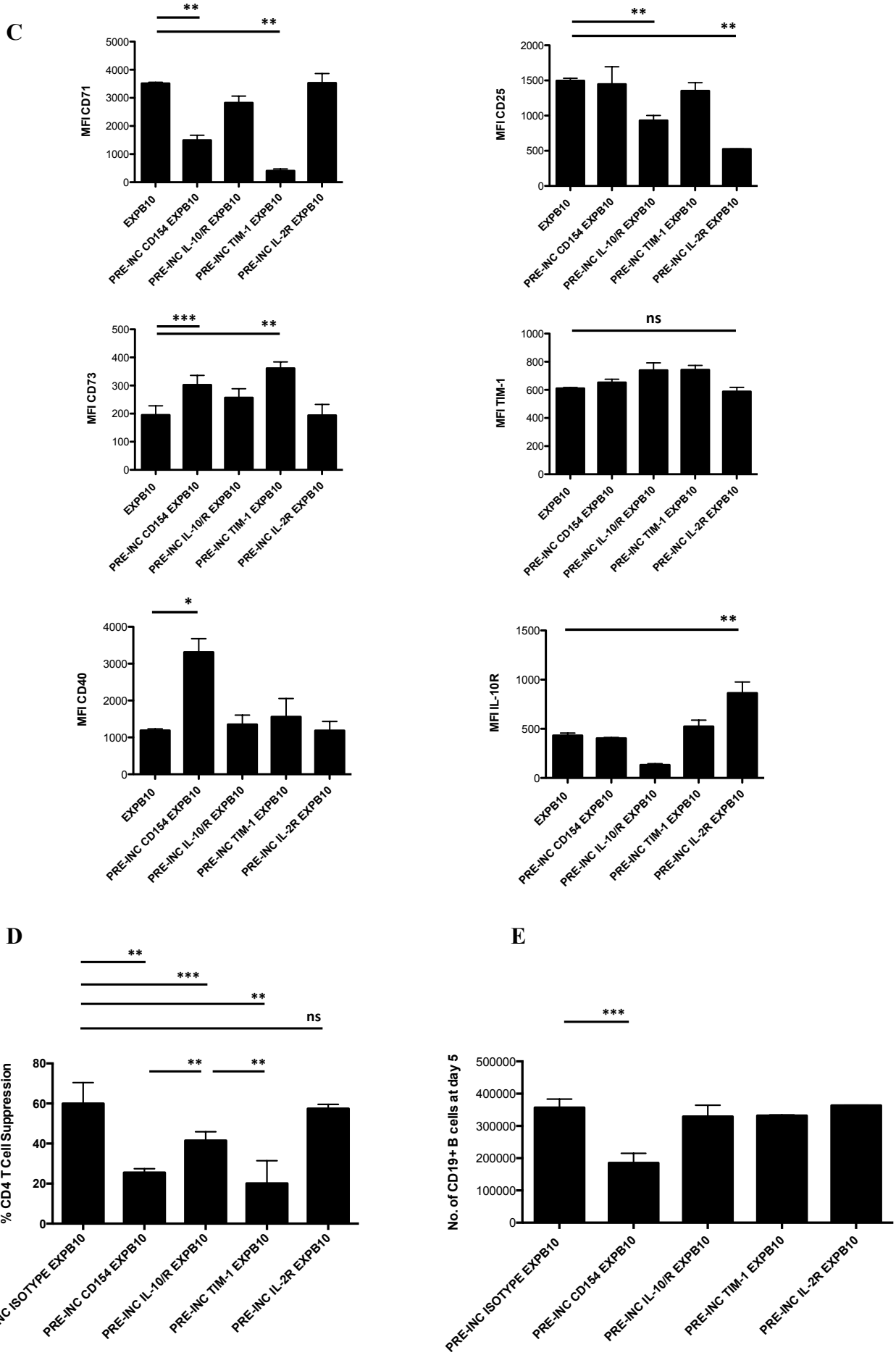
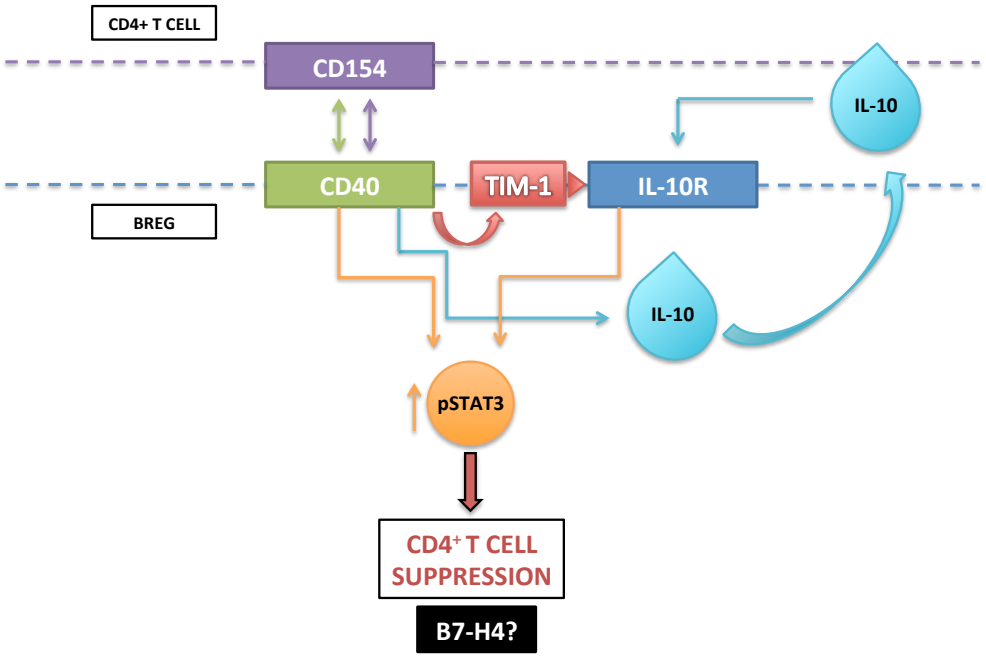

C
MFI CD71
MFI CD25
MFI CD73
MFI TIM-1
MFI CD40
MFI IL-10R
EXPB10
PRE-INC CD154 EXPB10
PRE-INC IL-10/R EXPB10
PRE-INC TIM-1 EXPB10
PRE-INC IL-2R EXPB10
ns
D
% CD4 T Cell Suppression
PRE-INC ISOTYPE EXPB10
E
No. of CD19+ B cells at day 5

Fig. 2.4.8 TIM-1 may modulate EXPB10 cellular responses to IL-10 and affect suppressive potency of EXPB10 cells

(a) Representative histograms of live $CD19^+$ B cells when stained for phosphorylated STAT3 (pSTAT3). EXPB10 cells were stained either immediately after harvest from expansion cultures or after an additional 1.5hr incubation period in cRPMI at 4°C. Prior to the staining process, $CD19^+$ B cells were incubated in media +/- exogenous hIL-10 for 10 minutes. Red line represents unstained control; blue line represents pSTAT3 staining. **(b)** % Increase in pSTAT3 levels expressed by EXPB10 and NON-EXPB10 cells after addition of exogenous hIL-10 relative to that of EXPB10 and NON-EXPB10 cells with no hIL-10 added (baseline). There was a significant decrease in levels of pSTAT3 expressed by PRE-INC TIM-1 EXPB10 and PRE-INC IL-10/R EXPB10 in response to hIL-10. Representative histograms of $CD19^+$ EXPB10 cells. Red line represents unstained control; blue line represents pSTAT3 staining. EXPB10 cells had been pre-incubated with an IgG isotype control (PRE-INC ISOTYPE), blocking CD154 mAb (PRE-INC CD154), IL-10/R mAb (PRE-INC IL-10/R), TIM-1 mAb (PRE-INC TIM-1) or IL-2/R mAbs (PRE-INC IL-2/R) for the last 48 hours of the 7-day expansion protocol. *$p<0.0001$, ¥$p<0.001$, paired t-test. **(c)** MFI of cell surface markers CD71, CD25, CD73, TIM-1, CD40, CD154, IL-10R, B7-H4 expressed by EXPB10 cells pre-incubated with IgG isotype mAb or other blocking mAbs. *$p<0.0001$, **$p<0.005$, ***$p<0.02$, paired t-test. **(d)** % Suppression of $CD4^+$T cell proliferation by EXPB10 cells pre-incubated with IgG isotype mAb or other blocking mAbs. **(e)** No. of $CD19^+$ B cells remaining at day 5 of the suppression assay when $CD4^+$ T cells and anti-CD3/CD28 beads were co-cultured with EXPB10 cells that had been pre-incubated with IgG isotype mAb or other blocking mAbs. ***$p<0.02$, paired t-test. Error bars in each panel represent Mean +/- SD. Data is representative of 4 independent experiments.

Fig 4.2.9

A


CD4+ T CELL
CD154
IL-10
CD40
TIM-1
IL-10R
BREG
IL-10
pSTAT3
CD4+ T CELL SUPPRESSION
B7-H4?

B

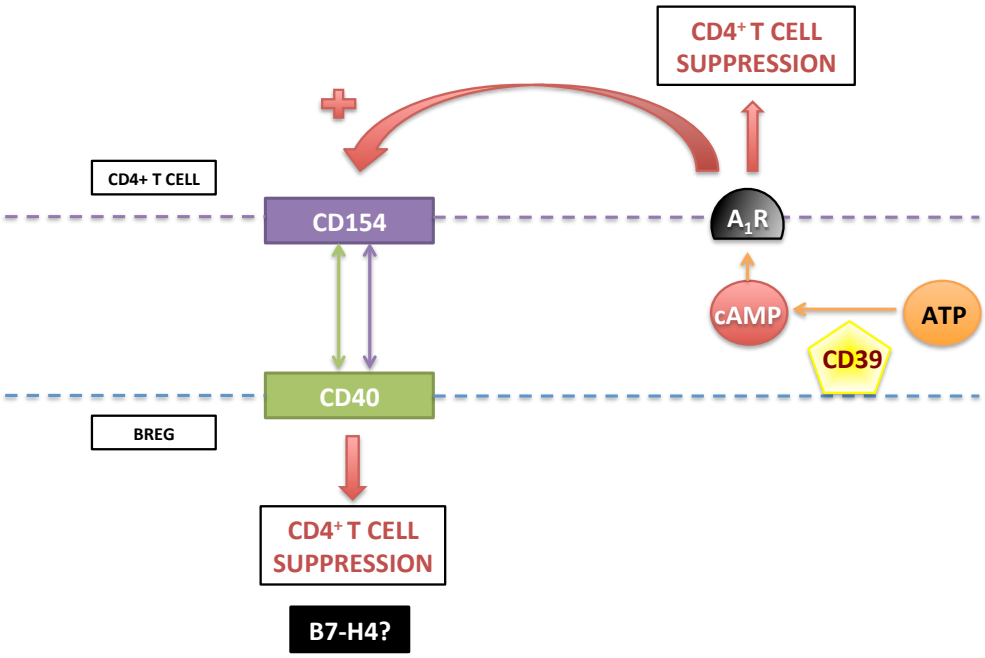

CD4+ T CELL SUPPRESSION
CD4+ T CELL
CD154
A1R
cAMP
ATP
CD39
CD40
BREG
CD4+ T CELL SUPPRESSION
B7-H4?

Fig 4.2.9 Potential mechanisms of suppression

(a) Putative CD154-TIM-1-IL10-pSTAT3 pathway: $CD4^+$ T cell up-regulates CD154 upon antigen-mediated activation and engages with CD40 on Breg. This interaction results in up-regulation of expression of TIM-1 and IL-10R as well as increased levels of pSTAT3 and IL-10 expression. TIM-1 modifies the IL-10R so that it is more responsive to IL-10. IL-10 is secreted from the Breg and binds to the IL-10R in an autocrine manner. This results in further up-regulation of pSTAT3 which subsequently initiates downstream immunosuppressive mechanisms that may include up-regulation of B7-H4 by Breg, to result in suppression of $CD4^+$ T cell proliferation. **(b)** Putative purinergic pathway: After CD154-CD40 engagement, Breg down-regulate CD73 and maintain CD39 expression. CD39 is able to catalyse breakdown of extracellular ATP to cAMP. CD73 has been down-regulated so there is no further catalysis of cAMP to Adenosine, and instead cAMP accumulates in the microenvironment. cAMP may then enter the $CD4^+$ T cell via the A_1R receptor expressed on $CD4^+$ T cells to subsequently up-regulate CD154 and/or prolong engagement of CD154-CD40, thus propagating the CD154-TIM-1-IL10-pSTAT3 pathway. Alternatively cAMP could act directly on the $CD4^+$ T cell to inhibit proliferation in a pathway independent of CD154-TIM-1-IL10-pSTAT3.

5.0 *IN VIVO* FUNCTION OF HUMAN EXPB10 CELLS

Earlier experiments have established that human $CD19^{+}IL\text{-}10^{+}$ B cells with regulatory function can be induced and expanded *in vitro* (*Chapter 3.0*). For the purpose of this project, such a cell population has been termed 'EXPB10.' Phenotypic analysis has demonstrated that this human Breg subset is $CD25^{+}CD71^{+}CD73^{-}$ and that the vast majority of these cells express TIM-1 and IL-10R (*Chapter 4.0*). Mechanistic data revealed that IL-10 may have a predominantly autocrine role in the function of EXPB10 cells, which rely on both cognate interactions, in part including CD154-CD40 engagement, as well as another unidentified soluble factor to fully suppress $CD4^{+}$ T cell proliferation and Th1 differentiation. TIM-1 may modulate Breg cellular responses to endogenous IL-10, and thus dictate suppressive function of EXPB10 cells. Endonucleotidases may also play a role in EXPB10-mediated suppression but this is yet to be confirmed.

Whilst it is important to understand how EXPB10 cells behave *in vitro*, it is critical to the future potential application of this population in transplantation and autoimmunity that we appreciate how EXPB10 cells may function in a much more complex and dynamic *in vivo* environment. A humanised Balb/c $Rag2^{-/-}$ $c\gamma^{-/-}$ mouse model of allograft rejection that was initially established by our group to investigate human Treg, also permits the interrogation of human EXPB10 cell function specifically in the context of transplantation [136, 137]. Here we aim to explore the following hypothesis:

- **EXPB10 cells can prolong allograft survival in a humanised mouse model of skin transplantation**

RESULTS

5.1 *In vivo* function of human EXPB10 cells in a humanised mouse model of transplantation

The Balb/c Rag2$^{-/-}$ cγ$^{-/-}$ mouse is devoid of mouse T cells, B cells and NK cells [136]. To establish a 'human immune system,' human peripheral blood mononuclear cells (PBMC) were injected intraperitoneally (ip), and the mice monitored were monitored for reconstitution of human lymphocytes. Successful reconstitution in this model was defined as presence of more than 1% viable human CD45^{+} (huCD45^{+}) lymphocytes of the total lymphocyte count in the spleen or 0.1% of the total lymphocyte count in peripheral blood [136, 137]. As EXPB10 cells could significantly deplete absolute numbers of CD4^{+} T cells *in vitro*, it was important to first confirm that EXPB10 would not inhibit successful reconstitution in this model. If reconstitution was significantly suppressed, it would be unlikely that rejection of human allograft would be achieved thus invalidating the model for our use. If reconstitution was unaffected, this model could then be used to interrogate the *in vivo* function of EXPB10 cells.

5.1.1 EXPB10 cells do not inhibit reconstitution of human CD45^{+} lymphocytes in a humanised Balb/c Rag2$^{-/-}$ cγ$^{-/-}$ mouse model

Balb/c Rag2$^{-/-}$ cγ$^{-/-}$ mice were injected intra-peritoneally (ip) with human PBMC +/- autologous EXPB10 either ip or intravenously (iv) at a 1:1 ratio (fig. 5.1.1a). When Treg were used in this model previously, they were usually injected ip alongside PBMC [136, 137]. Two different administrative routes of EXPB10 cell injection were initially investigated in this study to determine whether an iv route with rapid access to the spleen, would favour EXPB10 survival without compromising PBMC reconstitution. Reconstitution of huCD45^{+} lymphocytes in peripheral blood was measured every 7 days until the day 28 endpoint, at which time reconstitution was generally achieved [137]. Spleen, peripheral blood, peritoneal fluid and bone marrow were examined at time of harvest.

EXPB10 cells do not inhibit successful reconstitution in the humanised Balb/c Rag2$^{-/-}$ cγ$^{-/-}$ mouse model, irrespective of route of injection.

huCD45^{+} lymphocytes in peripheral blood achieved >0.1% reconstitution in all groups by day 7 post-adoptive transfer (fig. 5.1.1b), although it should be noted that by the endpoint, reconstitution was highly variable within each experimental group. Successful splenic reconstitution was also achieved across all groups at 28 days (fig. 5.1.1c), with no difference in % huCD45^{+} lymphocytes between any group. By day 28, huCD45^{+} lymphocytes were identified in all tissues harvested, comprising blood, spleen, peritoneal fluid and bone marrow. Again there was no difference in percentage of huCD45^{+} lymphocytes between any experimental group of mice, although in all groups the majority of human cells were found in the peritoneal cavity.

huCD45^{+}CD20^{+} B cells can be detected in all tissues harvested

Very low percentage frequencies of huCD45^{+}CD20^{+} B cells were detected in all tissues harvested across all experimental groups, with the lowest percentage of huCD45^{+}CD20^{+} B cells in spleen and blood detected when EXPB10 cells were injected iv; the majority of huCD45^{+} cells in all tissues consisted of CD3^{+} T cells (fig. 5.1.1d, >97% in all groups).

5.1.2 EXPB10 cells do significantly prolong allograft survival in a humanised Balb/c Rag2$^{-/-}$ cγ$^{-/-}$ mouse model of skin transplantation

To investigate whether EXPB10 cells could regulate an immune response to an allograft *in vivo*, a humanised mouse model of human skin transplantation already developed in our laboratory was utilised; protocol as outlined in fig. 5.1.2a [137]. Human skin was transplanted onto Balb/c Rag2$^{-/-}$ cγ$^{-/-}$ mice and left to engraft for 35 days. After 5 weeks, 5x10^{6} human PBMC from a different, HLA-mismatched human donor was injected ip into the engrafted mouse. Mice which had been injected with PBMC alone served as a negative control. Mice which were also injected simultaneously with 5x10^{6} NON-EXPB10 cells autologous to the PBMC, formed a positive control. Mice in the experimental group received 5x10^{6} PBMC and 5x10^{6}

EXPB10 cells from the same human donor. All mice were serially bled every 7 days, with the endpoint comprising either time of rejection or 100 days post-reconstitution. Rejection itself was a macroscopic diagnosis already validated in previous work [137]; diagnosis was blinded and carried out by myself and another researcher experienced with this mouse model. The experiment was conducted four times, with four different, HLA-mismatched human donor pairs. Human donors of skin were denoted with the prefix 'SSGD,' followed by a reference number; human donors of lymphocytes were denoted with the prefix 'S,' followed by a reference number.

In all experiments, mice which had received PBMC and EXPB10 cells demonstrated significantly prolonged allograft survival when compared to those which had received PBMC alone or PBMC and NON-EXPB10 cells (fig. 5.1.2b, *$p<0.03$, **$p<0.0004$). In each experiment, rejection dynamics in the control groups varied, such that with the human donor pair S020-SSGD45, both negative and positive control groups rejected between 26 and 48 days, whereas with the human donor pair S022-SSGD50, both control groups rejected very quickly between 10 and 13 days. Furthermore, with mice which had received lymphocytes from human donor pair S020-SSGD45, the experiment had to be stopped prematurely as all mice succumbed to GVHD by day 65. However in all experiments, EXPB10 cells did appear to significantly prolong allograft survival.

There is no difference in rate of reconstitution between mice which have received PBMC alone, PBMC + NON-EXPB0, or PBMC + EXPB10 cells

Since mice which received skin and lymphocytes from human donor pair S022-SSGD50 rejected from as early as day 10, serial tail bleeds were not possible for all mice from each experimental group. Therefore reconstitution of $huCD45^+$ lymphocytes in peripheral blood across time is only displayed for human donor pairs S020-SSGD45, S028-SSGD49 and S038-SSGD53 (fig. 5.1.2c). There was no difference in rate of reconstitution of $huCD45^+$ lymphocytes in peripheral blood between mice that which received EXPB10 cells and PBMC compared to the two control groups. Furthermore, there did not appear to be a correlation between rate of rejection and rate of reconstitution in any human donor pair experiment.

huCD45$^+$CD20$^+$ B cells are detectable in peripheral blood of mice which have received PBMC + EXPB10 cells but results are variable

Peripheral blood was examined by FACS analysis every 7 days up until the rejection endpoint. In those mice which had received human tissue from human donor pair S020-SSGD45, huCD45$^+$CD20$^+$ B cells were not only detectable in mice which had been reconstituted with PBMC and EXPB10 cells, but this percentage also increased with time (fig. 5.1.2d). However, in mice which had received human tissue from human donor pair S028-SSGD49 and S038-SGD53, minimal levels of huCD45$^+$CD20$^+$ B cells were detected in peripheral blood, and furthermore, huCD45$^+$CD20$^+$ B cells were seen in mice across all experimental groups.

Percentage of huCD45$^+$CD4$^+$ T cells in peripheral blood decreases with time after reconstitution in mice which have received PBMC + EXPB10 cells

The huCD45$^+$ cell compartment was examined for human T cells in mouse peripheral blood. The percentage of huCD45$^+$CD4$^+$ T cells in the peripheral blood of mice which had received PBMC and EXPB10 cells decreased over time when compared to that of mice which had been reconstituted with PBMC alone or PBMC and NON-EXPB10 cells (fig. 5.1.2e).

In mice which had received human tissue from human donor pair S020-SSGD45, there was no difference in percentage of huCD45$^+$CD8$^+$ T cells between mice which had received PBMC and EXPB10 cells compared to mice in control groups (fig. 5.1.2f). In mice which had received human tissue from human donor pairs S028-SSGD49 and S030-SSGD53 however, there may have been a slight increase in percentage of huCD45$^+$CD8$^+$ T cells over time in mice which had been reconstituted with PBMC and EXPB10 cells when compared to control groups. The disparity here may be accounted for by the variability in detection of huCD45$^+$CD20$^+$ B cells (fig. 5.1.2c) across the three different human donor pairs: in the experiment involving S020-SSGD45 human tissue, the increase in percentage of huCD45$^+$CD20$^+$ B cells was offset by a concomitant decrease in percentage of huCD45$^+$CD4$^+$ T cells; in the experiments involving S028-SSGD49 and S038-SSGD53 human tissue, there was no significant increase of huCD45$^+$CD20$^+$ B cells, and so the decrease in percentage of

huCD45^{+}CD4^{+} T cells was instead offset by a slight increase in percentage of huCD45^{+}CD8^{+} T cells over time.

5.1.3 Absolute number of huCD45^{+}CD4^{+} T cells is significantly decreased across all tissues harvested at rejection in mice which have received PBMC + EXPB10 cells compared to control groups

No allograft in any group survived to the 100-day endpoint, and all mice were instead harvested at the rejection time-point. Mice which received human tissue from human donor pair S020-SSGD45 were excluded from further analysis due to the incidence of GVHD. Blood, spleen and skin allograft were all examined by FACS analysis. Unfortunately, it was not feasible to analyse draining or contralateral lymph nodes in this model: given the nature of Balb/c Rag2$^{-/-}$ c$\gamma^{-/-}$ mice, accurate and consistent detection of lymph nodes was not possible. Similar to serial blood measurements over time, both percentage and absolute number of huCD45^{+}CD4^{+} T cells were significantly decreased in peripheral blood, spleen and skin allograft of mice which had been reconstituted by PBMC and EXPB10 cells, in comparison to control groups (fig. 5.1.3a-c, *p<0.03, ***p<0.005, $^{¥}$p<0.001). Absolute number and percentage of huCD45^{+}CD8^{+} T cells remained unchanged between experimental groups in spleen and skin; in blood however, absolute number and percentage of huCD45^{+}CD8^{+} T cells were significantly increased in mice that had received PBMC + EXPB10 cells compared to mice that had received PBMC alone (fig. 5.1.3a, **p<0.0004).

Significant increase in percentage of putative huCD45^{+}CD4^{+}CD25^{+}CD127^{lo} Treg is observed in skin allografts of mice reconstituted with PBMC + EXPB10 cells in comparison to control groups

There is growing evidence that Breg, both mouse and human, may support the induction and expansion of Treg [27, 28, 39, 43, 47]. FACS analysis of the huCD45^{+}CD4^{+} T cell compartment revealed a significant increase in percentage of putative huCD45^{+}CD4^{+}CD25^{+}CD127^{lo} Treg within the skin allografts of mice reconstituted with PBMC and EXPB10 cells, when compared to those of mice reconstituted with either PBMC alone or PBMC and NON-EXPB10 cells (fig. 5.1.3d,

¥¥p<0.01). There was a corresponding increase in absolute numbers of huCD45$^+$CD4$^+$CD25$^+$CD127^{lo} Treg in skin, despite the finding that total number of huCD45$^+$CD4$^+$ T cells in the skin allograft was lower in mice which have received PBMC and EXPB10 cells when compared to control groups (fig. 5.1.3c). There was a significant reduction in total number of putative huCD45$^+$CD4$^+$CD25$^+$CD127^{lo} Treg in both spleen and peripheral blood (fig. 5.1.3d, *p<0.03, ***p<0.005).

Significant increase in percentage of huCD45$^+$CD4$^+$FoxP3$^+$ T cells is observed in skin allografts of mice reconstituted with PBMC + EXPB10 cells in comparison to control groups

Since there may have been an increase in human Treg in the skin of allograft, FoxP3 expression was also analysed. Similar to findings with the putative huCD45$^+$CD4$^+$CD25$^+$CD127^{lo} Treg phenotype, a significant increase in percentage of huCD45$^+$CD4$^+$FoxP3$^+$ T cells was observed in skin allografts of mice reconstituted with PBMC and EXPB10 cells, when compared to those of mice in control groups (fig. 5.1.3.e, *p<0.03).

5.1.4 Percentage and absolute number of huCD45$^+$CD20$^+$ B cells are significantly increased in spleen and skin allograft of mice which have been reconstituted with PBMC + EXPB10 cells in comparison to control groups

A significant increase in percentage and total number of huCD45$^+$CD20$^+$ B cells in spleen and skin allograft was seen in those mice reconstituted with PBMC and EXPB10 cells compared to mice reconstituted with PBMC alone or PBMC and NON-EXPB10 cells (fig. 5.1.4a *p<0.03). The percentage and total number of huCD45$^+$CD20$^+$ B cells found in peripheral blood at time of rejection was highly variable between human donor pairs, but there was no difference between mice that have received EXPB10 cells compared to either control group.

Large proportion of huCD45$^+$CD20$^+$ B cells in mice reconstituted with PBMC + EXPB10 cells are TIM-1$^+$ but do not express the CD25$^+$CD71$^+$CD73$^-$ EXPB10 phenotype

The phenotype of huCD45$^+$CD20$^+$ B cell found in mice receiving PBMC and EXPB10 cells was analysed by FACS. Human B cells in this experimental group did not appear to retain the CD25$^+$CD71$^+$CD73$^-$ EXPB10 phenotype. However, a large proportion of huCD45$^+$CD20$^+$ B cells in blood (when present), spleen and skin did express TIM-1 (fig. 5.1.4b).

5.1.5 Immunoglobulin and Cytokines

Serum of mice that had been reconstituted with PBMC only, PBMC + NON-EXPB10 or PBMC + EXPB10 cells was sampled at time of rejection and analysed for both human cytokines and immunoglobulin, using cytokine bead arrays.

There are no significant differences in serum levels of human immunoglobulin between mice that have received EXPB10 cells compared to control groups

Both total human IgG and human IgM were detectable in the serum of fully reconstituted, humanised mice at the time of allograft rejection. However, there were no significant differences in immunoglobulin levels between experimental groups (fig. 5.1.5a).

Pro-inflammatory human cytokines IL-2 and IL-17a are significantly reduced at time of rejection in mice that have received EXPB10 cells compared to control groups

Analysis by cytokine bead array demonstrated that whilst there were no significant differences in serum levels of human cytokines IL-4, IL-6, TNFα, IFNγ and IL-10 across the different experimental groups, mice that had received EXPB10 cells did demonstrate significantly lower levels of IL-2 and IL-17a compared to controls (fig. 5.1.5b, *$p<0.003$, **$p<0.03$). There was also a trend to a slight increase in levels of

IL-10 in the EXPB10 group; however there were large outliers and this result was not significant.

5.2 Induction and expansion of human $CD4^+CD25^+CD127^{lo}$ Treg by autologous EXPB10 cells *in vitro*

We have observed an apparent increase in the percentage of putative $huCD45^+CD4^+CD25^+CD127^{lo}$ Treg and FoxP3 expression *in vivo* (fig. 5.1.3d-e), within skin allografts of humanised mice that have been reconstituted with PBMC and EXPB10 cells allogeneic to the allograft. Due to limitations of the model, it was not possible to extract sufficient numbers of this apparent Treg population from allograft tissue to then enable testing of suppressive function directly. Therefore we looked back to the *in vitro* system to explore whether human EXPB10 cells could indeed expand or induce functional Treg from autologous $CD4^+$ T cells.

5.2.1 Co-culture of EXPB10 cells with autologous $CD4^+$ T cells and anti-CD3/CD28 beads results in an increase in percentage of human $CD4^+CD25^+CD127^{lo}$ T cells

Suppression assays were performed as previously described (*Chapter 2.0*). Briefly, EXPB10 cells were co-cultured with autologous, polyclonally stimulated $CD4^+$ T cells at a 1:1 ratio for 5 days. On day 5, cells were harvested, stained and subjected to FACS analysis.

There was a significant increase in percentage of $CD4^+CD25^+CD127^{lo}$ T cells when autologous, polyclonally stimulated $CD4^+$ T cells were co-cultured with EXPB10 cells, when compared to polyclonally stimulated $CD4^+$ T cells cultured alone or with NON-EXPB10 cells (fig. 5.2.1a, *$p<0.03$, ¥¥$p<0.01$). However, the absolute number of $CD4^+CD25^+CD127^{lo}$ T cells was decreased in the presence of EXPB10 cells due to the overall decrease in total number of human $CD4^+$ T cells which occurred during the suppression assay (fig. 5.2.1b, *$p<0.03$).

Proliferation of human $CD4^+CD25^+CD127^{lo}$ T cells is suppressed by EXPB10 cells to equivalent levels as total $CD4^+$ T cells

$CD4^+CD25^+CD127^{lo}$ T cells appeared to preferentially survive or expand in the presence of EXPB10 cells. When proliferation profiles of this $CD4^+$ T cell subset were examined by analysing VPD dilution, it was found that proliferation of putative Treg was suppressed to equal levels as total $CD4^+$ T cells and putative $CD4^+CD25^+CD127^+$ T effectors (Teff) (fig. 5.2.1b). These data indicate that whilst the overall percentage of potential Treg has increased in the presence of EXPB10 cells, this is more likely due to preferential survival or induction, rather than expansion.

A significant increase in percentage of $CD4^+CD25^+CD127^{lo}$ T cells in the presence of EXPB10 cells is still observed when EXPB10-mediated suppression is abrogated by CD154- or TIM-1-blockade

Earlier experiments have demonstrated that the presence of a blocking mAb to CD154 in suppression co-cultures, or the pre-incubation of EXPB10 cells with the blocking mAb to TIM-1 (PRE-INC TIM-1 EXPB10) for the last 48 hours of the expansion protocol, resulted in significantly reduced EXPB10-mediated suppression (*Chapter 4.0* fig. 4.2.4a & 4.2.8d). In order to clarify whether it was the increase in putative Treg that which accounts for EXPB10-mediated suppression of $CD4^+$ T cell proliferation, suppression assays were set up with the addition of blocking mAb CD154 or with PRE-INC TIM-1 EXPB10. One would predict that if there was no longer an increase in percentage of putative $CD4^+CD25^+CD127^{lo}$ Treg in the presence of EXPB10 cells when suppression was significantly reduced, then it would be unlikely that generation of Treg was the principle mechanism by which EXPB10 cells mediated suppression. However, despite significant reduction in suppressive potency under these conditions, an associated increase in percentage of $CD4^+CD25^+CD127^{lo}$ T cells was still seen in the presence of EXPB10 cells, equivalent to those in culture conditions where suppression was not blocked (fig. 5.2.1c).

EXPB10 cells can significantly suppress proliferation of polyclonally stimulated $CD4^+CD25^-$ T cells

To elucidate whether EXPB10 cells were able to suppress autologous $CD4^+$ T cell responses in the absence of an already existing Treg population i.e. in the absence of Treg expansion, suppression assays were conducted by co-culturing EXPB10 cells at a 1:1 ratio with $CD4^+CD25^-$ T cells and anti-CD3/CD28 beads for 5 days. $CD4^+CD25^-$ T cells were initially isolated through sequential negative selection of $CD4^+$ T cells followed by positive selection of $CD25^+$ T cells. FACS analysis of co-cultures harvested at day 5 revealed that EXPB10 cells were able to suppress $CD4^+CD25^-$ T cells at equal potency to total $CD4^+$ T cells (fig. 5.2.1d).

Co-culture of $CD4^+CD25^-$ T cells with EXPB10 cells is associated with a significant increase in percentage of $CD4^+CD25^+CD127^{lo}$ T cells

The phenotype of $CD4^+$ T cells was analysed at day 5 of co-culture of EXPB10 cells with $CD4^+CD25^-$ T cells and beads. Similar to co-culture with total $CD4^+$ T cells, the presence of EXPB10 cells was associated with a significant increase in percentage of $CD4^+CD25^+CD127^{lo}$ T cells when compared to $CD4^+CD25^-$ T cells cultured with beads alone or with beads and NON-EXPB10 cells (fig. 5.2.1e, $^{**}p<0.0004$, $^{***}p<0.005$). Again total numbers of $CD4^+CD25^+CD127^{lo}$ T cells in the presence of EXPB10 cells was reduced due to the overall reduction in total $CD4^+$ T cell numbers ($^*p<0.03$). These data support the theory that EXPB10 cells may be able to support the preferential induction or survival of $CD4^+CD25^+CD127^{lo}$ Treg.

5.2.2 EXPB10-generated $CD4^+CD25^+CD127^{lo}$ T cells are functional Treg that can suppress an autologous immune response *in vitro*

Although previous data presented in this chapter suggested that EXPB10 cells could induce or expand Treg, it was important to clarify whether the observed percentage increase in $CD4^+CD25^+CD127^{lo}$ T cells represented an increase in an actually functional regulatory cell population. Suppression assays were set up, with autologous $CD4^+$ T cells co-cultured for 5 days with anti-CD3/CD28 beads alone,

with beads and NON-EXPB10 cells, or with beads and EXPB10 cells at a 1:1 ratio. After 5 days, anti-CD3/CD28 beads were removed by magnetic separation, and $CD4^+CD25^+CD127^{lo}$ T cells were then FACS-sorted to >94% purity from each condition (fig. 5.2.2a). The resulting T cell populations were subsequently re-stained with VPD and were co-cultured with anti-CD3/CD28 beads and autologous, CFSE-stained PBMC at decreasing ratios for 5 days.

Analysis of CFSE dilution revealed that $CD4^+CD25^+CD127^{lo}$ T cells isolated from all co-culture conditions were functional Treg which were able to potently suppress proliferation of autologous $CD4^+$ T cell responses *in vitro* (fig. 5.2.2b). $CD4^+CD25^+CD127^{lo}$ Treg which had been generated in the presence of EXPB10 cells were significantly albeit slightly less suppressive than those Treg generated in the absence of $CD19^+$ B cells or with NON-EXPB10 cells at most ratios of PBMC to Treg (fig. 5.2.2b, **p<0.0004).

These data illustrate that on a cell per cell basis, EXPB10-generated $CD4^+CD25^+CD127^{lo}$ Treg may be slightly less suppressive or less stable than $CD4^+CD25^+CD127^{lo}$ Treg generated in the absence of $CD19^+$ B cells, or in the presence of NON-EXPB10 cells. However, a significantly greater proportion of $CD4^+$ T cells appear to be functional, potent Treg when co-cultured with EXPB10 cells when compared to controls (fig. 5.2.1a).

EXPB10 cells do not appear to generate Tr1 cells *in vitro*

EXPB10 cells could produce IL-10 (fig. 3.1.1f) and were able to preferentially support the generation of functional $CD4^+CD25^+CD127^{lo}$ Treg (fig. 5.2.2b). Other IL-10^+ antigen presenting cells such as IL-10^+ dendritic cells have been shown to induce IL-10^+ Tr1 cells [266]. To investigate whether EXPB10 cells were able to induce or expand Tr1 cells, suppression assays were set up with the standard conditions described above. At day 5 of co-culture, wells were harvested and $CD4^+$ T cells FACS sorted to >95% purity (*data not shown)*. Isolated $CD4^+$ T cells that had been previously co-cultured with anti-CD3/CD28 beads and in the absence or presence of NON-EXPB10 or EXPB10 cells, were then stimulated in fresh media with PMA and ionomycin for 6 hours. Supernatant were subsequently harvested and

analysed by a cytokine bead array for IL-10. Minimal levels of IL-10 were detected in supernatants of stimulated $CD4^+$ T cells; there was no significant difference in levels of IL-10 measured in supernatants of $CD4^+$ T cells that had previously been co-cultured with anti-CD3/CD28 beads alone, with anti-CD3/CD28 beads and NON-EXPB10 cells, or anti-CD3/CD28 beads and EXPB10 cells. Thus it appears that EXPB10 cells do not generate Tr1 cells *in vitro*, when a Tr1 cell is defined as one that expresses IL-10 upon stimulation with PMA and ionomycin for 6 hours.

5.3 Identification of $CD19^+CD25^+CD71^+CD73^-$ B cells in human peripheral blood

$CD19^+CD25^+CD71^+CD73^-$ B cells have already been identified in peripheral blood and have been found to be enriched in human individuals who are immunologically tolerant to bee venom [53]. Given that EXPB10 cells were $CD25^+CD71^+CD73^-$ and expressed both TIM-1 and CD154, both of which appeared to dictate potency of this *ex vivo*-generated human Breg population (*Chapter 4.0*), we examined PBMC isolated from peripheral blood of 10 healthy individuals for distribution of these markers, both within whole $CD19^+$ B cells and B cell subsets which have been reported in the literature to be enriched for IL-10^+ Breg [20, 48]. The gating strategy for $CD19^+CD73^-CD25^+CD71^+$ B cells, $CD19^+CD24^{hi}CD38^{hi}$ B cells and $CD19^+CD24^{hi}CD27^+$ B cells is illustrated in fig. 5.1.3a. All gating was performed on mf-1 controls.

5.3.1 $CD19^+CD25^+CD71^+CD73^-$ B cells are present in peripheral blood of healthy subjects, including within $CD24^{hi}CD38^{hi}$ B cell and $CD24^{hi}CD27^+$ B cell subsets

Expression of CD73, CD25 and CD71 by live $CD19^+$ B cells, as well as by known Breg-enriched $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^+$ B cell subsets, was analysed (fig. 5.1.3a). $CD19^+CD25^+CD71^+CD73^-$ B cells were present in all 10 samples, and furthermore, this B cell subset was identified in both the transitional and memory B cell compartments. Percentage frequency of $CD19^+CD25^+CD71^+CD73^-$ B cells was

similar in both whole $CD19^+$ B cells and the $CD24^{hi}CD27^+$ B cell subset; however percentage frequency of $CD19^+CD25^+CD71^+CD73^-$ B cells was significantly reduced in the transitional $CD24^{hi}CD38^{hi}$ B cell compartment (fig. 5.3.1b, **p<0.0004).

TIM-1 expression is significantly enriched in $CD19^+CD25^+CD71^+CD73^-$ B cells

TIM-1 expression in $CD19^+CD25^+CD71^+CD73^-$ B cells was significantly enriched when compared to that of whole $CD19^+$ B cells as well as both $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^+$ B cell subsets (fig. 5.1.3c, ¥¥¥p<0.05, **p<0.0004).

CD154 expression is significantly enriched in $CD19^+CD25^+CD71^+CD73^-$ B cells

CD154 expression was also significantly elevated in the $CD19^+CD25^+CD71^+CD73^-$ B cell subset in comparison to that of whole $CD19^+$ B cells as well as both $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^+$ B cell populations (fig. 5.1.3d, **p<0.0004).

5.3.2 $CD19^+CD25^+CD71^+CD73^-$ B cells are enriched in patients with squamous cell carcinoma (SCC) and long-term kidney transplant recipients (LTKTRs)

Human Treg and Breg populations have been identified in patients with cancer and those who have received kidney transplants and are operationally tolerant [12, 13, 107]. PBMC samples were obtained from patients that had either been diagnosed with a squamous cell carcinoma (SCC) of the skin (n=8), or cancer-free patients who had undergone a kidney transplant between 15-25 years ago and who had stable graft function (n=6). Demographics of these patients are illustrated in fig. 5.3.2a. Analysis of peripheral blood that had been taken from patients diagnosed with SCC of the skin revealed a significant increase in percentage frequency of $CD19^+CD25^+CD71^+CD73^-$ B cells when compared to unmatched, healthy controls (fig. 5.3.2a, **p<0.0004). There was no significant difference in levels of $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^+$ B cell populations between these two subject groups.

In addition, analysis of peripheral blood from long-term kidney transplant recipients with stable graft function (LKTRs) demonstrated a significant increase in percentage levels of $CD19^{+}CD25^{+}CD71^{+}CD73^{-}$ B cells when compared to unmatched, healthy controls (fig. 5.3.2b, **$p<0.0004$). There was again no significant difference in levels of $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^{+}$ B cell populations between these two subject groups. As demonstrated in fig. 5.3.2.a, the LKTRs sampled were taking a broad array of immunosuppressants, but due to the small sample size it was not possible to stratify according to different regimens.

DISCUSSION

Earlier work in this project established that human $CD19^{+}IL\text{-}10^{+}$ B cells with regulatory function could be generated *ex vivo*. This EXPB10 cell population was $CD25^{+}CD71^{+}CD73^{-}$, and a large proportion expressed both TIM-1 and IL-10R. Suppression of polyclonally stimulated $CD4^{+}$ T cell responses required cognate interactions, in part dependent on CD154-CD40 engagement, as well as soluble factors including IL-10 and possibly cAMP.

However, it had not yet been established how EXPB10 cells would behave when in a much more complex, *in vivo* environment. EXPB10 cells were polyclonally activated by $CD154^{+}$ CHO cells and so were unlikely to be antigen-specific. In addition, it has been demonstrated that EXPB10 cells were highly activated and were capable of stimulating $CD4^{+}$ T cell proliferation when in the absence of a prolonged activating signal provided by anti-CD3/CD28 beads (fig. 3.2.1g, 4.1.1a, 4.2.4d). Furthermore, when CD154-CD40 interactions were blocked, EXPB10 cells down-regulated CD71 and CD25 expression, demonstrating labile stability (fig. 4.2.4c).

The humanised mouse model provided a means by which some of these potential issues could be explored. In the first instance, it was important to clarify whether this particular Balb/c $Rag2^{-/-}c\gamma^{-/-}$ mouse model was appropriate to use in the initial interrogation of EXPB10 cell function. In order to permit human alloresponses to human tissue, successful reconstitution of human PBMC had to be achieved in both peripheral blood (>0.1%) and spleen (>1%) [136, 137]. Although EXPB10 cells were able to profoundly suppress $CD4^{+}$ T cell number *in vitro*, when EXPB10 cells were injected either alongside autologous human PBMC ip or separately iv, there was no adverse effect on rate of reconstitution of $huCD45^{+}$ lymphocytes in peripheral blood over 28 days, nor on reconstitution in spleen, peritoneal fluid or bone marrow when compared to mice which received PBMC only (fig. 5.1.1b & 5.1.1c). The majority of $huCD45^{+}$ lymphocytes were found in peritoneal fluid across all experimental groups at 28 days, possibly reflecting the ip administration of PBMC in all mice. Human lymphocytes are likely to have been absorbed much more slowly into the circulation from the peritoneal cavity, in comparison to direct iv injection.

Once it was established that EXPB10 cells did not inhibit successful human leucocyte chimerism in this model, it was possible to ask whether EXPB10 cells were able to exert suppressive function *in vivo*. Since slightly higher percentages of huCD45$^+$CD20$^+$ B cells were identified in the blood and spleen of mice that had received EXPB10 cells ip rather than iv (fig. 5.1.1d), this route of EXPB10 cell administration was opted for during transplantation experiments. Ip injection of both PBMC and EXPB10 at a 1:1 ratio into mice that had been engrafted with human skin resulted in significant prolongation of allograft survival when compared to mice that had received either PBMC alone or PBMC and NON-EXPB10 cells (fig. 5.1.2b). There was no difference in allograft survival in mice which received PBMC + NON-EXPB10 cells when compared to those which had been reconstituted with PBMC alone, indicating that unmanipulated CD19$^+$ B cells did not confer any effect, positive or negative, on T cell function in this model. In addition, there appeared to be no difference in rate of reconstitution between mice which had received PBMC + EXPB10 cells compared to control groups in the presence of allograft (5.1.2c). These findings are important in that they demonstrated that EXPB10 cells were able to suppress alloresponses to human tissue *in vivo,* whilst having no effect on homeostatic proliferation required to achieve sufficient human leucocyte chimerism in this model.

The rate of rejection of both controls and mice which had received EXPB10 cells did however vary substantially between human donor pairs (fig 5.1.2b). There was no significant disparity between HLA mismatches of the donor pairs (median mismatch of 5 across HLA-A, -B and DRβ1) to account for this difference. Given that PBMC was human donor-derived, this difference may instead have been due to intrinsic variation between the human donors used, particularly given that rate of reconstitution also differed between human donor pairs and had no bearing on rate of rejection (fig. 5.1.2b & 5.2.1c). As all donor blood was obtained from NHSBT, subject demographics were anonymised and not available; therefore it was not possible to evaluate these data in the context of human donor age or medical history, both of which may affect the dynamics of an immune response [267, 268]. It may have been valuable to analyse the composition of T cell and B cell compartments of PBMC in the different human donors used, and establish any obvious differences prior to injection. Alternatively, this difference in rejection rate may have been due to

technical discrepancies during ip injection, such that variable numbers of PBMC +/- EXPB10/NON-EXPB10 cells were injected between experiments involving different human donor pairs.

The detection of $CD20^+$ B cells was highly variable in peripheral blood between human donor groups during reconstitution in the presence of allograft (fig. 5.1.2d). Mice which received PBMC, EXPB10 cells and skin allograft from human donor pair S020-SSGD45, demonstrated a significant increase in $huCD45^+CD20^+$ B cells in peripheral blood with time compared to control groups, unlike in other human donor pairs (fig. 5.1.2d). However three of five mice which received EXPB10 cells in this experiment also went on to develop GVHD between days 62-65 (fig. 5.1.2b). There was no correlation between percentage level of $huCD45^+CD20^+$ B cells and those mice that developed GVHD in this experimental group, but it is not clear from this result whether the two findings were linked. GVHD in this model is the term given to human anti-mouse responses, and is a long-recognised complication of human PBMC-engrafted immunodeficient mice [269]. The phenomenon manifests with symptoms such as severe and sudden weight loss (>20%) and anaemia, requiring the animal to be euthanised. The three mice that did exhibit GVHD, did so with an intact allograft, suggesting that EXPB10 cells have suppressed only those $CD4^+$ T cells responses specific to the allograft. It is of course possible that EXPB10 cells themselves were contributing to GVHD: EXPB10 cells were highly activated and expressed pro-inflammatory cytokines. However, serum analysis of human cytokines from mice that received EXPB10 cells revealed a significant decrease in both IL-2 and IL-17a compared to that of controls, and no difference in IL-6, IFNγ and TNFα at time of rejection (fig. 5.1.5b). GVHD was not seen in subsequent experiments with other human donor pairs, perhaps again reflecting the varying immune responses of PBMC from different human donors. As this GVHD result represented an inconsistency that could not be readily explained, this particular human donor pair experiment was excluded from subsequent analyses.

Examination of the T cell compartments in blood, spleen and skin allograft at time of rejection, revealed a significant decrease in both percentage and total number of $huCD45^+CD4^+$ T cells in the presence of EXPB10 cells (fig. 5.1.3a-c); there was no significant difference in $huCD45^+CD8^+$ T cell percentage and number in spleen and

skin in those mice that had received EXPB10 cells compared to control groups, but a significant increase was seen in blood of mice that had received EXPB10 cells (fig. 5.1.3a). Similar observations were made in peripheral blood from serial measurements over the first 28 days following reconstitution (fig. 5.1.2e-f). Given that total number of huCD45$^+$CD4$^+$ T cells was reduced as well as percentage at time of rejection, it is unlikely that this decrease was simply the consequence of an additional cell population being present (EXPB10 cells), thus giving the appearance of T cell depletion. Furthermore, total numbers of huCD45$^+$CD20$^+$ B cells present in both spleen and blood were still relatively low in comparison to total numbers of huCD45$^+$CD3$^+$ T cells in mice which had received EXPB10 cells (fig. 5.1.3a-b & 5.1.4a); therefore it is less likely that EXPB10 cells simply had a greater survival or proliferative capacity than NON-EXPB10 cells and huCD45$^+$CD3$^+$ T cells, and so were selectively crowding out huCD45$^+$CD4$^+$ T cells. It is more probable therefore, that the observed reduction in numbers of CD4$^+$ T cells in blood, spleen and skin allograft in the presence of EXPB10 cells represented a true result documenting suppression, corresponding with increased allograft survival. EXPB10 cells may have achieved increased allograft survival through the inhibition of proliferation and function of CD4$^+$ T cells, supporting earlier *in vitro* observations (*Chapters 3.0 & 4.0*).

Serum levels of pro-inflammatory cytokines IL-2 and IL-17a were significantly reduced in mice which received PBMC + EXPB10 cells, despite these samples being taken at time of rejection (fig. 5.1.5b). Unlike findings indicated by *in vitro* data, serum levels of IFNγ and TNFα were not significantly decreased in these mice. Serum levels of IL-10 were increased in mice which received PBMC + EXPB10 cells when compared to control groups; however this result was not statistically significant due to large outliers. As these samples were taken at time of rejection, it is difficult to interpret the true meaning of such results. The significant reduction in levels of IL-2 and IL-17a may reflect EXPB10-mediated suppression, as may the potential increase in IL-10 levels; alternatively the differences seen may represent differential sampling after or during rejection episodes, such that what has been captured is actually the subsiding inflammatory response. Earlier serum samples should be measured in order to clarify this.

The greatest proportion of huCD45$^+$CD20$^+$ B cells at time of rejection was found in the skin, whilst the greatest number of huCD45$^+$CD20$^+$ B cells at time of rejection was found in the spleen (fig. 5.1.4a). This was true across all experimental groups, although there was a significantly greater percentage and total number of huCD45$^+$CD20$^+$ B cells in those mice which have received PBMC + EXPB10 cells when compared to control groups, in spleen and skin at time of rejection. It is not evident where suppression of the alloresponse was taking place, from these experiments. Total numbers of huCD45$^+$CD4$^+$ T cells were decreased in blood, spleen and skin of mice which had received PBMC + EXPB10 cells (fig. 5.1.4a), but these measurements were taken at the time of rejection. It would be valuable to analyse the different compartments at a time-point earlier than that of rejection to determine where the majority of huCD45$^+$CD20$^+$ B cells have infiltrated prior to allograft rejection. However given that different human donor pairs were associated with different rates of rejection, this time-point may be difficult to ascertain.

Since it has been established *in vitro* that EXPB10 cells require CD154-CD40 engagement to exert suppressive function, it is tempting to speculate that EXPB10 cells were only able to suppress allospecific CD4$^+$ T cells when they themselves had been presented with antigen and had up-regulated CD154. Indeed, huCD45$^+$CD4$^+$ T cells are unlikely to express CD154 unless activated in an antigen-specific manner [270]. Passenger dendritic cells originating from within the skin allograft are thought to present antigen directly to allospecific huCD45$^+$CD4$^+$ T cells at the draining lymph node and possibly in the allograft itself (*F. Issa, personal communication*). EXPB10 cells were highly activated and expressed high levels of CD80 and CD86; it is possible that allospecific EXPB10 cells processed alloantigen and indirectly presented to allospecific CD4$^+$ T cells, either in the allograft or lymphoid tissue, thereby engaging the CD154-CD40 interaction and initiating suppressive function. Alternatively, EXPB10 cells may have engaged with activated CD4$^+$ T cells after antigen presentation had already occurred. Lymph node analysis would be helpful to determine whether antigen presentation occurs at this site, as is thought with Treg and T effectors [137]. However it was not possible to accurately and consistently identify peripheral lymphoid tissue in these experiments, possibly due to the *Rag2*$^{-/-}$ *cγ*$^{-/-}$ background of these mice [271].

The largest number of human lymphocytes was detected, perhaps unsurprisingly, in the spleens of reconstituted Balb/c $Rag2^{-/-}$ $c\gamma^{-/-}$ mice (fig. 5.1.3b & fig. 5.1.4a). It is possible that human lymphoid follicles were able to form within the mouse spleen, resulting in CD154-CD40 engagement between activated $huCD45^+CD4^+$ T cells and EXPB10 cells, and subsequent polarisation of an allospecific EXPB10 suppressive response; although the lack of human BAFF and HLA expression in this murine model makes the formation of a potentially robust lymphoid structure less likely [271, 272]. Immunohistochemical analysis of the spleen at a time-point preceding rejection may help to answer this possibility.

A large proportion of $huCD45^+CD20^+$ B cells in mice which had been reconstituted with PBMC + EXPB10 cells were TIM-1^+, much like $CD25^+CD71^+CD73^-$ EXPB10 cells prior to *in vivo* administration (fig. 5.1.4b). Although $huCD45^+CD20^+$ B cells in these mice were not $CD25^+CD71^+CD73^-$ at time of rejection (*data not shown*), the expression of TIM-1 suggests that some stability may have been retained by EXPB10 cells in an *in vivo* environment. The *in vitro* observation that down-regulation of CD71 and CD25 expression correlated with loss of EXPB10 cell suppression function does raise the possibility that the $huCD45^+CD20^+$TIM-1^+ B cells detected in the skin were no longer suppressive. It would be ideal to extract $huCD45^+CD20^+$ B cells from these mice and re-test suppressive function, both in terms of polyclonal suppressive function and allospecificity. Unfortunately, the total numbers of $huCD45^+CD20^+$ B cells, extracted either from spleen or skin, were too low to render this experiment possible. In future experiments, it may be possible to obtain sufficient numbers of $huCD45^+CD20^+$ B cells for tetramer or pentamer-based analysis of the BCR, to determine whether the BCR repertoire has narrowed and the putative EXPB10 cells been polarised to allospecificity [273, 274].

Analysis of the $CD4^+$ T cell phenotype revealed a significant, almost three fold increase in percentage of putative $huCD45^+CD4^+CD25^+CD127^{lo}$ Treg and $huCD45^+CD4^+FoxP3^+$ T cells in the skin allografts of mice which received PBMC + EXPB10 cells compared to that of mouse controls (fig. 5.1.3d). Total numbers of putative $huCD45^+CD4^+CD25^+CD127^{lo}$ Treg were also increased in these mice when compared to those that had received PBMC alone or PBMC + NON-EXPB10 cells (fig. 5.1.3e). A significant decrease in this putative Treg subset was found in both

spleen and blood of mice that had received EXPB10 cells, probably reflecting the overall reduction in huCD45$^+$CD4$^+$ T cell number in these tissues when EXPB10 cells were injected into these mice. Previous studies both *in vitro* and by using this humanised mouse model of transplantation, have demonstrated that human CD4$^+$CD25$^+$CD127^{lo} Treg are able to potently suppress alloimmune responses, even at ratios as low as 1:0.2 PBMC to Treg *in vivo* [136, 137]. It may be that the balance of T effectors to Treg, rather than the actual number of Treg, determines Treg potency, as has been suggested by *in vivo* Treg studies in both mouse models and patients [136, 137, 141, 275, 276]. EXPB10 cells may have prolonged human skin graft survival by promoting Treg survival whilst inhibiting T effector function and proliferation.

Before one can consider this inference, it is important to determine the suppressive function of this putative Treg population. FoxP3 expression may not be a reliable indicator of Treg phenotype in these conditions, as it has now been firmly established that human CD4$^+$ T effector cells also transiently express FoxP3 when activated [277, 278]. Demethylation of the Treg-specific demethylated region (TSDR) within the *FoxP3* locus is a much more reliable indicator of human Treg function and stability [279-281]. Ultimately however, the definitive way in which to confirm that huCD45$^+$CD4$^+$CD25$^+$CD127^{lo} T cells detected in the allograft were in fact Treg, was to test for suppressive function. It was not possible to extract sufficient numbers of this subset from skin allografts, so we turned to the *in vitro* system to help determine the nature of these human T cells. 5-day co-culture of EXPB10 cells with autologous CD4$^+$ T cells and beads revealed a very similar result to that found *in vivo*: an almost two fold increase in percentage of CD4$^+$CD25$^+$CD127^{lo} T cells when compared to CD4$^+$ T cells and beads cultured either alone or with NON-EXPB10 cells (fig. 5.2.1a). Interestingly, there was no difference in proliferation profiles of this subset vs. CD4$^+$CD25$^+$CD127$^+$ cells or whole CD4$^+$ T cells, demonstrating that proliferation putative Treg was as equally suppressed as T effector cells in this system when compared to control conditions (fig. 5.2.1b). One can extrapolate from these findings that if this CD4$^+$CD25$^+$CD127^{lo} T cell sub-population was indeed regulatory, the increased percentage was more likely a result of Treg induction or preferential survival, rather than expansion or proliferation.

Mouse Breg have previously been shown to support the maintenance of mouse Treg; a B cell-specific deletion of IL-10 was associated with a deficiency of Treg after induction of collagen-induced arthritis [27, 28]. Moreover, transfer of wild type T2-MZP Breg to arthritic IL-$10^{-/-}$ mice increased the frequency of $FoxP3^{+}$ Treg and reduced levels of pro-inflammatory Th1 and Th17 cells, whilst inhibiting inflammation. More recently, adoptive transfer of mouse Breg induced under tolerising conditions in the presence of alloantigen, resulted in the long-term survival of pancreatic islet cells and reduction in autoimmune uveitis in transplant and autoimmune models respectively, as well as the increased frequency of mouse Treg [39, 40]. Breg-mediated tolerance was demonstrated to be dependent on mouse Treg, as depletion of Treg completely abrogated Breg-mediated prolongation of graft survival [39]. Adoptive transfer of Breg with naïve $CD4^{+}Foxp3^{-}GFP^{-}$ T cells demonstrated that the increased frequency of Treg was due to Breg-dependent induction. Indeed, co-culture of EXPB10 cells with $CD4^{+}CD25^{-}$ T cells and anti-CD3/CD28 beads confirmed that EXPB10 cells were able to suppress $CD4^{+}$ T cell proliferation in the absence of a pre-existing Treg population (fig. 5.2.1d); furthermore, the observation that an increased percentage of putative $CD4^{+}CD25^{+}CD127^{lo}$ Treg was again found when EXPB10 cells were cultured with $CD4^{+}CD25^{-}$ T cells demonstrated that EXPB10 cells supported the induction and/or preferential survival of this regulatory subset, rather than expansion of a pre-existing Treg population (fig. 5.2.1e). Work with IL-10^{+} dendritic cells has previously demonstrated that Tr1 cells can be induced through antigen presentation in the presence of IL-10 [266]. However, cytokine analysis of $CD4^{+}$ T cells that had been co-cultured in the presence or absence of EXPB10 cells, suggested that Tr1 cells could not be expanded or induced by EXPB10 cells. The low levels of IL-10 produced by EXPB10 cells (fig. 4.1.3a), compared to those produced by IL-10^{+} dendritic cells capable of inducing Tr1 cells [266], may account for this difference. It is difficult to speculate further, given that different stimulatory conditions were used to induce IL-10 production in EXPB10 cells and IL-10^{+} dendritic cells.

FACS-sorting of $CD4^{+}CD25^{+}CD127^{lo}$ T cells and subsequent *in vitro* suppression assays confirmed that this population did indeed represent functional Treg (fig. 5.2.2b), supporting the view that the $huCD45^{+}CD4^{+}CD25^{+}CD127^{lo}$ T cells identified in skin allografts were also regulatory. Although Treg generated in the presence of

EXPB10 cells did not appear to be more potent than those generated in the presence of NON-EXPB10 cells, and absolute numbers of EXPB10-generated Treg were reduced compared to those Treg generated in the presence of NON-EXPB10 or with no $CD19^+$ B cells due to global suppression of $CD4^+$ T cell proliferation by EXPB10 cells (fig. 5.2.1a), this data demonstrates that EXPB10 cells were associated with the preferential survival or induction of functional Treg. The balance of T effectors and Treg both *in vitro* and *in vivo* had been altered. Human IL-10^+ Breg have previously been found to be associated with an increase in FoxP3 expression on activated $CD4^+$ T cells *in vitro* [47]. As already discussed however, unlike in the mouse, FoxP3 expression in stimulated human T cells is not necessarily indicative of suppressive function. To our knowledge, this is the first evidence demonstrating that human Breg can promote survival or induction of functional human Treg.

Interestingly, significant reduction of EXPB10-mediated suppression by the blockade of CD154 or TIM-1 did not abrogate the observed increase in Treg percentage *in vitro* (fig. 5.2.1c). EXPB10 cells appeared to support Treg generation by a mechanism that was independent of the potential CD154-IL-10-TIM-1 suppressive pathway outlined in *Chapter 4.0*. Furthermore, unlike in mouse Breg models which demonstrated Treg induction [27, 28, 39], these data indicate that EXPB10-mediated suppression of $CD4^+$ T cells was not primarily dependent on $CD4^+CD25^+CD127^{lo}$ Treg, at least in the *in vitro* setting. Similar to EXPB10 cells, mouse Breg that were able to induce Treg in an islet allograft model, expressed both LAP and TIM-1 (fig. 4.1.1d) [39]. Using blocking mAbs, Lee et al. illustrated that Breg-mediated tolerance to islet allografts was TGFβ-dependent; furthermore, that this blockade was associated with a significant, if not total, reduction in levels of $FoxP3^+$ Treg. However *in vitro* at least, EXPB10 cells did not appear to suppress $CD4^+$ T cell responses by secretion of TGFβ (fig. 4.2.6a-b). It may be that EXPB10 cells did not employ this suppressive mechanism. Alternatively, given the conditions required for LAP cleavage and TGFβ release [246], it may be that this mechanism was more relevant in the *in vivo* setting. The increase in proportion of $CD4^+$ T cells that exhibited a Treg phenotype *in vivo* when in the presence of EXPB10 cells (fig. 5.1.3d-e) may represent a fine-tuning of immune regulation. EXPB10 cells may not only initiate active suppression of an alloimmune response, but may also support and promote maintenance of the regulatory immune system to create a broader, tolerising environment.

It must be noted that this particular mouse model is one that offers an extremely hostile environment to human $CD19^+$ B cells. Humanised mouse models with a $Rag2^{-/-}c\gamma^{-/-}$ background and which utilise human PBMC have been well-documented to promote very poor human APC survival and human B cells generally fail to engraft [282, 283]. This probably accounts for such low levels of $huCD45^+CD20^+$ B cells detected in peripheral blood and spleen of all mice studied (fig. 5.1.4a). Furthermore the low levels of human immunoglobulin detected in humanised mouse sera may not reflect the true potential or breadth of antibody production by EXPB10 cells when in more conducive *in vivo* conditions (fig. 5.1.5a). Van de Veen et al. identified an IL-10-enriched B cell population in the peripheral blood of immunotolerant human subjects, which expressed a similar phenotype, $CD19^+CD25^+CD71^+CD73^-$, to EXPB10 cells [53]. Production of the potentially anti-inflammatory antibody IgG_4, was confined to this endogenous and antigen-specific B cell population within immunotolerant human subjects. Given the low levels of $huCD45^+CD20^+$ human B cells and human Ig found in reconstituted, humanised mice used in this project, it was unfortunately not possible to accurately identify the *in vivo*, antibody-producing capacity of EXPB10 cells.

Whilst an excellent model by which to study human T cell responses, such as that of acute cellular rejection in transplantation [136, 137], this model was not necessarily the optimal *in vivo* environment in which to study human B cell function. One could argue that the observation that EXPB10 cells were able to survive and suppress a human T cell response despite such poor conditions for human B cell survival, would suggest that EXPB10 cells were potently suppressive or at least demonstrated a great capacity to persist. This may be due to the highly activated nature of EXPB10 cells, which have already received substantial 'T cell help' in the form of $CD154^+$CHO cells; thus EXPB10 cells may not necessarily have required the additional T cell support usually needed by B cells to engraft in this model. The data demonstrate initial proof of principle that *ex vivo*-generated human Breg can suppress a human immune response and prolong human allograft function *in vivo*.

However, one must interpret these results with caution: it is not clear whether EXPB10 cells will maintain phenotype and suppressive function when in a much

more hospitable and physiological environment. We cannot extrapolate from this work that EXPB10 cells will continue to be regulatory in conditions that promote memory B cell formation, plasma cell differentiation and robust antibody responses. There are now newer humanised mouse models emerging that may be more useful for a subsequent in depth study of EXPB10 cells, particularly with respect to phenotypic stability and antigen specificity [271, 282-284].

Mice with a severe combined immunodeficiency background (SCID mice) have been injected with haematopoietic stem cells (HSC) rather than adult PBMC to reliably generate a naive human immune system comprised of all immune cell lineages including human B cells, APCs and myeloid cells [283, 284]. However, in this model, human T cells were educated on mouse thymic epithelium and were H2-restricted, rather than HLA-restricted; furthermore, although human lymphocytes, megakaryocytes and red blood cells were present in the bone marrow, only low numbers circulate in peripheral blood [285]. HSC injection in irradiated, adult SCID mice together with human foetal liver and thymus fragments implanted underneath the renal capsule, have been able to achieve a complete human immune system with higher levels of engraftment than with HSC injection alone [286, 287]. Importantly, human T cells in this model were HLA-restricted, thus minimising xenogeneic GVHD responses. However, splenic germinal centres were poorly organised, preventing the development of a robust antigen-specific antibody response [286]. Antigen-specific human IgM antibody responses have been generated following the immunisation of HSC-engrafted mice, and memory B cells from these mice have been used to generate IgM hybridomas *in vitro*, but the achievement of affinity maturation and class-switching from the IgM to the IgG isotype has proven to be particularly difficult [288]. A recent study has suggested that most human B cells that developed in HSC-engrafted humanised mice were immature $CD5^+$ B cells [289]. Furthermore, these models did require human foetal tissue and surgical expertise, raising practical issues in relation to the ease with which they could be used.

Two alternative approaches have been tried with some success to enable robust study of human B cell function within the confines of an intact human immune system *in vivo*. Mouse BAFF, which is a crucial B cell differentiation and survival factor, is ineffective on human B cells; there is a single amino acid difference between the

human and murine BLyS proteins in the portion of the molecule recognised by BAFF-R [272, 290-292]. The administration of recombinant human BAFF enhanced B cell engraftment and survival in SCID mice that were reconstituted with human PBMC (in which human B cells usually fail to efficiently engraft, much like in the model used in this project); human lymphocytes in the spleen comprised up to 50% $CD20^+$ B cells when human BAFF was administered for 14 days [272]. Human BAFF-treated recipients had on average 40-fold higher levels of serum Ig than controls and mounted a *de novo* antibody response to the thymus-independent antigens in a pneumovax vaccine. In addition, providing both human BAFF and human IL-7 may have further enhanced the antibody response in humanised mice [272, 293]. Use of recombinant human BAFF after transplantation of human allograft in the Balb/c $Rag2^{-/-}c\gamma^{-/-}$ humanised mouse may support B cell survival and differentiation, affinity maturation of the antibody response and class-switching from IgM to IgG. This may be a simple and effective modification of the mouse model already validated in our laboratory, although xenogeneic GVHD responses may still remain an issue.

Alternatively, a transgenic mouse that has been modified such that all mouse cells express human HLA-DR4, may offer a more robust approach. The engraftment of $HLA\text{-}DR4^+$ donor HSCs into NSG Tg(HLA-DR4) or NOG Tg(HLA-DR4) mice reconstituted serum levels of human cytokines and human IgM, IgG (all four sub-classes), IgA, and IgE comparable to humans, and elicited high titres of specific human IgG antibodies upon tetanus toxoid vaccination [294, 295]. When infused with $HLA\text{-}DR4^+$ HSC, these transgenic mice were able to generate antigen-specific B and T cell responses but without the need for human foetal tissue or an advanced surgical procedure; furthermore the incidence of xenogeneic GVHD was obviated as human immune cells were HLA-restricted. Van de Veen et al. have demonstrated that human $CD25^+CD71^+CD73^-$ Breg can produce the potentially anti-inflammatory immunoglobulin IgG_4 [53]; mouse B10 cells transiently express IL-10 before differentiating into antibody-producing plasma cells [66]; mouse regulatory plasma cells have been shown to regulate immune responses through the secretion of IL-35 [43]. An HLA-DR4 transgenic mouse model, with or without the exogenous administration of human BAFF, may offer the optimal approach by which to further interrogate EXPB10 cell function in terms of Ig production and differentiation before clinical application can be considered.

An improved understanding of endogenous Breg present in both the human circulation and lymphoid tissue would also be useful in translational work, in particular with regards to later pharmacological manipulation in the clinic. Analysis of peripheral blood in ten healthy individuals confirmed the findings of Van de Veen et al. [53], and demonstrated that $CD25^{+}CD71^{+}CD73^{-}$ B cells could be identified at low frequencies in the circulation (fig. 5.3.1b). In addition, $CD25^{+}CD71^{+}CD73^{-}$ B cells could also be identified at similar levels within the memory $CD24^{hi}CD27^{+}$ B cell subset reported to be enriched for IL-10^{+} Breg (fig. 5.3.1b) [48]. Whilst there was a significant reduction in percentage frequency of $CD25^{+}CD71^{+}CD73^{-}$ B cells within the transitional $CD24^{hi}CD38^{hi}$ subset also thought to be enriched for IL-10^{+} Breg [20], a small fraction was present (fig. 5.3.1b, **$p<0.0004$). This is in keeping with other work that reports that IL-10^{+} B cells can be induced in $CD24^{lo}CD38^{lo}$ and $CD24^{int}CD38^{int}$ B cells as well as in the $CD24^{hi}CD38^{hi}$ compartment [20, 53]. It may be that $CD25^{+}CD71^{+}CD73^{-}$ B cells represent a unique Breg phenotype which is developmentally distinct from those IL-10^{+} Breg previously identified in $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^{+}$ B cell subsets. Alternatively, $CD25^{+}CD71^{+}CD73^{-}$ B cells may represent the underlying, unifying Breg subset to which all other IL-10^{+} B cells belong. It will be difficult to establish which if either of these possibilities is the reality. Sorting and testing function of $CD25^{+}CD71^{+}CD73^{-}$ B cells from whole $CD19^{+}$ B cells in peripheral blood to confirm regulatory function would be valuable, as would be sorting $CD25^{+}CD71^{+}CD73^{-}$ B cells within the $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^{+}$ B cell compartments to determine whether they too were suppressive compared to B cells within these subsets which were not $CD25^{+}CD71^{+}CD73^{-}$. However, extremely low numbers of this phenotypic subset within the transitional and memory compartments in human peripheral blood will prove a difficult technical challenge to these experiments.

Analysis of EXPB10 cells illustrated that $CD25^{+}CD71^{+}CD73^{-}$ EXPB10 cells up-regulated TIM-1 and CD154 (*Chapter 4.0* fig. 4.1.1d & 4.2.5a). Cell surface expression of these two molecules appeared to be relevant to suppressive potencies of EXPB10 cells (*Chapter 4.0* fig. 4.2.5c & 4.2.8d). Analysis of the $CD25^{+}CD71^{+}CD73^{-}$ compartment of $CD19^{+}$ B cells within peripheral blood of healthy individuals demonstrated a similar enrichment of TIM-1 and CD154, both in

comparison to $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^{+}$ B cell subsets (fig. 5.1.3c & 5.1.3d). Given the similar phenotypic properties of the $CD25^{+}CD71^{+}CD73^{-}$ B cell compartment within both freshly isolated $CD19^{+}$ B cells (i.e. NON-EXPB10 cells) and EXPB10 cells, it tempting to speculate that the two B cell populations are one and the same: the former being endogenous (or naïve), and the latter being induced.

Such speculation is reinforced by the finding that percentage frequency of $CD25^{+}CD71^{+}CD73^{-}$ B cells in the circulating blood of patients diagnosed with SCC of the skin, was significantly raised when compared to that of healthy individuals, unlike the $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^{+}$ B cell subsets (fig. 5.3.2b, **$p<0.0004$). One must interpret these data with extreme caution however, as group sizes were small and the controls were not age or sex-matched; control samples were obtained from leucocyte cones provided by NHSBT, such that no subject demographics were available. The median age of patients with an SCC was 82.5 years, with an interquartile range of 74.25-86.75 years (fig. 5.3.2a); it may be that age is a confounding factor in this comparison. The same caveats should be applied when comparing the B cell compartment of healthy controls with LTKTRs. A significant increase in $CD25^{+}CD71^{+}CD73^{-}$ B cells was again seen in LTKTRs with stable graft function when compared to healthy controls (fig. 5.3.2b, *$p<0.0001$). However, given that there were no additional comparator groups of LTKTRs with acute rejection, LTKTRs with stable function in the absence of immunosuppressants nor patients taking long-term immunosuppressants in the absence of allograft, it is impossible to infer from these data whether the observed increase in $CD25^{+}CD71^{+}CD73^{-}$ B cells was due to the transplanted kidney, stable graft function, use of an immunosuppressant or indeed all three parameters. It may be that the percentage increase seen in $CD25^{+}CD71^{+}CD73^{-}$ B cells in patients with a newly diagnosed cancer or with stable graft function is indicative of an over-regulated or well-regulated immune system respectively, but this conclusion cannot be reached with these results. However, such pilot data is encouraging and could form the basis of larger, well-controlled observational cohort studies.

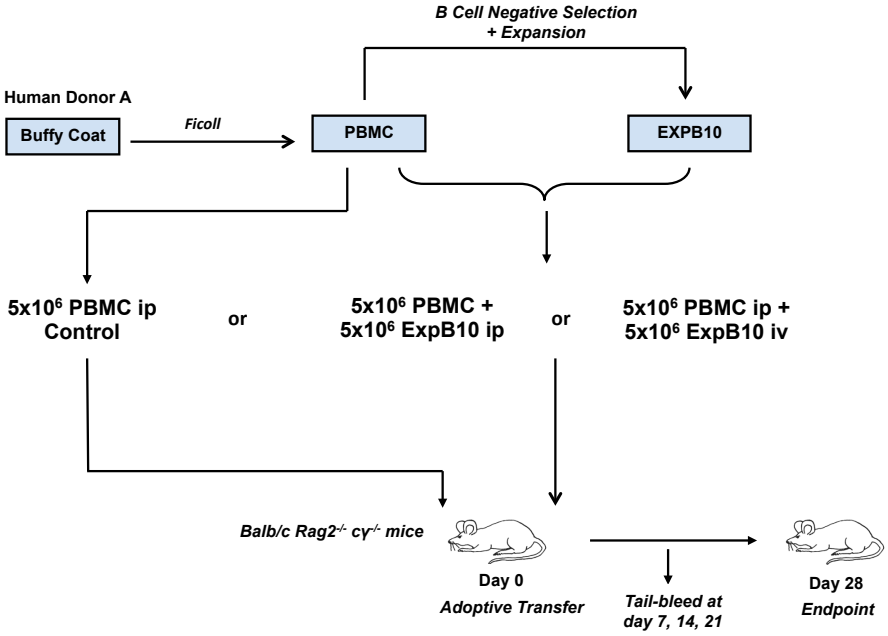
SUMMARY

EXPB10 cell were able to significantly prolong allograft survival in a humanised mouse model of skin transplantation, providing proof of concept data that *ex vivo* generated human Breg were able to suppress an alloimmune response *in vivo*. This suppressive effect was associated with a significant decrease in absolute number of human $CD4^+$ T cells as well as a significant decrease in levels of pro-inflammatory cytokines IL-2 and IL-17a. Furthermore, the balance of T effectors to Treg was shifted in the presence of EXPB10 cells, such that we saw an increase in the proportion of putative Treg in the human allograft at time of rejection. *In vitro data* indicated that this Treg subset was functionally suppressive. EXPB10 cells appeared to retain TIM-1 expression *in vivo*, but more nuanced humanised mouse models are needed to fully probe the full phenotypic stability and function of this Breg subset. Furthermore, given the recent forays into Treg clinical trials, it may be valuable to evaluate the combined *in vivo* suppressive potency of EXPB10 cells and *ex vivo* expanded Treg.

EXPB10 cells were identified in the peripheral blood of healthy human subjects, both in the whole $CD19^+$ B cell population as well as in transitional $CD24^{hi}CD38^{hi}$ and memory $CD24^+CD27^+$ B cell subsets that have been reported to be enriched for IL-10^+ Breg. $CD25^+CD71^+CD73^-$ B cells in peripheral blood were enriched for both CD154 and TIM-1, much like EXPB10 cells, indicating that they may represent a population with similar functional characteristics. A significant increase in percentage frequency of $CD25^+CD71^+CD73^-$ B cells was also seen in patients with either an SCC or a long-term kidney transplant with stable graft function, when compared to unmatched, healthy controls. *Ex vivo* isolation of this subset directly from peripheral blood is warranted to investigate suppressive function in this potentially naturally occurring Breg population. It may be that $CD25^+CD71^+CD73^-$ TIM-1^+ B cells represent the unifying Breg subset that is present across multiple lineages of B cell populations.

Fig 5.1.1

A


B Cell Negative Selection
+ Expansion
Human Donor A
Buffy Coat
Ficoll
PBMC
EXPB10
5x10⁶ PBMC ip
Control
or
5x10⁶ PBMC +
5x10⁶ ExpB10 ip
or
5x10⁶ PBMC ip +
5x10⁶ ExpB10 iv
Balb/c Rag2-/- cγ-/- mice
Day 0
Adoptive Transfer
Tail-bleed at
day 7, 14, 21
Day 28
Endpoint

B

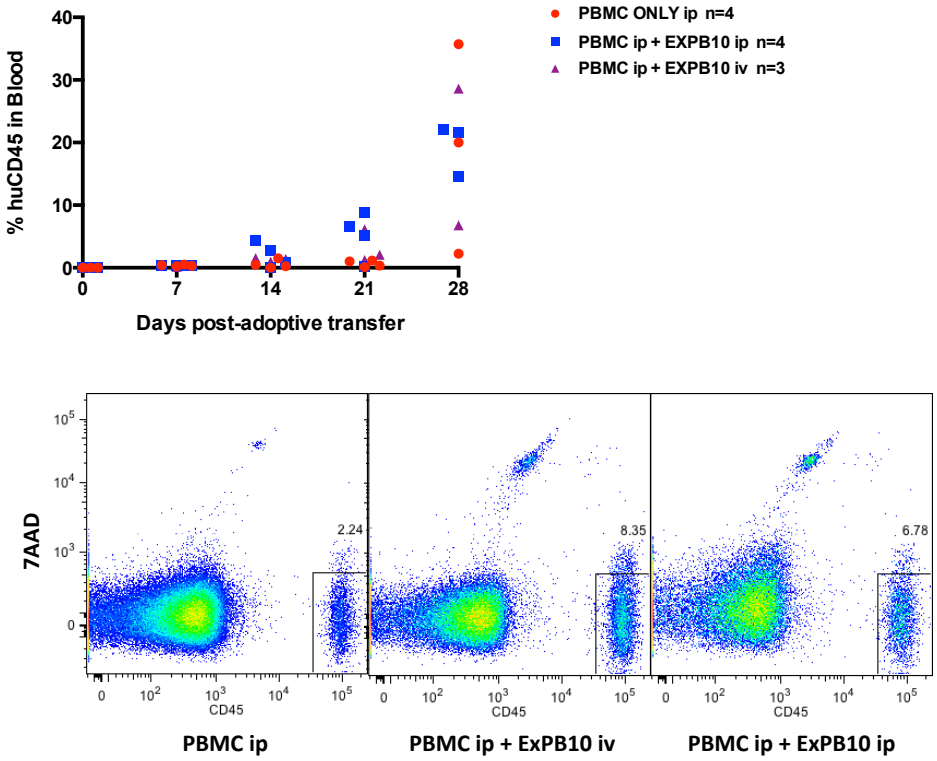
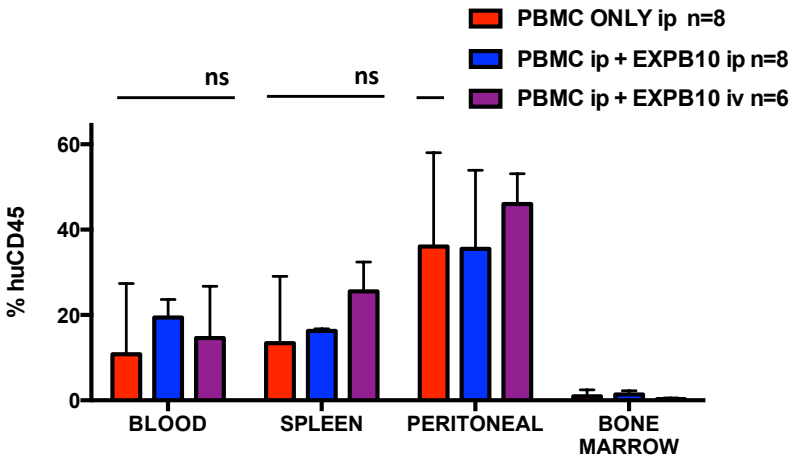
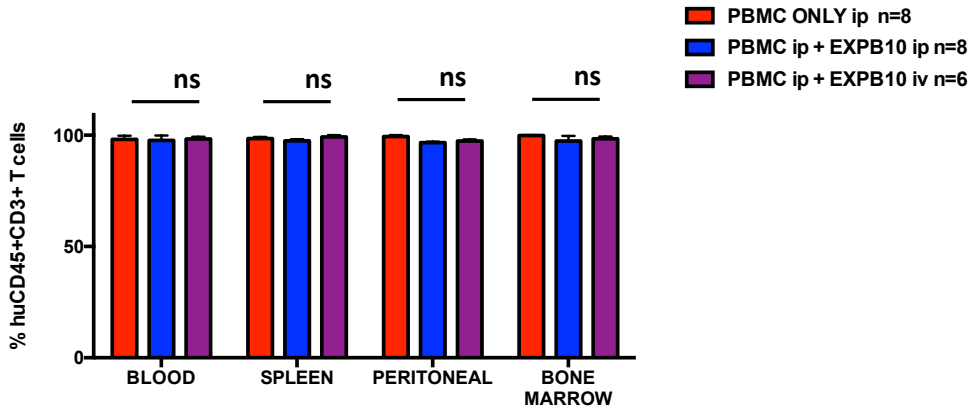

PBMC ONLY ip n=4
PBMC ip + EXPB10 ip n=4
PBMC ip + EXPB10 iv n=3
% huCD45 in Blood
Days post-adoptive transfer
7AAD
CD45
2.24
8.35
6.78
PBMC ip
PBMC ip + ExPB10 iv
PBMC ip + ExPB10 ip

Fig 5.1.1 (cont.)

C



D



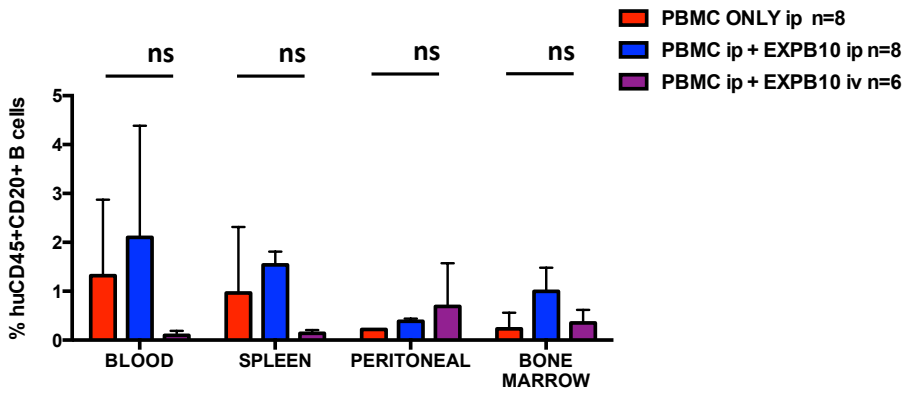
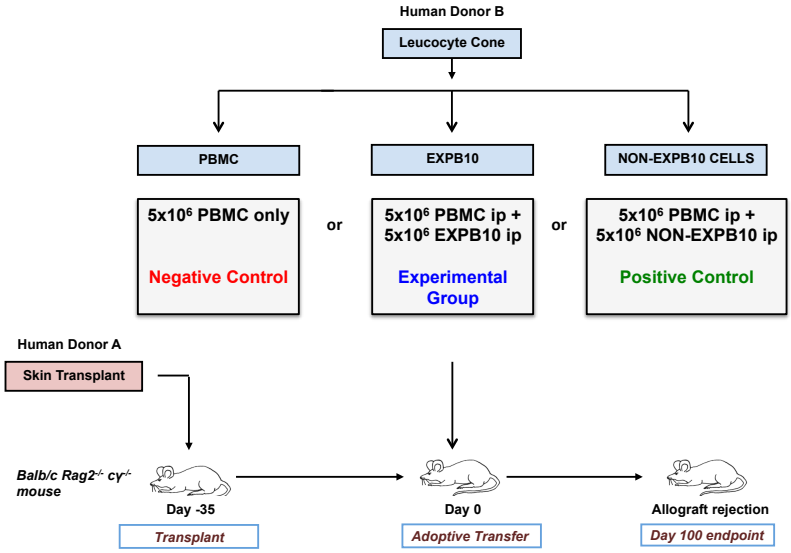


Fig 5.1.1 EXPB10 cells do not inhibit reconstitution of human CD45^{+} lymphocytes in a humanised Balb/c Rag2$^{-/-}$ cγ$^{-/-}$ mouse model

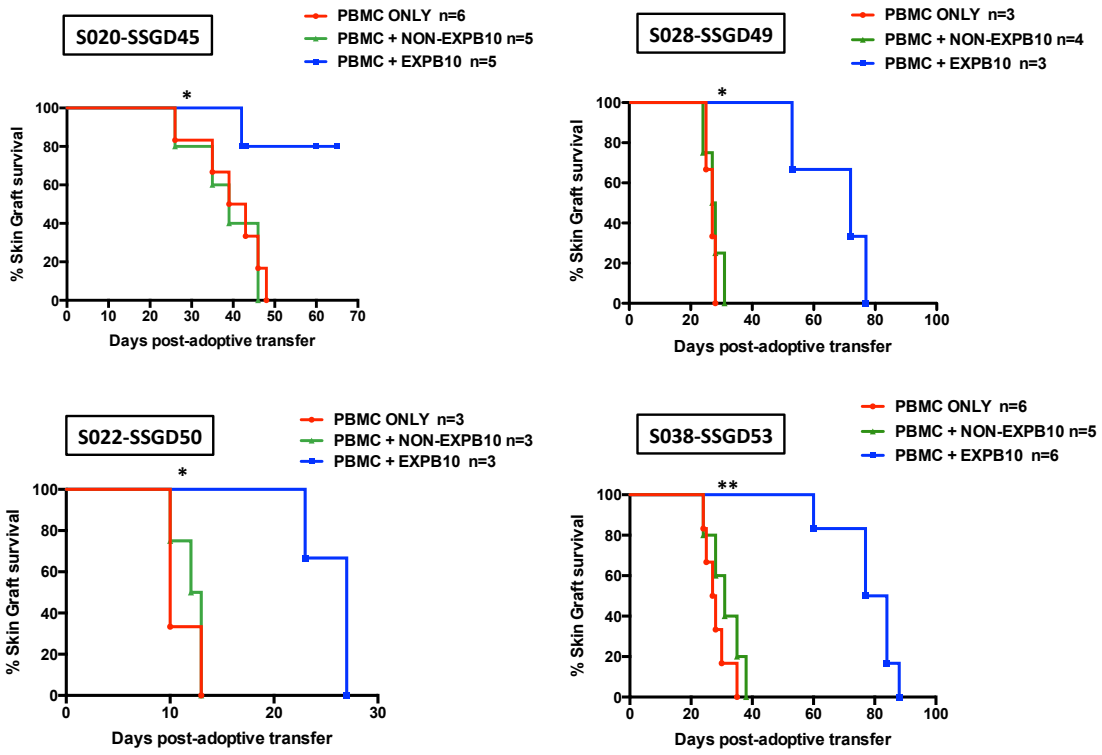
(a) Flow diagram of experimental set-up: CD19^{+} B cells were negative selected from PBMC isolated via Ficoll-Paque centrifugation. PBMC were adoptively transferred intraperitoneally (ip) into Balb/c Rag2$^{-/-}$ cy$^{-/-}$ mice, +/- ip EXPB10 or intravenous (iv) EXPB10 at a 1:1 ratio. % huCD45^{+} reconstitution in peripheral blood was measured at 7-day intervals until the day 28 endpoint. **(b)** EXPB10 cells did not inhibit reconstitution of human CD45^{+} lymphocytes in a humanised Balb/c Rag2$^{-/-}$ cγ$^{-/-}$ mouse model. Graph illustrates reconstitution of huCD45^{+} lymphocytes in peripheral blood over 28 days. Representative FACS plots at day 28, of live huCD45^{+} lymphocytes in PBMC ip only controls (n=4), PBMC ip + EXPB10 iv (n=3), PBMC ip + EXPB10 ip (n=4). **(c)** Graph to illustrate huCD45^{+} lymphocyte reconstitution in blood, spleen, peritoneal fluid and bone marrow at day 28, across all experimental groups. Paired t-test. **(d)** Graphs to illustrate % frequency of huCD3^{+} T cells and % frequency of huCD20^{+} B cells within the huCD45^{+} lymphocyte population across blood, spleen, peritoneal fluid and bone marrow in all mouse groups. Paired t-test. Error bars in each panel represent Mean +/- SD. PBMC and EXPB10 were autologous in each experiment, and 2 human donors were used across the 3 experimental groups.

Fig. 5. 1.2

A



B

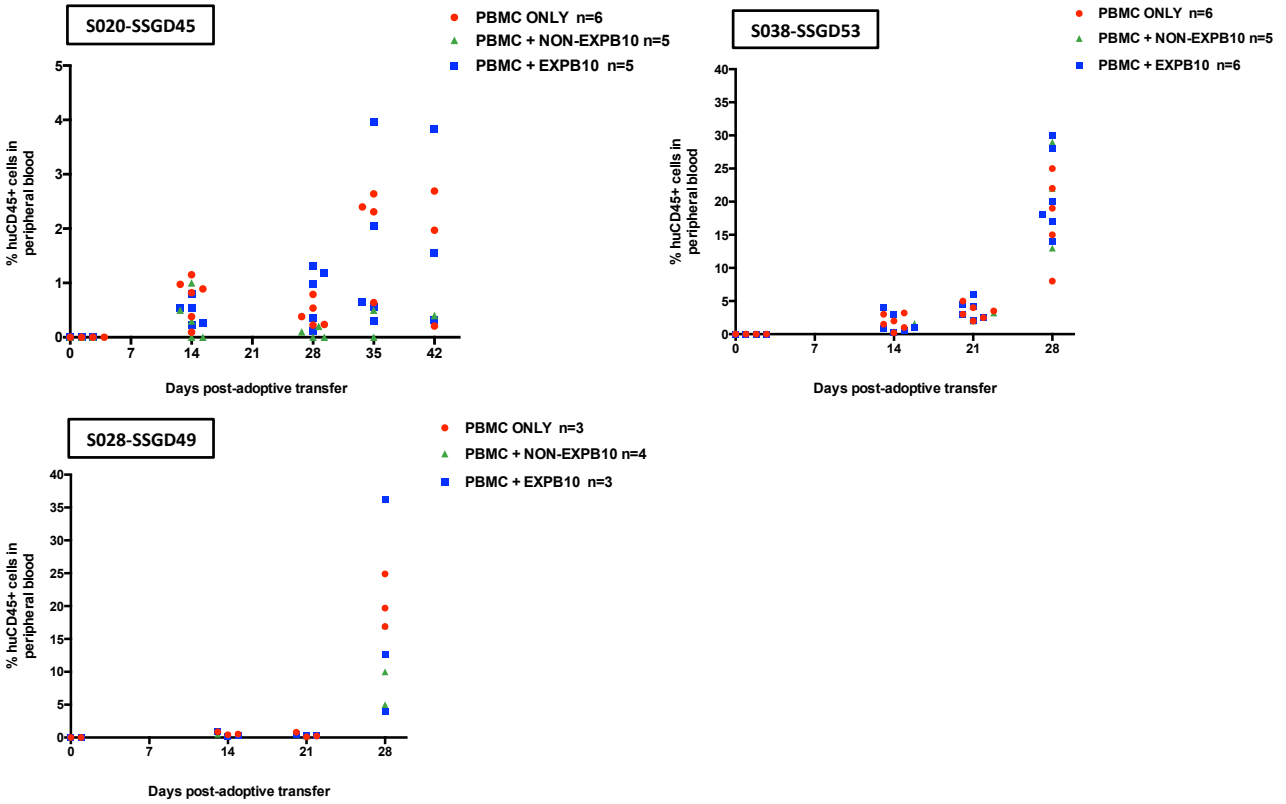


	Number of mice per group (n)		
	PBMC ONLY	PBMC + NON-EXPB10	PBMC + EXPB10
S022-SSGD50	3	3	3
S038-SSGD53	6	5	6
S028-SSGD49	3	4	3
Total number of mice	12	12	12

	Median survival time (MST) of allograft		
	PBMC ONLY	PBMC + NON-EXPB10	PBMC + EXPB10
S022-SSGD50	10	12.5	27
S038-SSGD53	27.5	31	80.5
S028-SSGD49	27	27.5	72

Fig. 5. 1.2 (cont.)

C



D

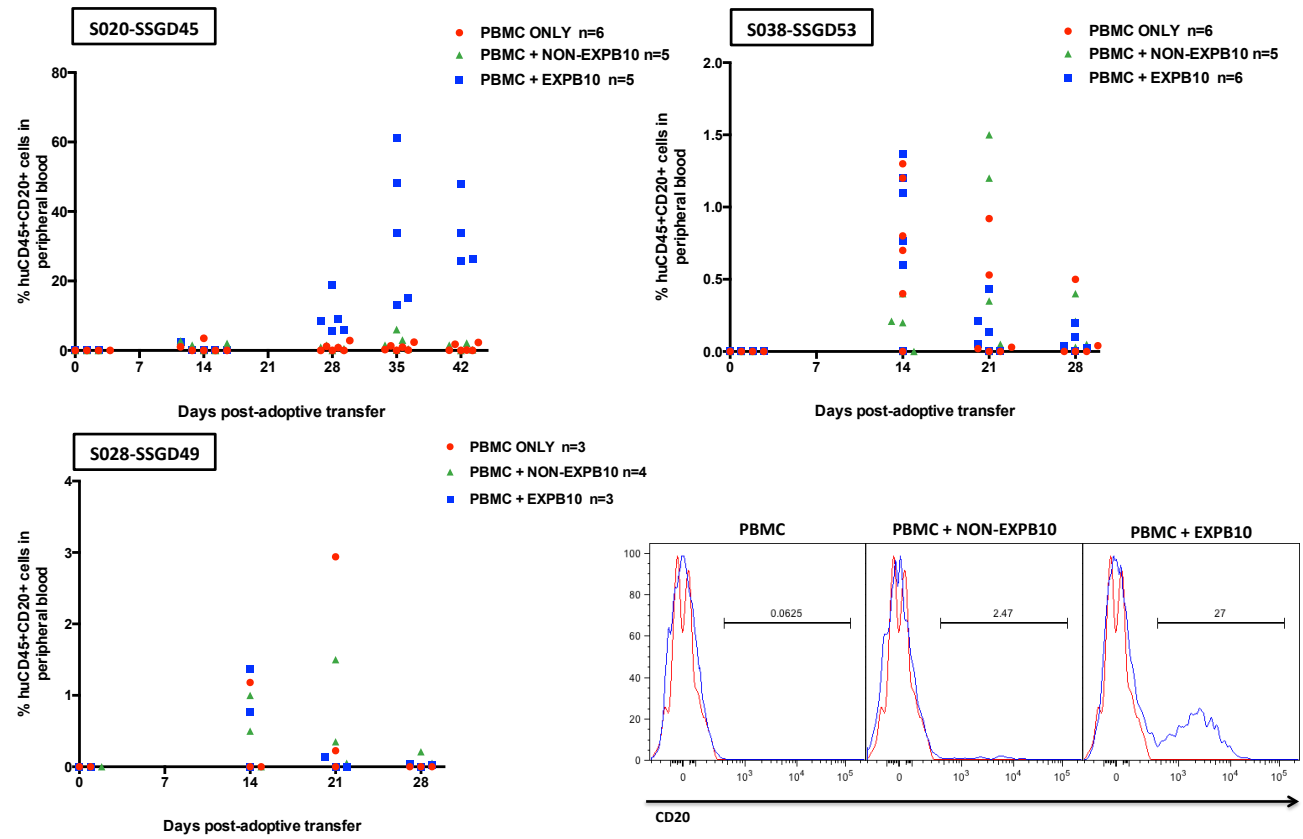


Fig. 5. 1.2 (cont.)

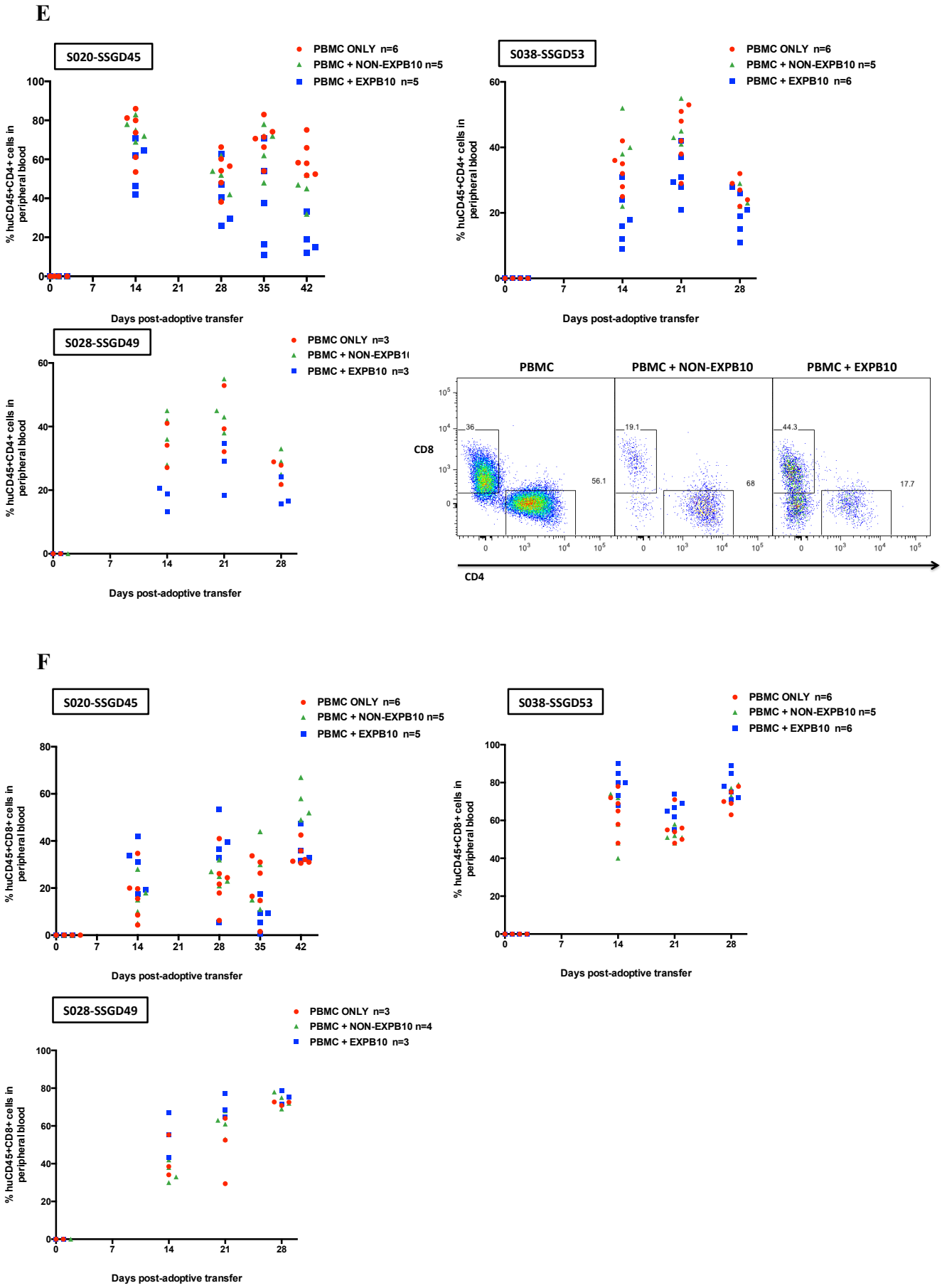
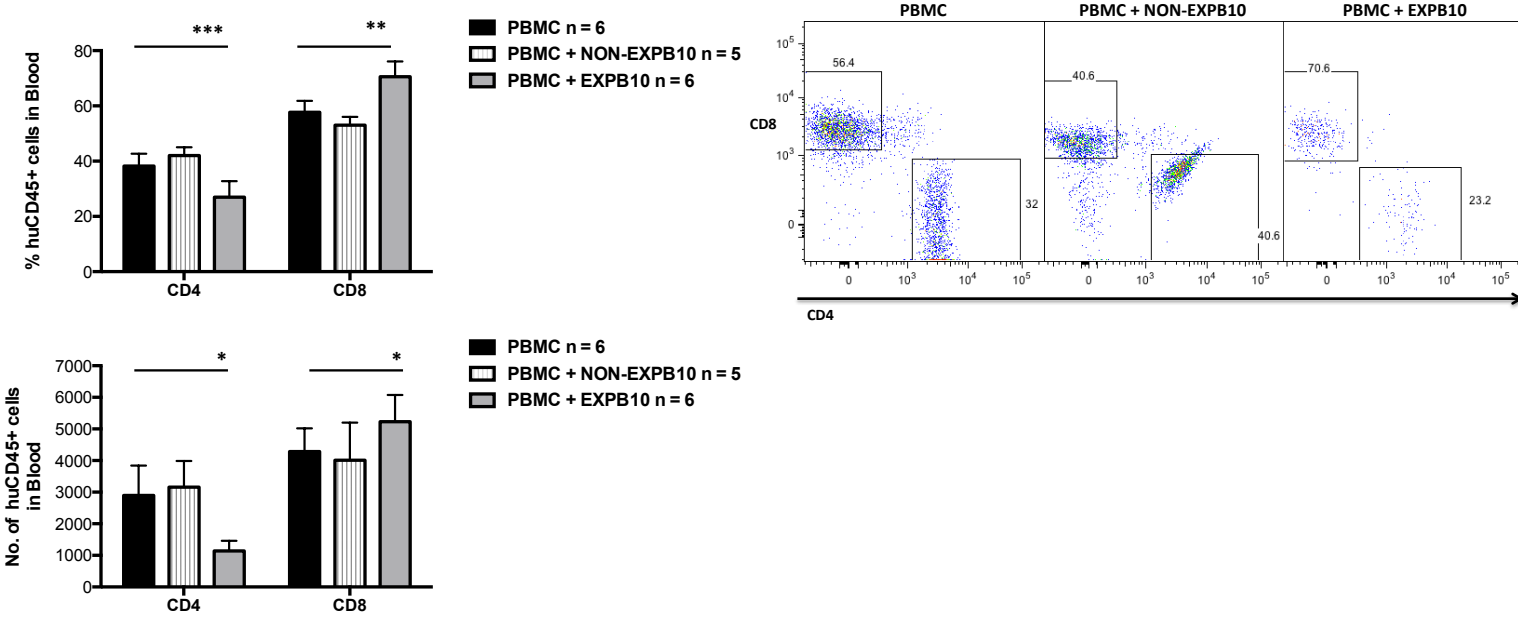

E
S020-SSGD45
PBMC ONLY n=6
PBMC + NON-EXPB10 n=5
PBMC + EXPB10 n=5
% huCD45+CD4+ cells in peripheral blood
Days post-adoptive transfer
S038-SSGD53
PBMC ONLY n=6
PBMC + NON-EXPB10 n=5
PBMC + EXPB10 n=6
% huCD45+CD4+ cells in peripheral blood
Days post-adoptive transfer
S028-SSGD49
PBMC ONLY n=3
PBMC + NON-EXPB1
PBMC + EXPB10 n=3
% huCD45+CD4+ cells in peripheral blood
Days post-adoptive transfer
PBMC
PBMC + NON-EXPB10
PBMC + EXPB10
CD8
CD4
36
56.1
19.1
68
44.3
17.7
F
S020-SSGD45
PBMC ONLY n=6
PBMC + NON-EXPB10 n=5
PBMC + EXPB10 n=5
% huCD45+CD8+ cells in peripheral blood
Days post-adoptive transfer
S038-SSGD53
PBMC ONLY n=6
PBMC + NON-EXPB10 n=5
PBMC + EXPB10 n=6
% huCD45+CD8+ cells in peripheral blood
Days post-adoptive transfer
S028-SSGD49
PBMC ONLY n=3
PBMC + NON-EXPB10 n=4
PBMC + EXPB10 n=3
% huCD45+CD8+ cells in peripheral blood
Days post-adoptive transfer

Fig. 5.1.2 EXPB10 cells significantly prolong graft survival in a humanised Balb/c Rag2$^{-/-}$ cγ$^{-/-}$ mouse model of human skin transplantation

(a) Flow diagram of experimental set-up: Balb/c Rag2$^{-/-}$ cγ$^{-/-}$ mice were transplanted with human skin from human donor A on day-35. After isolating lymphocytes from allogeneic human donor B, PBMC were adoptively transferred intraperitoneally into Balb/c Rag2$^{-/-}$ cy$^{-/-}$ mice, +/- EXPB10 or NON-EXPB10 cells at a 1:1 ratio. Grafts, spleens and blood were harvested at time of allograft rejection or the 100-day endpoint. **(b)** Skin allograft survival curves for 4 independent experiments using 4 different HLA-mismatched human donor pairs. 'SSGD' denotes human donor A from which human skin was transplanted; 'S0' denotes human donor B from which human lymphocytes were used. Red line represents mice which received PBMC only; green line represents mice which received PBMC + NON-EXPB10 cells; blue line represents mice which received PBMC + EXPB10 cells. Experiment using human donor tissue from S020-SSGD45 was stopped prematurely due to onset of GVHD in presence of functioning allograft. Tables summarising total number of mice used in each experimental group (n) and median survival of allograft (MST) in each experimental group per human donor pair. S020-SSGD45 is excluded from this summary due to GVHD incidence. *$p<0.03$, **$p<0.0004$, Log-rank (Mantel-Cox) test. **(c)** % Frequency of huCD45^{+} lymphocytes in peripheral blood over time in mice reconstituted with PBMC only (red), PBMC + NON-EXPB10 cells (green) or PBMC + EXPB10 cells (blue). **(d)** % huCD45^{+}CD20^{+} B Cells in peripheral blood over time in mice reconstituted with PBMC only (red), PBMC + NON-EXPB10 cells (green) or PBMC + EXPB10 cells (blue). Representative histograms of live huCD45^{+} lymphocytes that have been stained for huCD20. Red line represents mf-1 control; blue line represents huCD20^{+} staining. **(e)** % huCD45^{+}CD4^{+} T Cells in peripheral blood over time in mice reconstituted with PBMC only (red), PBMC + NON-EXPB10 cells (green) or PBMC + EXPB10 cells (blue). Representative FACS plots of live huCD45^{+} lymphocytes in peripheral blood, which have been stained for huCD4 and huCD8. **(f)** % huCD45^{+}CD8^{+} T Cells in peripheral blood over time in mice reconstituted with PBMC only (red), PBMC + NON-EXPB10 cells (green) or PBMC + EXPB10 cells (blue). **(c)-(f)** Data representative of 3 independent experiments using 3 different HLA-mismatched human donor pairs.

Fig. 5.1.3

A


***
**
% huCD45+ cells in Blood
CD4
CD8
PBMC n = 6
PBMC + NON-EXPB10 n = 5
PBMC + EXPB10 n = 6
PBMC
PBMC + NON-EXPB10
PBMC + EXPB10
CD8
CD4
56.4
32
40.6
40.6
70.6
23.2
*
*
No. of huCD45+ cells in Blood

B

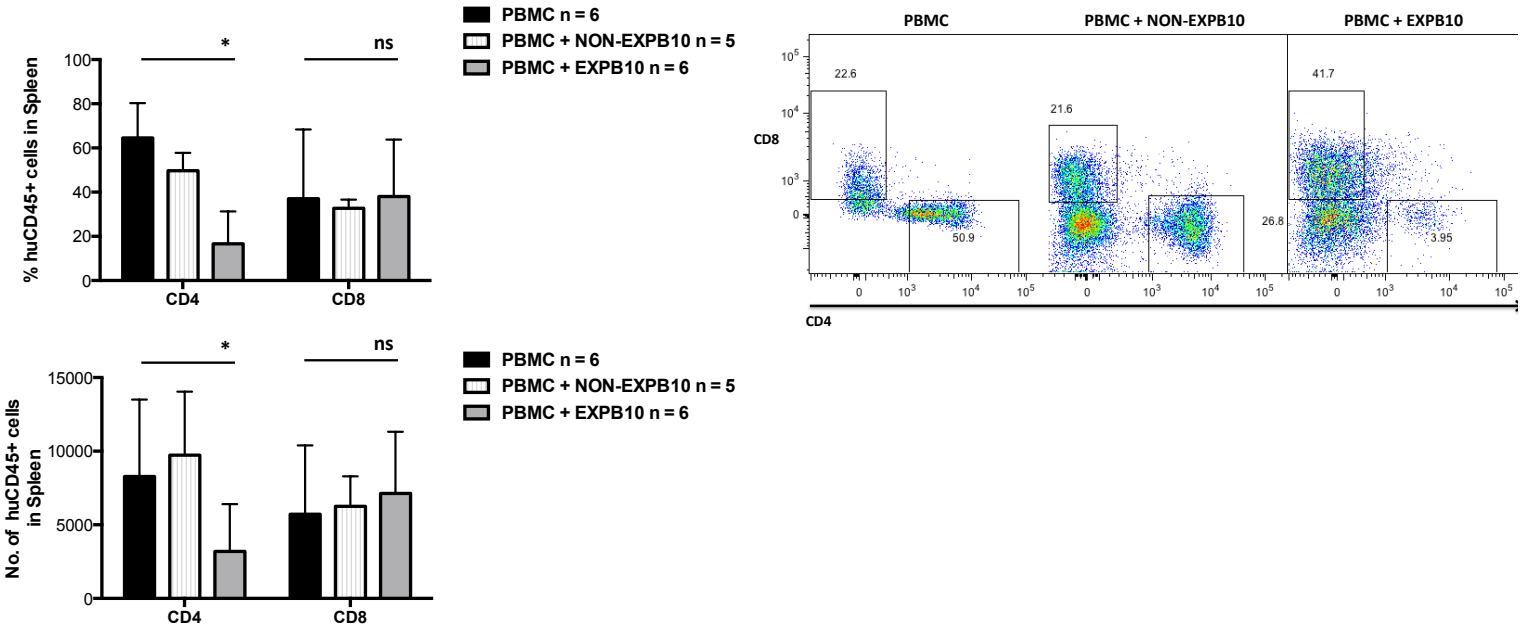

*
ns
% huCD45+ cells in Spleen
CD4
CD8
PBMC n = 6
PBMC + NON-EXPB10 n = 5
PBMC + EXPB10 n = 6
PBMC
PBMC + NON-EXPB10
PBMC + EXPB10
CD8
CD4
22.6
50.9
21.6
41.7
26.8
3.95
*
ns
No. of huCD45+ cells in Spleen

Fig. 5.1.3 (cont.)

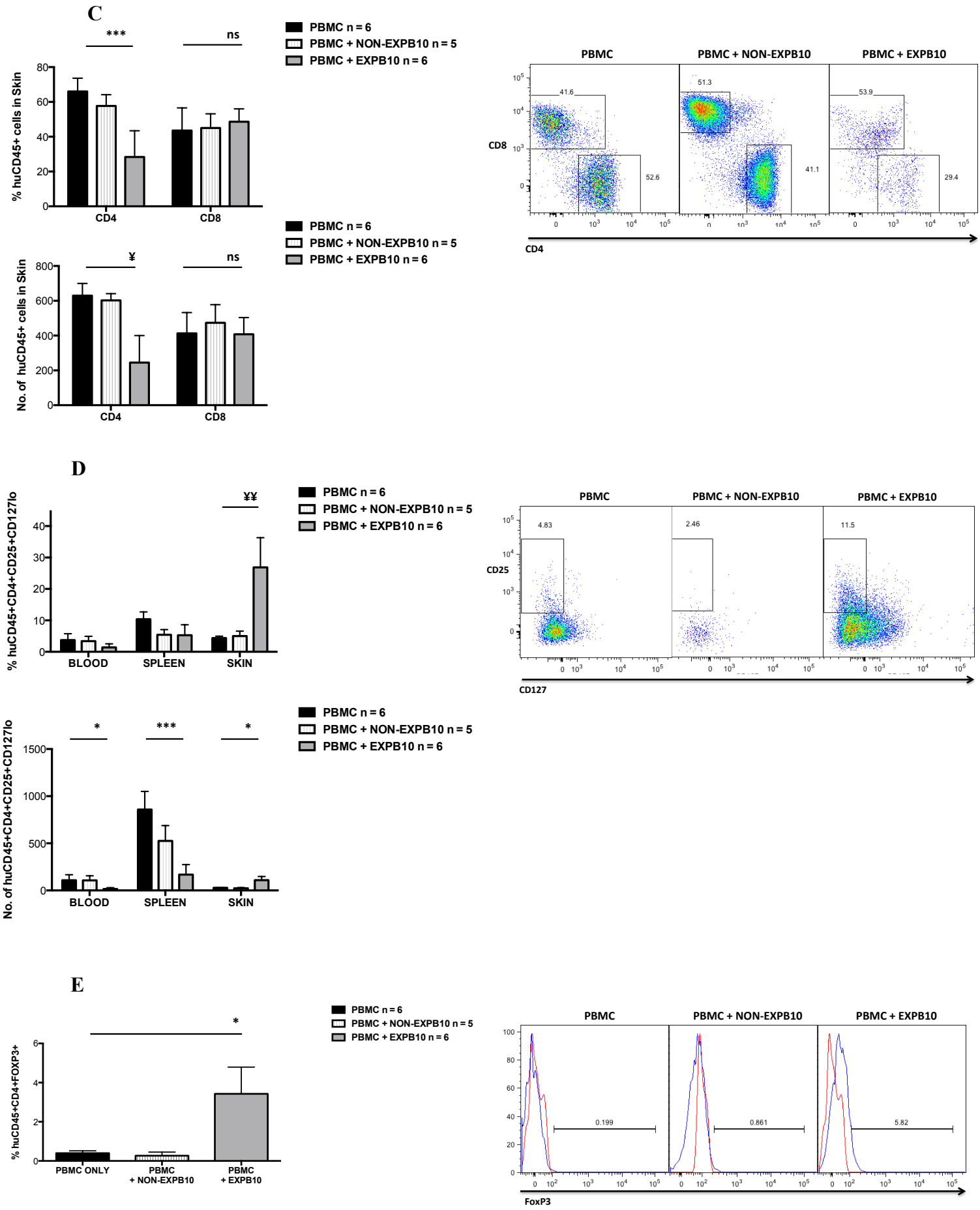

C
***
ns
PBMC n = 6
PBMC + NON-EXPB10 n = 5
PBMC + EXPB10 n = 6
% huCD45+ cells in Skin
CD4
CD8
¥
No. of huCD45+ cells in Skin
PBMC
PBMC + NON-EXPB10
PBMC + EXPB10
41.6
51.3
53.9
52.6
41.1
29.4
CD8
CD4
D
¥¥
% huCD45+CD4+CD25+CD127lo
BLOOD
SPLEEN
SKIN
4.83
2.46
11.5
CD25
CD127
*
***
No. of huCD45+CD4+CD25+CD127lo
E
% huCD45+CD4+FOXP3+
PBMC ONLY
PBMC + NON-EXPB10
PBMC + EXPB10
0.199
0.861
5.82
FoxP3

Fig. 5.1.3 Absolute number of huCD45$^+$CD4$^+$ T cells is significantly decreased across all tissues harvested at rejection in mice which have received PBMC + EXPB10 cells compared to control groups

(a)-(c) Number and percentage of huCD45$^+$CD4$^+$ and huCD45$^+$CD8$^+$ cells in blood, spleen and human skin allograft at time of rejection, of mice that have received PBMC alone, PBMC and NON-EXPB10 or PBMC and EXPB10 cells. Representative FACS plots of live huCD45$^+$ cells demonstrating staining of huCD45$^+$CD4$^+$ and huCD45$^+$CD8$^+$ cells in blood, spleen and human skin allograft across all experimental groups. **(d)** % and number of CD4$^+$CD25$^+$CD127^{lo} putative Treg found in blood, spleen and skin allograft of mice that have received PBMC alone, PBMC and NON-EXPB10 or PBMC and EXPB10 cells. Representative FACS plots of live huCD45$^+$CD4$^+$ T cells demonstrating staining of putative huCD45$^+$CD4$^+$CD25$^+$CD127^{lo} Treg in human skin allograft of mice across all experimental groups. Gating is based on mf-1 controls. *p<0.03, **p<0.0004, ***p<0.005, ¥p<0.001, ¥¥p<0.01, paired t-test. **(e)** % CD4+FoxP3$^+$ T cells found in human skin allograft of mice that have received PBMC alone, PBMC and NON-EXPB10 or PBMC and EXPB10 cells. Representative histograms of live huCD45$^+$CD4$^+$ T cells demonstrating FoxP3 staining in human skin allograft of mice across all experimental groups. Red line represents isotype control; blue line represents FoxP3 staining. Error bars in each panel represent Mean +/- SD. Data representative of 3 independent experiments with 3 different human donor pairs; in total, mice which received PBMC alone n=12; mice which received PBMC + NON-EXPB10 n=12; mice which received PBMC + EXPB10 n=12.

Fig. 5.1.4

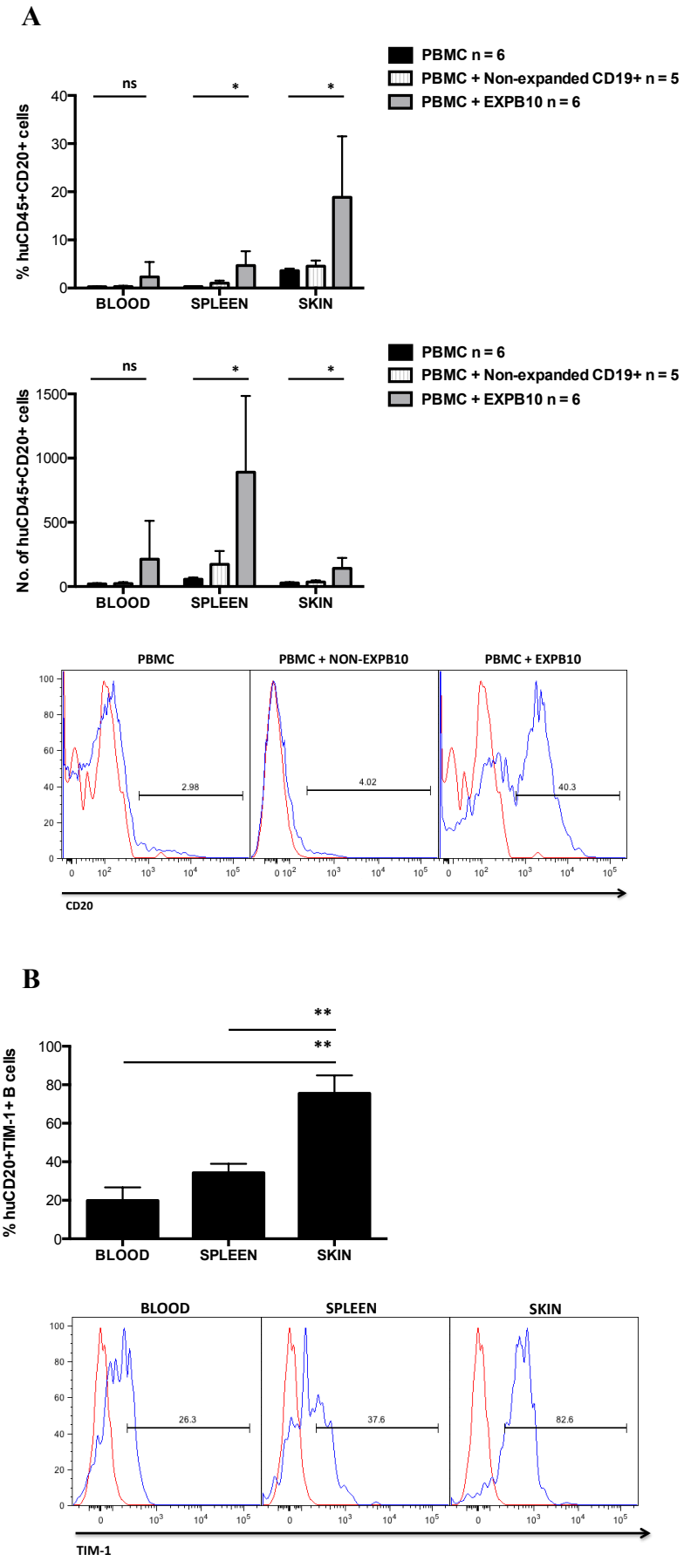
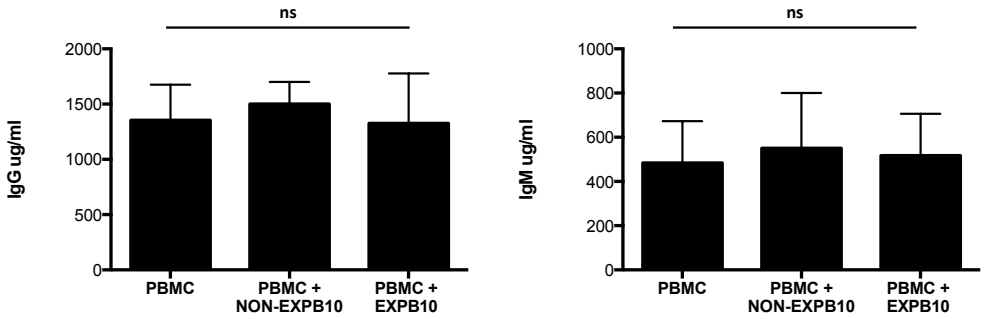

A
PBMC n = 6
PBMC + Non-expanded CD19+ n = 5
PBMC + EXPB10 n = 6
% huCD45+CD20+ cells
ns
*
*
BLOOD
SPLEEN
SKIN
No. of huCD45+CD20+ cells
PBMC
PBMC + NON-EXPB10
PBMC + EXPB10
2.98
4.02
40.3
CD20
B
% huCD20+TIM-1+ B cells
**
**
BLOOD
SPLEEN
SKIN
26.3
37.6
82.6
TIM-1

Fig. 5.1.4 Percentage and absolute number of huCD45$^+$CD20$^+$ B cells are significantly increased in spleen and skin allograft of mice which have been reconstituted with PBMC + EXPB10 cells in comparison to control groups

(a) Number and percentage of huCD45$^+$CD20$^+$ B cells in blood, spleen and human skin allograft at time of rejection, of mice that have received PBMC alone, PBMC and NON-EXPB10 or PBMC and EXPB10 cells. Representative histograms of live huCD45$^+$ lymphocytes demonstrating CD20 staining in human skin allograft of mice across all experimental groups. Red line represents mf-1 control; blue line represents CD20 staining. **(b)** % Frequency of huCD20$^+$TIM-1$^+$ B cells in blood, spleen and skin of mice that have received PBMC and EXPB10 cells. Representative histograms of live huCD45$^+$CD20$^+$ lymphocytes demonstrating TIM-1 staining in human skin allograft of mice that have received PBMC and EXPB10 cells. Red line represents mf-1 control; blue line represents TIM-1 staining. *$p<0.03$, **$p<0.0004$, paired t-test. Error bars in each panel represent Mean +/- SD. Data representative of 3 independent experiments using 3 different HLA-mismatched human donor pairs.

Fig. 5.1.5

A


ns
IgG ug/ml
2000
1500
1000
500
0
PBMC
PBMC + NON-EXPB10
PBMC + EXPB10
ns
IgM ug/ml
1000
800
600
400
200
0
PBMC
PBMC + NON-EXPB10
PBMC + EXPB10

B

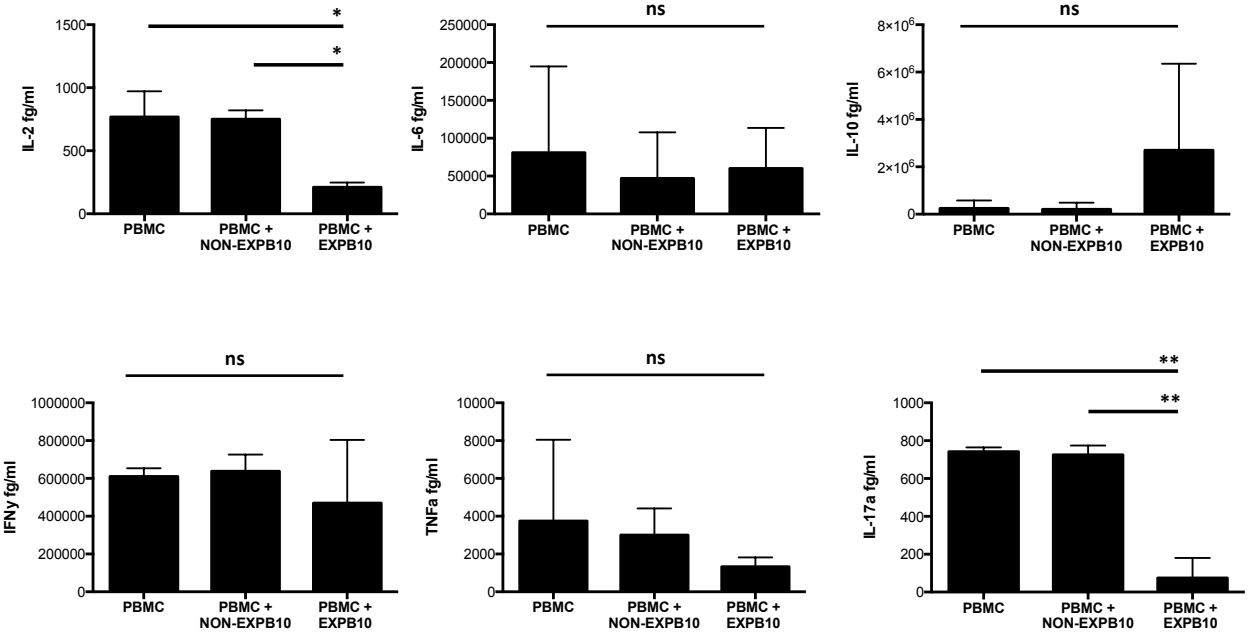

*
*
IL-2 fg/ml
1500
1000
500
0
PBMC
PBMC + NON-EXPB10
PBMC + EXPB10
ns
IL-6 fg/ml
250000
200000
150000
100000
50000
0
PBMC
PBMC + NON-EXPB10
PBMC + EXPB10
ns
IL-10 fg/ml
8×10^6
6×10^6
4×10^6
2×10^6
0
PBMC
PBMC + NON-EXPB10
PBMC + EXPB10
ns
IFNy fg/ml
1000000
800000
600000
400000
200000
0
PBMC
PBMC + NON-EXPB10
PBMC + EXPB10
ns
TNFa fg/ml
10000
8000
6000
4000
2000
0
PBMC
PBMC + NON-EXPB10
PBMC + EXPB10
**
**
IL-17a fg/ml
1000
800
600
400
200
0
PBMC
PBMC + NON-EXPB10
PBMC + EXPB10

Fig. 5.1.5 Immunoglobulin and Cytokines

(a) Levels of human immunoglobulin IgM and IgG in serum of humanised mice that had received PBMC only, PBMC and NON-EXPB10 or PBMC and EXPB10 cells, at time of rejection. **(b)** Levels of human cytokines IL-2, IL-6, IL-10, IFNγ, TNFα and IL-17a in serum of humanised mice that had received PBMC only, PBMC and NON-EXPB10 or PBMC and EXPB10 cells, at time of rejection. *$p<0.03$, **$p<0.0004$, paired t-test. Error bars in each panel represent Mean +/- SD. Data representative of 3 independent experiments using 3 different HLA-mismatched human donor pairs.

Fig. 5.2.1

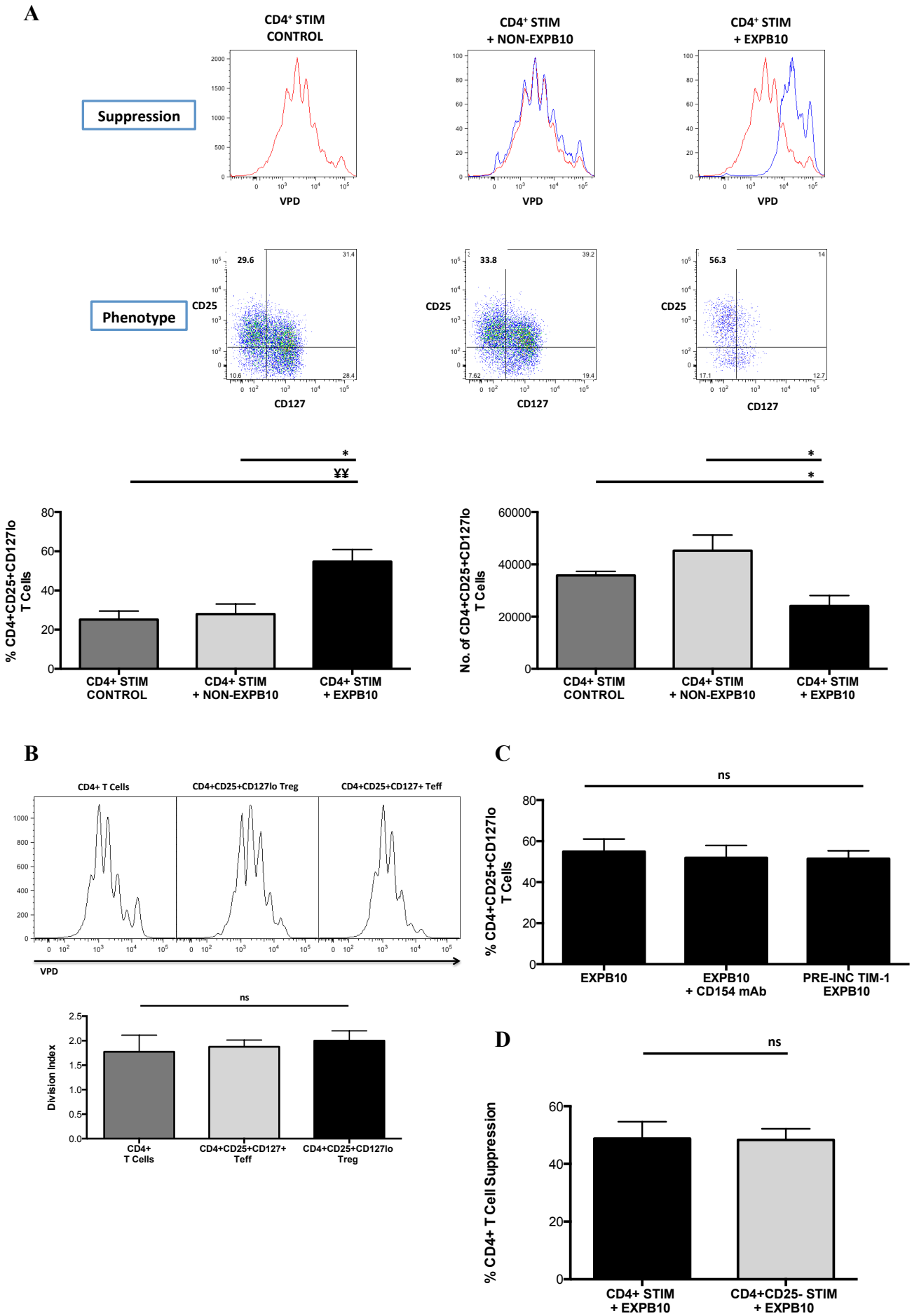

A
CD4+ STIM CONTROL
CD4+ STIM + NON-EXPB10
CD4+ STIM + EXPB10
Suppression
VPD
Phenotype
CD25
CD127
29.6
33.8
56.3
% CD4+CD25+CD127lo T Cells
No. of CD4+CD25+CD127lo T Cells
CD4+ STIM CONTROL
CD4+ STIM + NON-EXPB10
CD4+ STIM + EXPB10
B
CD4+ T Cells
CD4+CD25+CD127lo Treg
CD4+CD25+CD127+ Teff
VPD
Division Index
CD4+ T Cells
CD4+CD25+CD127+ Teff
CD4+CD25+CD127lo Treg
C
ns
% CD4+CD25+CD127lo T Cells
EXPB10
EXPB10 + CD154 mAb
PRE-INC TIM-1 EXPB10
D
ns
% CD4+ T Cell Suppression
CD4+ STIM + EXPB10
CD4+CD25- STIM + EXPB10

Fig. 5.2.1 (cont.)

E

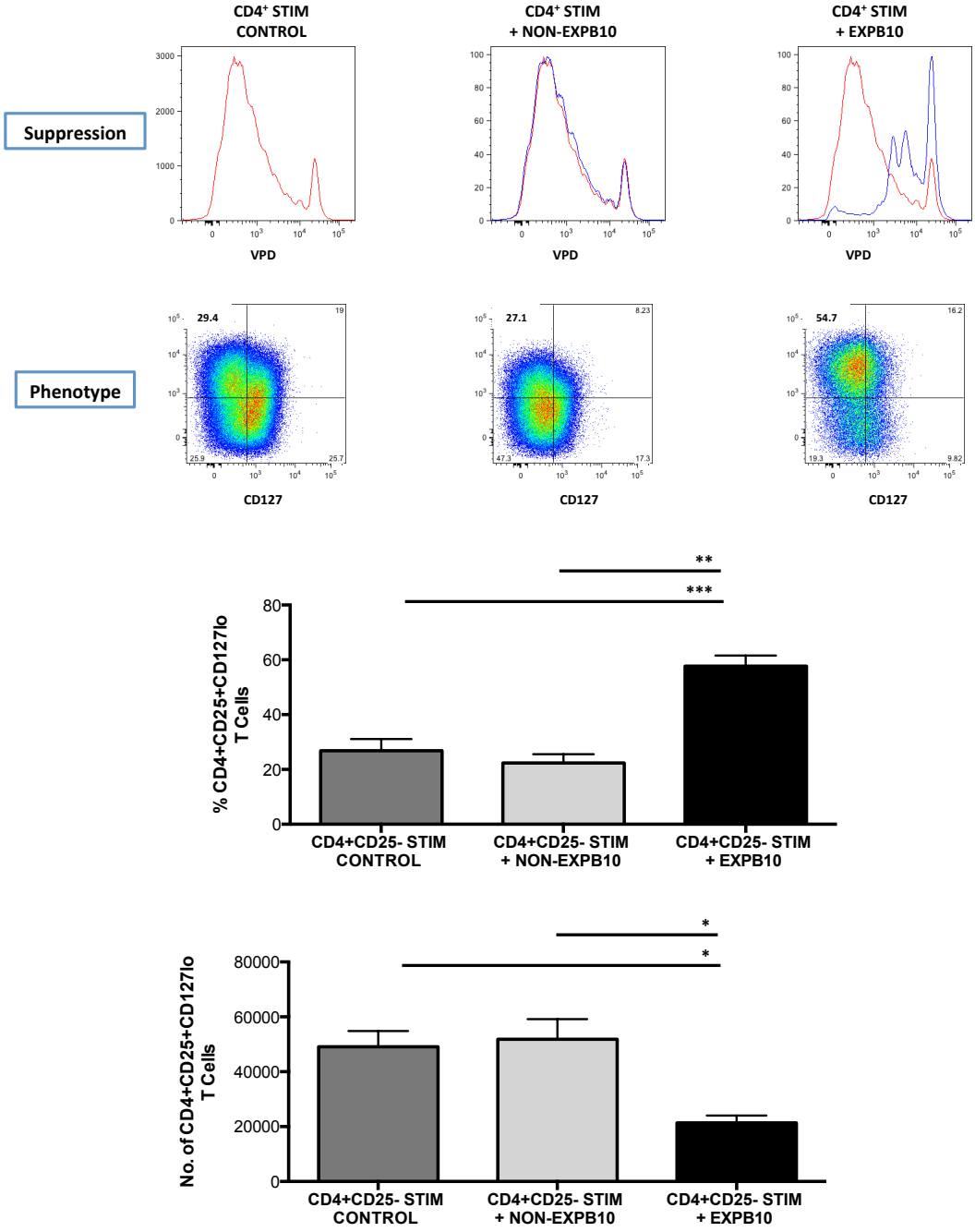
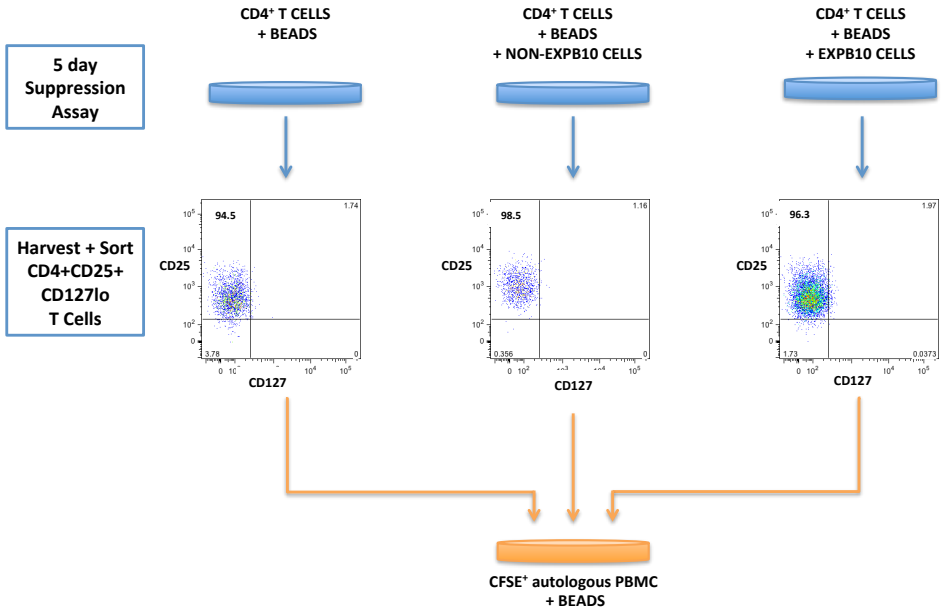


Fig. 5.2.1 Co-culture of EXPB10 cells with autologous $CD4^+$ T cells and anti-CD3/CD28 beads results in an increase in percentage of human $CD4^+CD25^+CD127^{lo}$ T cells

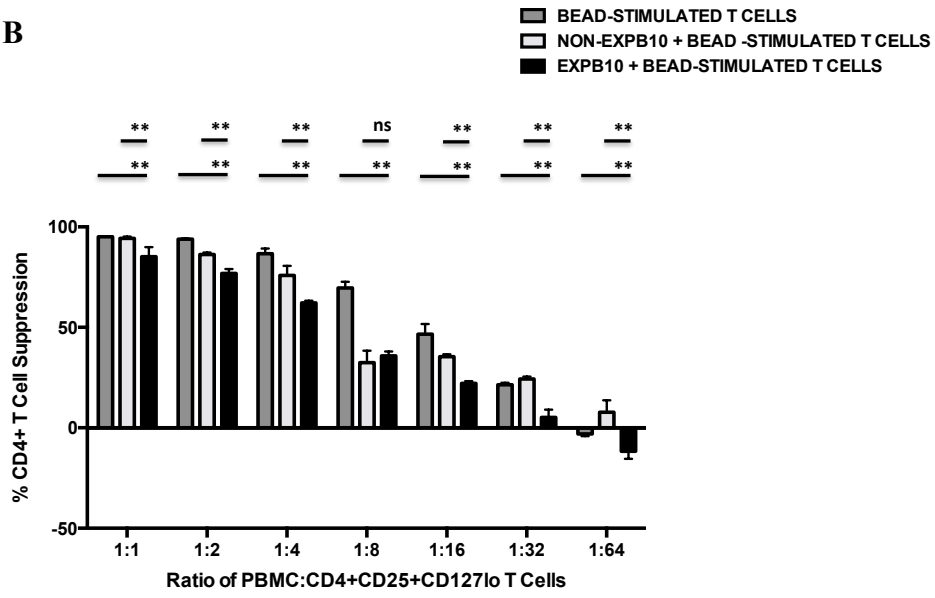
(a) % and number of putative $CD4^+CD25^+CD127^{lo}$ Treg at day 5 of suppression assay when $CD4^+$ T cells were co-cultured with anti-CD3/CD28 beads alone ($CD4^+$ STIM CONTROL), with anti-CD3/CD28 beads and NON-EXPB10 cells ($CD4^+$ STIM + NON-EXPB10), or with anti-CD3/CD28 beads and EXPB10 cells ($CD4^+$ STIM + EXPB10) for 5 days. Representative histograms of live $CD4^+VPD^+$ T cells demonstrate proliferation. Red line represents $CD4^+$ STIM CONTROL condition; blue line represents $CD4^+$ STIM + NON-EXPB10 or $CD4^+$ STIM + EXPB10 conditions. Representative FACs plots of live $CD4^+$ T cells at day 5 of suppression assay displaying expression of CD25 and CD127. Gating was based on mf-1 controls. *$p<0.03$, ¥¥$p<0.01$, paired t-test. **(b)** Proliferation profile and division index of whole $CD4^+$ T cells, putative $CD4^+CD25^+CD127^{lo}$ Treg and $CD4^+CD25^+CD127^+$ T effectors (Teff) at day 5 of suppression assay when $CD4^+$ T cells were co-cultured with anti-CD3/CD28 beads and EXPB10 cells ($CD4^+$ STIM + EXPB10) for 5 days. Representative histograms are of live $CD4^+$, $CD4^+CD25^+CD127^{lo}$ and $CD4^+CD25^+CD127^+$ T cells at day 5 of suppression assay. **(c)** % $CD4^+CD25^+CD127^{lo}$ Treg at day 5 of suppression assay when $CD4^+$ T cells were co-cultured with anti-CD3/CD28 beads and EXPB10 cells ($CD4^+$ STIM + EXPB10) for 5 days in the absence or presence of blocking mAbs to CD154 at 10ug/ml, or with EXPB10 cells that have been pre-incubated with blocking mAb to TIM-1 during the last 48 hours of expansion protocol (PRE-INC TIM-1 EXPB10). **(d)** % Suppression of $CD4^+$ T cell proliferation when either whole $CD4^+$ T cells or $CD4^+CD25^-$ T cells were co-cultured with anti-CD3/CD28 beads and EXPB10 cells for 5 days. **(e)** % and number of putative $CD4^+CD25^+CD127^{lo}$ Treg at day 5 of suppression assay when $CD4^+CD25^-$ T cells were co-cultured with anti-CD3/CD28 beads alone, with NON-EXPB10 cells, or with EXPB10 cells for 5 days. Representative histograms of live $CD4^+VPD^+$ T cells demonstrating proliferation. Red line represents $CD4^+$ STIM CONTROL condition; blue line represents $CD4^+$ STIM + NON-EXPB10 or $CD4^+$ STIM + EXPB10 conditions. Representative FACs plots of live $CD4^+$ T cells at day 5 of suppression assay displaying expression of CD25 and CD127. Gating was based on mf-1 controls. *$p<0.03$, **$p<0.0004$, ***$p<0.005$, paired t-test. Error bars in each panel represent Mean +/- SD. Data representative of 4 independent experiments.

5.2.2

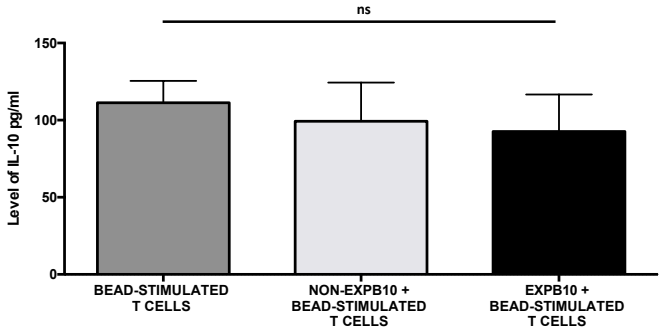
A


CD4+ T CELLS
+ BEADS
CD4+ T CELLS
+ BEADS
+ NON-EXPB10 CELLS
CD4+ T CELLS
+ BEADS
+ EXPB10 CELLS
5 day
Suppression
Assay
Harvest + Sort
CD4+CD25+
CD127lo
T Cells
94.5
98.5
96.3
CD25
CD127
CFSE+ autologous PBMC
+ BEADS

B


BEAD-STIMULATED T CELLS
NON-EXPB10 + BEAD -STIMULATED T CELLS
EXPB10 + BEAD-STIMULATED T CELLS
ns
% CD4+ T Cell Suppression
Ratio of PBMC:CD4+CD25+CD127lo T Cells
1:1
1:2
1:4
1:8
1:16
1:32
1:64

C

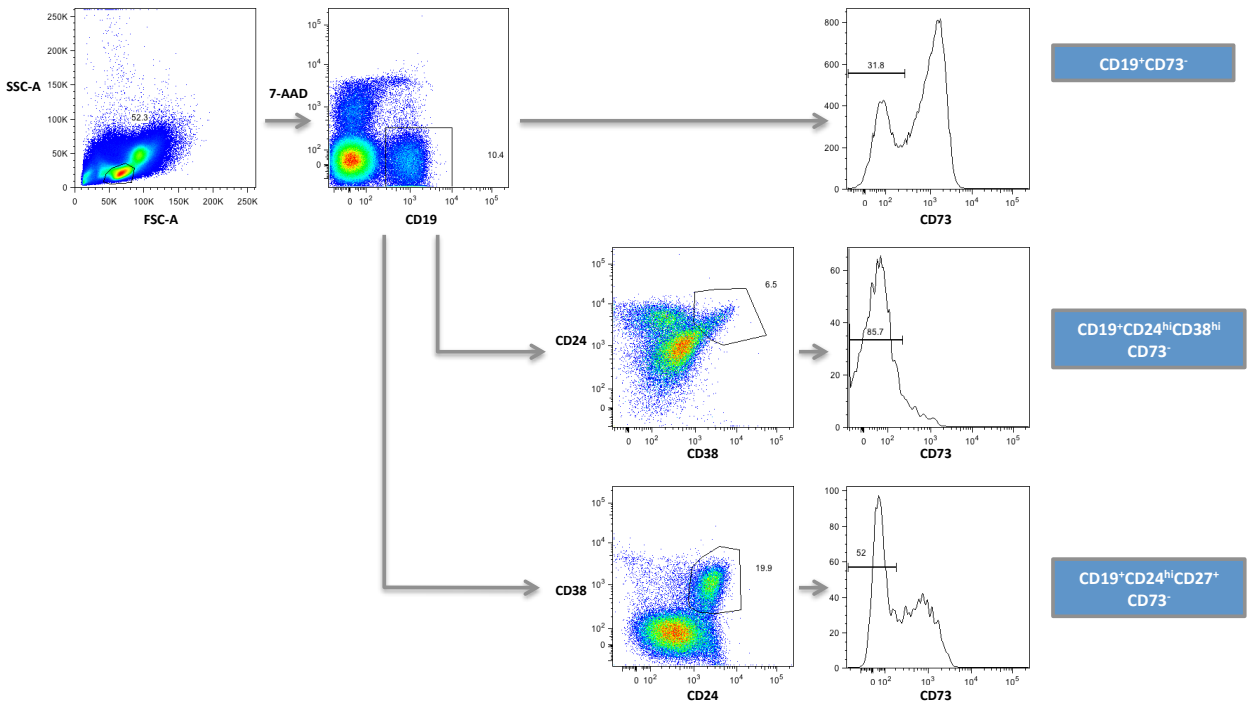

ns
Level of IL-10 pg/ml
BEAD-STIMULATED
T CELLS
NON-EXPB10 +
BEAD-STIMULATED
T CELLS
EXPB10 +
BEAD-STIMULATED
T CELLS

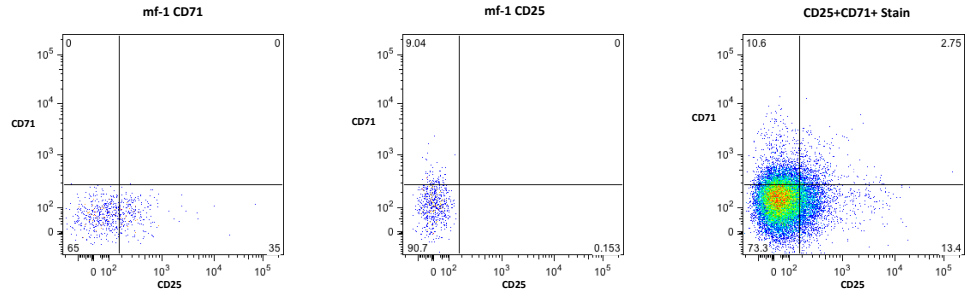
5.2.2 EXPB10-generated $CD4^+CD25^+CD127^{lo}$ T cells are functional Treg which can suppress an autologous immune response *in vitro*

(a) Schematic diagram outlining *in vitro* Treg suppression assay. Autologous $CD4^+$ T cells were co-cultured with anti-CD3/CD28 beads alone +/- NON-EXPB10 cell or EXPB10 cells, for 5 days. At day 5, beads were removed by magnet and $CD4^+CD25^+CD127^{lo}$ T cells were FACS-sorted to >94% purity from suppression co-cultures. Sorted T cell populations were stained with VPD and co-cultured with CFSE-stained autologous PBMC and anti-CD3/CD28 beads at reducing ratios for 5 days. **(b)** % Suppression of $CD4^+$ T cells by FACS-sorted Treg at decreasing ratios of PBMC to Treg, when $CD4^+CD25^+CD127^{lo}$ T cells had been isolated from co-cultures of $CD4^+$ T cells and beads only (BEAD-STIMULATED T CELLS), or from co-cultures of $CD4^+$ T cells, beads and NON-EXPB10 cells (NON-EXPB10 + BEAD-STIMULATED T CELLS) or from co-cultures of $CD4^+$ T cells, beads and EXPB10 cells (EXPB10 + BEAD-STIMULATED T CELLS). **$p<0.0004$, paired t-test. **(c)** Levels of IL-10 produced by whole $CD4^+$ T cells that had been isolated on day 5 of suppression assay when $CD4^+$ T cells had been FACS-sorted from co-cultures of $CD4^+$ T cells and beads only +/- NON-EXPB10 cells or EXPB10 cells. After FACS-sorting, whole $CD4^+$ T cells were stimulated with PMA and ionomycin for 6 hours and supernatants analysed by cytokine bead array for IL-10. Error bars in each panel represent Mean +/- SD. Data representative of 3 independent experiments.

Fig. 5.3.1

A


SSC-A
FSC-A
52.3
7-AAD
CD19
10.4
CD73
31.8
CD19+CD73-
CD24
CD38
6.5
85.7
CD19+CD24hiCD38hi CD73-
CD24
19.9
52
CD19+CD24hiCD27+ CD73-


mf-1 CD71
CD71
CD25
65
35
mf-1 CD25
9.04
90.7
0.153
CD25+CD71+ Stain
10.6
2.75
73.3
13.4

B

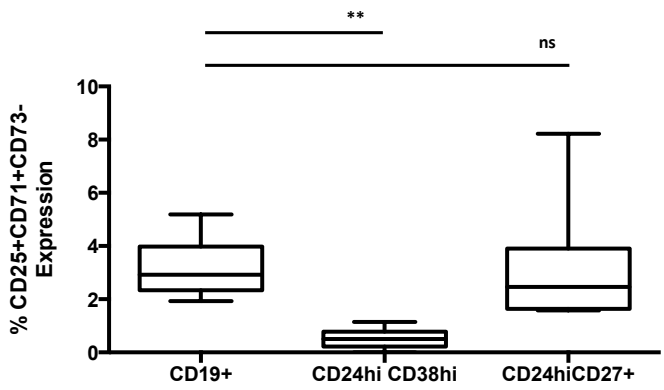
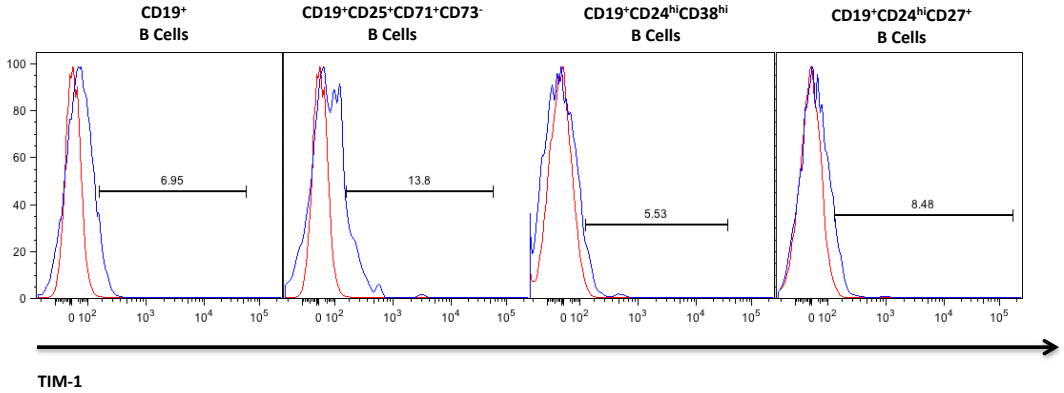
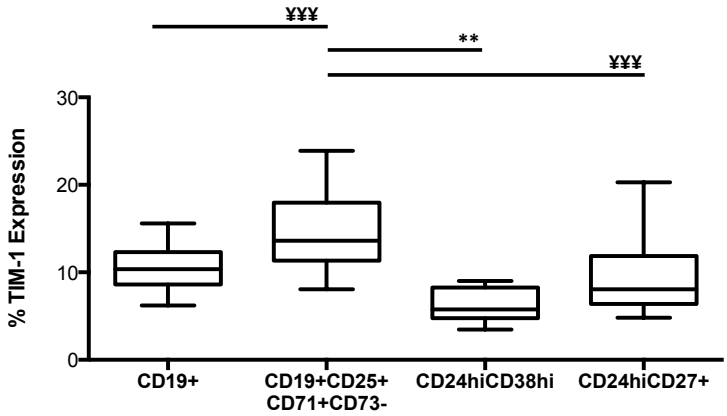

**
ns
% CD25+CD71+CD73- Expression
CD19+
CD24hi CD38hi
CD24hiCD27+

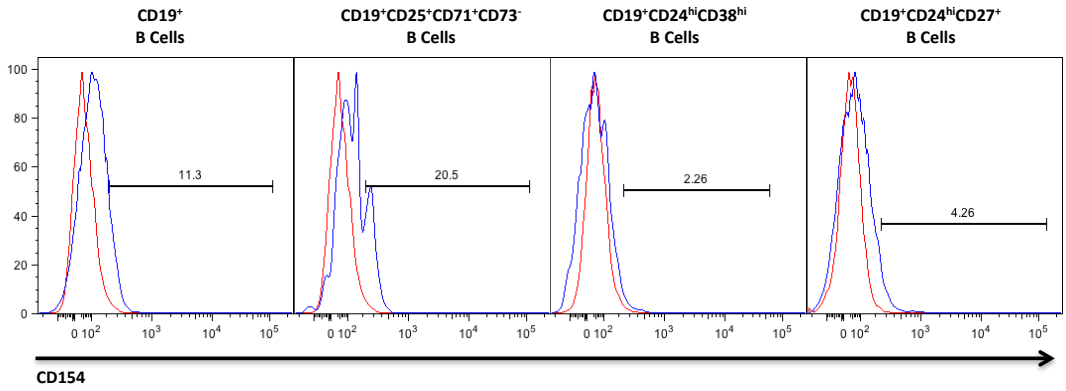
Fig. 5.3.1 (cont.)

C





D



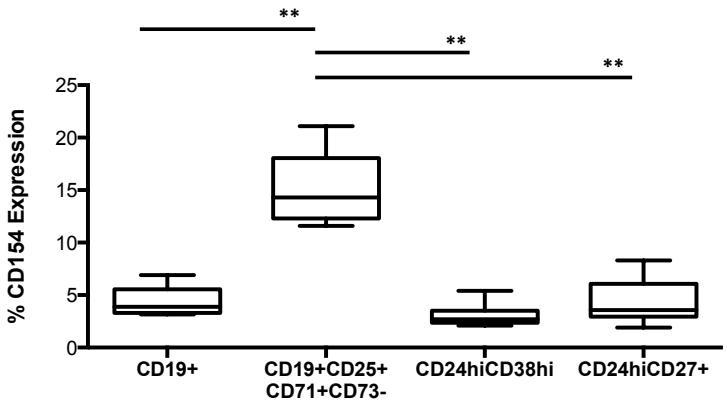


Fig. 5.3.1 $CD19^+CD25^+CD71^+CD73^-$ B cells are present in peripheral blood of healthy subjects, including within $CD24^{hi}CD38^{hi}$ B cell and $CD24^{hi}CD27^+$ B cell subsets

(a) Gating strategy used to analyse PBMC from peripheral blood of healthy individuals for live $CD19^+CD25^+CD71^+CD73^-$ B cells. Live $CD19^+$ B cells were first identified by CD19 and 7-AAD staining following selection of lymphocyte gate based on cell size. $CD19^+CD73^-$ B cells were then either gated directly from whole $CD19^+$ B cells or from $CD24^{hi}CD38^{hi}$ or $CD24^{hi}CD27^+$ B cell subsets. The subsequent $CD19^+CD73^-$ cells were stained for CD25 and CD71. All gating was based on mf-1 controls. **(b)** % $CD25^+CD71^+CD73^-$ B cells in whole $CD19^+$ B cells or in $CD24^{hi}CD38^{hi}$ or $CD24^{hi}CD27^+$ B cell subsets from peripheral blood of healthy individuals. **p<0.0004, Mann-Whitney test. **(c)** % TIM-1 expression by whole $CD19^+$ B cells, $CD25^+CD71^+CD73^-$ B cells, $CD24^{hi}CD38^{hi}$ B cells or $CD24^{hi}CD27^+$ B cells from peripheral blood of healthy individuals. Representative histograms of live $CD19^+$ B cells demonstrating TIM-1 expression. Red line represents mf-1 control; blue line represents TIM-1 staining. ¥¥p<0.05, **p<0.0004, Mann-Whitney test. **(d)** % CD154 expression by whole $CD19^+$ B cells, $CD25^+CD71^+CD73^-$ B cells, $CD24^{hi}CD38^{hi}$ B cells or $CD24^{hi}CD27^+$ B cells from peripheral blood of healthy individuals. Representative histograms of live $CD19^+$ B cells demonstrating CD154 expression. Red line represents mf-1 control; blue line represents CD154 staining. **p<0.0004, Mann-Whitney test. n=10 healthy individuals in all analyses.

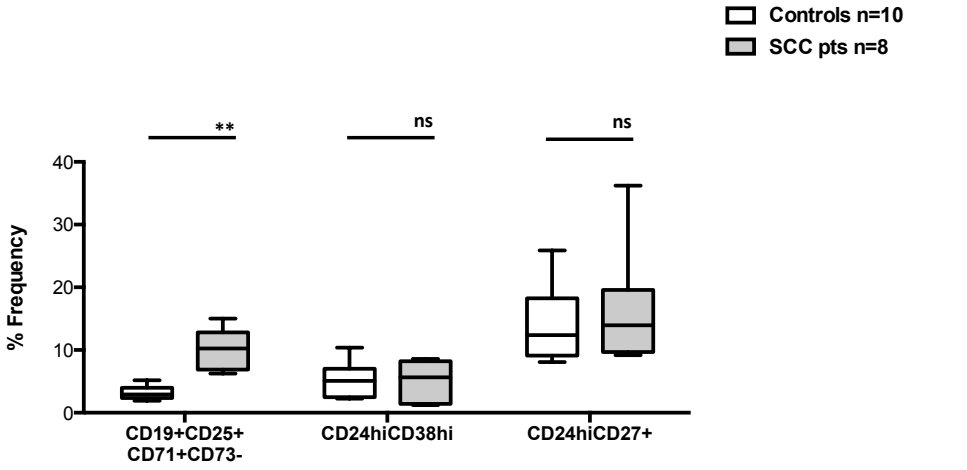
Fig. 5.3.2

A

SCC Pts n=8	Age (yrs) 82.5 (74.25-86.75)
MB01	90
MB02	74
MB03	83
MB06	72
MB07	75
MB08	88
MB11	83
MB17	82

LTKTRs n=6	Age (yrs) 67 (55.5-74.5)	Duration of IS (mo) 248 (214.8-279.5)	Immunosuppression (IS)
K021	65	242	Tacrolimus, azathioprine, prednisolone
K052	51	226	Ciclosporin
K071	76	293	Ciclosporin, azathioprine
K079	57	254	Ciclosporin, azathioprine, prednisolone
K119	69	275	Ciclosporin, azathioprine
K516	74	181	Sirolimus, prednisolone

B



C

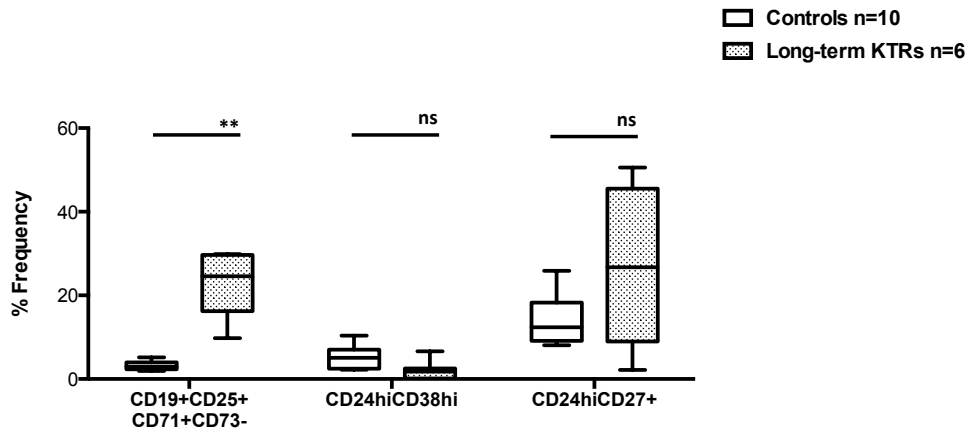


Fig. 5.3.2 $CD19^{+}CD25^{+}CD71^{+}CD73^{-}$ B cells are enriched in patients with squamous cell carcinoma and long-term kidney transplant recipients

(a) Tables summarising patient (pt) demographics including age in years (yrs), duration and details of any immunosuppression (IS) taken. Patients were included who had been diagnosed with a squamous cell carcinoma of the skin (SCC), or who had a kidney transplant for over 15 year and who have stable graft function (LKTR). **(b)** % of $CD25^{+}CD71^{+}CD73^{-}$, $CD24^{hi}CD38^{hi}$ or $CD24^{hi}CD27^{+}$ B cell subsets in whole $CD19^{+}$ B cells from peripheral blood of healthy individuals or patients with an SCC. *p<0.0004, Mann-Whitney test. **(c)** % of $CD25^{+}CD71^{+}CD73^{-}$, $CD24^{hi}CD38^{hi}$ or $CD24^{hi}CD27^{+}$ B cell subsets in whole $CD19^{+}$ B cells from peripheral blood of healthy individuals or LKTRs. **p<0.0004, Mann-Whitney test.

6.0 CONCLUSIONS AND FUTURE DIRECTIONS

Human B cells have long been accepted as a vital arm of the adaptive defence system, largely through their capacity to present antigen and to generate antibody. However, over the last decade, evidence of an alternate B cell function has emerged; that is, of regulation. Initially identified as an IL-10^{+} B cell population in mice [19, 21-24], the fundamental role of IL-10^{+} Breg in immune homeostasis has since been demonstrated in animal models of autoimmune disease, chronic inflammation and transplantation. Evidence of human Breg emerged as late as 2010, with the identification of $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^{+}$ B cell subsets in human peripheral blood, which were enriched with IL-10^{+} B cells with regulatory function [20, 48]. Whole genome expression analysis subsequently revealed another putative human Breg subset with the $CD25^{+}CD71^{+}CD73^{-}$ phenotype, associated with immunotolerant human subjects in the context of allergy [53]. The clinical relevance of human Breg in transplantation was sharply highlighted by the identification of a B cell signature of tolerance in operationally tolerant human kidney transplant recipients [12, 13].

In depth study of human Breg has been severely hampered by the rarity of such B cells in peripheral blood and the difficulty by which they can be reliably identified. Human B cell subsets enriched with putative Breg are of low frequencies, they are difficult to isolate and only a small portion of such populations actually comprise IL-10^{+} B cells. Furthermore, different human Breg phenotypes emerge depending on what mode of cellular stimulation is used to generate IL-10 production. Before application of human Breg cellular therapy or pharmaceutical manipulation of endogenous human Breg can be contemplated, much more detailed study of these cells is required, as well as the capacity to produce such cells in large numbers.

This current work has attempted to generate human Breg *ex vivo*, and to subsequently study the function of such cells both *in vitro* and *in vivo* by use of a humanised mouse model of skin transplantation developed in our laboratory [137, 138]. Several studies had previously demonstrated the requirement of the CD154-CD40 interaction for the

induction of mouse and human Breg [20, 47, 119]. Indeed, co-culture of human $CD19^+$ B cells with irradiated $CD154^+$ CHO cells resulted in proliferation of human B cells, increased expression of IL-10, and the generation of suppressive human B cells subsequently referred to as EXPB10 cells. Optimal conditions comprising repeated stimulation with $CD154^+$ CHO cells and exogenous cytokines human IL-2, IL-4 and IL-10 for 14 days, were able to achieve greater than an 800 fold expansion of EXPB10 cells.

In vitro interrogation demonstrated that EXPB10 cells could significantly suppress proliferation of autologous $CD4^+$ T cells in a dose-dependent manner. Furthermore, EXPB10 cells could significantly inhibit expression of pro-inflammatory cytokines interferon-γ **(**IFNγ) and tumour necrosis factor α (TNFα) by $CD4^+$ T cells, when compared to unmanipulated $CD19^+$ B cells (NON-EXPB10 cells). Importantly, proliferation and survival of EXPB10 cells did not vary with suppressive potency of EXPB10 cells, lending support to the view that these findings were not simply due to artefact of *in vitro* culture.

The engagement of CD154 expressed on activated T cells with CD40 constitutively expressed on B cells is widely known to result in diverse outcomes dependent on multiple parameters surrounding the interaction i.e. soluble or membrane-bound form of CD154, specificity and valency of CD154, activation status of the B cell as well as the intensity by which the CD40 epitope is targeted. Indeed this was highlighted most effectively by the differential suppressive potency of EXPB10 cells achieved when EXPB10 cells were generated under conditions where exposure to CD154 was in some way varied: co-culture of different ratios of $CD154^+$ CHO cells to $CD19^+$ B cells; co-culture of $CD19^+$ B cells with $CD154^{hi}$ or $CD154^{lo}$ CHO cells; re-stimulation of EXPB10 cells at day 7 of expansion with additional $CD154^+$ CHO cells. The degree of CD154 stimulation also dictated, in part, the rate of proliferation of $CD19^+$ B cells in this *in vitro* system. The use of different CD40 ligands in expansion co-cultures, either membrane-bound or soluble, may help to determine whether it is this particular form of CD154 expressed on CHO cells which is targeting a specific CD40 epitope, that in turn triggers downstream signalling to direct suppressive function. Identification of the targeted CD40 epitope by X-ray crystallography may also illuminate this interaction.

The finding that there were no differences in suppressive potencies of EXPB10 cells when different exogenous human cytokines had been added to expansion co-cultures reinforced the importance of the CD154-CD40 interaction in EXPB10 cell generation; only the rate of expansion varied under different cytokine conditions. Thus it was the extent of CD154 stimulation, not the exogenous cytokines to which B cells were exposed which determined suppressive potency. However both the extent of CD154 stimulation and the presence or absence of specific cytokines did appear to affect percentage IL-10 expression and levels of IL-10 production. In recent years, other cytokines and growth factors have been found to promote mouse Breg development, such as B cell survival factor BAFF, pro-inflammatory cytokine IL-21 and anti-inflammatory cytokine IL-35 [40, 98]. It will be valuable to establish in future experiments whether the addition of all or some of these factors could improve either expansion rate or suppressive function of EXPB10 cells in this *in vitro* system.

The relevance of CD154 to EXPB10 cell function as well as EXPB10 cell generation was revealed by the use of blocking mAbs to CD154 and CD40 in *in vitro* suppression assays; such blockade significantly reduced suppression of $CD4^+$ T cell proliferation by EXPB10 cells. Furthermore, blockade of this interaction as late as day 4 of the 5-day suppression assay could still significantly reduce EXPB10-cell mediated suppression, demonstrating that not only was suppression reversible but also that suppression was dependent on constant CD154-CD40 engagement. Interestingly, a more detailed study of the daily dynamics within the suppression assay revealed that EXPB10 cells initially promoted $CD4^+$ T cell proliferation before gaining suppressive function; the change in EXPB10 cell function correlated with CD154 expression by $CD4^+$ T cells within the co-culture, suggesting that increased exposure to a signal from activated $CD4^+$ T cells induced or promoted the suppressive function of EXPB10 cells. These observations were supported by the finding that EXPB10 cells appeared to stimulate $CD4^+$ T cell proliferation when in the absence of activating anti-CD3/CD28 beads, whilst suppressing such proliferation in the presence of anti-CD3/CD28 beads.

The observation that persistent and high levels of stimulation by CD154 were required to maintain EXPB10 cell suppressive function together with the finding that

the $CD25^{+}CD71^{+}CD73^{-}$ phenotype appeared to be less stable in less suppressive and less activated EXPB10 cells at the end of the suppression assay or when in the presence of CD154 blockade, suggested that activation status and suppressive capability of EXPB10 cells were inexorably linked. What is not clear from this data is whether all human $CD19^{+}$ B cells, when stimulated to an 'activation threshold,' could develop suppressive function or whether a progenitor EXPB10 cell exists which could respond uniquely to such activation and develop into a functionally suppressive EXPB10 cell. The downstream suppressive mechanisms may be varied, with IL-10 being one of several depending on the extracellular microenvironment. In essence, the human B cell may have regulated an otherwise overactive immune response through an automatic feedback loop by activating a mechanism intrinsic to either all human B cells, or to only a lineage-defined human Breg cell.

The sorting of different starting B cell populations before expansion may help to answer this question. In addition, the direct study of endogenous human $CD25^{+}CD71^{+}CD73^{-}$ B cells in comparison to other putative human Breg subsets may also aid in clarifying the universal Breg phenotype, if indeed one exists. Observational human data described in *Chapter 5.0* demonstrated that not only were $CD25^{+}CD71^{+}CD73^{-}$ B cells present in peripheral blood of healthy human subjects, but that these cells were also present within two B cell subsets already described to be enriched for human Breg: $CD24^{hi}CD38^{hi}$ transitional B cells and $CD24^{hi}CD27^{+}$ memory B cells. Previous studies have demonstrated that whilst $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^{+}$ B cell subsets comprise a high frequency of IL-10^{+} B cells, B cells capable of expressing IL-10 are also present in other human B cell subsets [20, 53]. It may be that human $CD25^{+}CD71^{+}CD73^{-}$ B cells represent the true human Breg phenotype. In support of this view, it was found that frequency of $CD25^{+}CD71^{+}CD73^{-}$ B cells was increased in the peripheral blood of patients diagnosed with squamous cell carcinoma of the skin when compared to unmatched healthy controls, unlike the frequency of $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^{+}$ B cell subsets.

It is tempting indeed to accept that the $CD25^{+}CD71^{+}CD73^{-}$ B cells identified in human peripheral blood hold the same suppressive properties as *ex vivo* generated $CD25^{+}CD71^{+}CD73^{-}$ EXPB10 cells; similarly enriched expression of CD154 and

TIM-1 in both B cell populations certainly lends support to this theory. However this is an assumption that requires clarification. FACS sorting of $CD25^{+}CD71^{+}CD73^{-}$ B cells from human $CD19^{+}$ B cells in peripheral blood and subsequent *in vitro* testing of suppressive function could help to elucidate whether this is the case. Investigation of human Breg lineage may prove to be more difficult. Ideally, one would like to test suppressive function of the isolated $CD25^{+}CD71^{+}CD73^{-}$ B cell population and remaining B cell fraction within the $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^{+}$ B cell subsets. Given the low frequencies of $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^{+}$ B cells found in human peripheral blood, however, this may not be technically possible.

Transwell experiments confirmed that EXPB10 cells did require a cognate interaction with $CD4^{+}$ T cells. One of these cognate interactions may well be the interaction of CD154 expressed on the activated T cell and CD40 expressed on EXPB10 cells. Transwell experiments also demonstrated that following this cognate interaction, a soluble factor was needed to mediate suppression of $CD4^{+}$ T cells by EXPB10 cells. The blockade of IL-10 and IL-10R resulted in partial abrogation of EXPB10-mediated suppression, suggesting that CD154 and IL-10 are these membrane-bound and soluble factors respectively. However, further *in vitro* study demonstrated that there were both CD154-dependent and CD154-independent pathways utilised by EXPB10 cells to regulate an immune response. The CD154-independent pathway appeared to also require cognate interactions or at least, very close proximity between $CD4^{+}$ T cells and EXPB10 cells. The ecto-ATPase pathway may well be a candidate for this mechanism, given the preliminary finding that inhibition of endonucleatidase function did significantly reduce, but did not wholly abrogate suppression of $CD4^{+}$ T cell proliferation by EXPB10 cells; an accumulation of immunosuppressive cAMP may have been partly responsible for EXPB10-mediated regulation in this *in vitro* system.

It is also possible that the action of endonucleatidases within the cellular microenvironment could actually affect the CD154-dependent pathway; previous studies have demonstrated prolonged CD154-CD40 interactions and indeed an increase in CD154 expression by activated T cells in the presence of increased levels of cAMP [248, 249]. Given that upon pre-incubation blockade with CD154 blocking mAb, EXPB10 cells up-regulated the expression of CD73 and were significantly less suppressive, a further alterative may also present itself: it may be that the metabolic

action of CD39 in the absence of CD73 is a downstream consequence of the CD154-CD40 interaction involving EXPB10 cells and is in fact the endpoint mechanism by which EXPB10 cells suppress a $CD4^+$ T cell response via CD154. Additional use of ecto-ATPase inhibitors within the transwell experiments should help to clarify this. Furthermore, levels of cAMP, adenosine as well as ATP could be measured within cell culture supernatants by mass spectrometry in order to establish whether these enzymes are indeed functionally active within this experimental setting.

Other immunoregulatory mechanisms which are able to disrupt cellular metabolism were not tested, such as reactive oxygen species (ROS) or indoleamine 2,3-dioxygenase (IDO); future experiments may warrant investigation of these more innate-associated processes. Immunosuppressive molecules up-regulated by EXPB10 cells, such as LAP, did not appear to be responsible for EXPB10 cell-mediated regulation *in vitro*. However, as highlighted previously, positive controls for the inhibitors used in these experiments were not often available; and certainly a potential *in vivo* action of TGFβ cannot be precluded.

B7-H4 is also an attractive candidate by which EXPB10 cells could ultimately suppress the $CD4^+$ T cell response. As described in *Chapter 4.0*, B7-H4 can be induced by both IL-10 and IL-6, both of which are secreted by EXPB10 cells. *In vitro* blockade of IL-10 and IL-10R demonstrated that it was the ability of the EXPB10 cell to respond to autocrine IL-10 which was critical to the cell's regulatory function, rather than the direct action of IL-10 on the responding $CD4^+$ T cell. In addition, the blockade of the CD154-CD40 interaction in the suppression assay not only significantly reduced suppressive potency of EXPB10 cells, but also significantly reduced expression of B7-H4 by EXPB10 cells. Unfortunately, a blocking mAb to human B7-H4 is not currently available to easily interrogate this hypothesis. However, emergent technologies such as morpholino oligonucleotides or clustered regularly-interspaced short palindromic repeats (CRISPR) may provide alternative methods by which B7-H4 expression by EXPB10 cells may be silenced and function evaluated.

To our knowledge this is the first report of the expression of TIM-1 by a population of *ex vivo* expanded human Breg population. Furthermore, this is the first description of

a role for TIM-1 in the regulatory pathway employed by a human Breg cell. Blockade of TIM-1 resulted in an abrogated response of EXPB10 cells to IL-10 as well as a significant reduction in suppressive potency. How TIM-1 affected this change in cellular responses to IL-10 is not yet clear. Conformational changes involving the IL-10R and further down-stream signalling are two possibilities. The down-regulation of CD71 expression upon blockade of either CD154 or TIM-1 indicates that TIM-1 may also have been important in maintaining high levels of activation within the cell; high levels of activation which, as discussed, have been shown to correlate with suppressive potency of EXPB10 cells in both *Chapters 3.0* and *4.0*.

It is of course possible that TIM-1 represents a more fundamental molecule in B cell metabolism, such that blockade results in a compromise in basic B cell function. However, measurement of EXPB10 cell numbers and proliferation in the absence and presence of TIM-1 blockade suggest that this is not the case. As described in *Chapter 4.0*, recent mouse studies have found that in the absence of TIM-1, mouse Breg produce less IL-10 and demonstrate a more pro-inflammatory cytokine profile. TIM-1 may act as a functional gateway for potential human Breg, modulating the balance between pro-inflammatory and regulatory differentiation through cellular activation and responses to IL-10. Future experiments should determine whether blockade of TIM-1 within this human Breg population would also affect cytokine production. An additional method of TIM-1 blockade, such as with the genetic techniques mentioned earlier here, may also be valuable in confirming our findings.

The use of a humanised mouse model of skin transplantation enabled us to examine the function of EXPB10 cells in a much more complex *in vivo* setting. Intraperitoneal injection of human EXPB10 cells significantly prolonged survival of an HLA-mismatched human skin graft in a fully reconstituted Balb/c $Rag2^{-/-}$ $c\gamma^{-/-}$ mouse, when compared to controls. No significant difference in the rate of reconstitution was found between those mice which had received PBMC only, PBMC and NON-EXPB10 cells, or PBMC and EXPB10 cells. A significant decrease in percentage and number of human $CD4^+$ T cells in peripheral blood, spleen and skin allograft was observed in mice which had received EXPB10 cells compared to control groups. However, an increased proportion of human $CD4^+$ T cells within the human skin

allografts of mice which had received EXPB10 cells, expressed the putative human Treg phenotype $CD4^+CD25^+CD127^{lo}$ as well as FoxP3. Although insufficient cell numbers of this subset could be extracted from human skin allografts at the time of harvest, *in vitro* experiments confirmed that EXPB10 cells were able to either expand or induce a population of functional human $CD4^+CD25^+CD127^{lo}$ Treg. Interestingly, this effect was found to be both CD154- and TIM-1-independent. To our knowledge, this is the first demonstration of the generation of functional human Treg by a human Breg population.

Measurement of serum cytokines within the humanised mouse model also revealed a significant decrease in levels of pro-inflammatory cytokines IL-2 and IL-17a within the peripheral blood of mice which had received EXPB10 cells when compared to controls. As these samples were taken at time of rejection, it is difficult to interpret the true meaning of such results. Earlier serum samples should be measured in order to clarify this; however, it may be that EXPB10 cells are able to promote a more regulatory environment, thus engendering effective modulation of the immune response through the use of multiple regulatory populations. It would be valuable to examine whether human EXPB10 cells could increase the suppressive potency of *ex vivo* generated human Treg when concomitantly introduced into this *in vivo* system at a sub-therapeutic concentration. Such studies could provide extremely relevant data, given that human Treg are currently being administered to human kidney transplant recipients in clinical trials. Adjuvant cellular therapy such as human Breg administration may confer further immunoregulatory stability or dose reduction, with all the clinical advantages that may accompany such modifications.

A significant increase in human $CD20^+$ B cells was seen in the spleen and skin of the EXPB10 group. In addition, a large proportion of the human $CD20^+$ B cells identified were TIM-1^+, suggesting that EXPB10 cells might retain some functional stability, although the majority of these human $CD20^+$ B cells did not carry the phenotype $CD25^+CD71^+CD73^-$. Given the nature of the humanised mouse model used, it was not possible to extract sufficient numbers of human $CD20^+$ B cells from mice after reconstitution in order to re-test function. This is highly pertinent to the potential use of such a regulatory B cell population in the clinic: EXPB10 cells were found to promote $CD4^+$ T cell proliferation at the early stages of the *in vitro* suppression assay,

and EXPB10-mediated suppression was shown to be reversible when CD154 was blocked. The evidence presented in this work supports the theory that suppression by EXPB10 cells requires on-going signaling from activated $CD4^+$ T cells, and so this raises questions about the stability of suppressive function of EXPB10 cells when acute inflammation has subsided *in vivo*. It is not certain how EXPB10 cells will behave in the absence of such pro-inflammatory signals, although it is reassuring that EXPB10 cells were able to significantly prolong allograft survival when injected 35 days after initial transplantation of the skin graft, when any acute phase response should have subsided [137].

Indeed, whilst the Balb/c $Rag2^{-/-}$ $c\gamma^{-/-}$ mouse has provided an excellent model in which the immediate immunosuppressive function of human B cells can be questioned, this *in vivo* system is not without limitation, as highlighted in *Chapter 5.0*. In order to more effectively address the long-term behaviour and development of EXPB10 cells *in vivo*, it may be better to examine this regulatory B cell subset in a humanised mouse model more suited to human B cell engraftment, such as the HLA-DR4 transgenic mouse model discussed at length in *Chapter 5.0*. Such a model may also promote the formation of tertiary lymphoid structures, enabling more in depth study of the dynamics of both rejection and regulation *in vivo*, as well as the production of antibody.

Antigen specificity of human Breg has not been explored within this work, but this will be a crucial factor in determining clinical value of such a therapy. Since it has been established *in vitro* that EXPB10 cells require CD154-CD40 engagement to exert suppressive function, it is tempting to speculate that EXPB10 cells were only able to suppress allospecific $CD4^+$ T cells when they themselves had been presented with antigen and had up-regulated CD154. Prior to clinical use, it will be imperative to explore how human EXPB10 cells behave in the presence of antigen both *in vitro* during the expansion protocol as well as *in vivo*; in particular whether the BCR repertoire will narrow, whether EXPB10 cells can become polarised to gain antigen specificity, and importantly whether they can retain regulatory function.

This work provides an initial method by which moderately large numbers of human Breg can not only be generated but also studied. Here we demonstrate the first

evidence that an *ex vivo* generated population of human $CD19^{+}CD25^{+}CD71^{+}CD73^{-}$ Breg can significantly regulate a human immune response *in vivo*, in particular, to prolong human allograft survival. Preliminary study has demonstrated that suppressive function of this particular *ex vivo* generated human Breg population is dependent on the interaction of CD154 with CD40 as well as stimulation by autocrine IL-10. *In vitro* experiments have also identified a role for TIM-1 in the mechanism by which human Breg modulate an immune response. Purinergic signalling may also play a part but further elucidation is required. The expansion or induction of human Treg may offer an additional method by which this human Breg population is able to regulate the immune response.

Cellular therapy is rapidly emerging as an exciting mode of biological management. Such advances may offer the promise of antigen-specific, individually tailored therapy for patients much in need, not only in the field of transplantation but also in those of autoimmune disease and chronic inflammatory conditions. Human Breg therapy is one such potential treatment that may help to shape the course of clinical immunoregulation. The expansion process described here could be further optimised to provide much greater yields, and to allow continued examination of this suppressive population both *in vitro* and *in vivo.* This study of *ex vivo* expansion of human Breg, or EXPB10 cells, may provide a platform from which a greater understanding and development of a clinically relevant, immunosuppressive human B cellular therapy can be realised.

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