

Oxford Transplant Foundation - September 2026 Report

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Title: Generation of antigen-specific human regulatory B cell therapy

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Stated Aims:

1. Optimisation of GFP gene delivery into human expBreg.
2. Generation of engineered antigen specific receptor-encoding constructs.
3. Delivery of genes encoding antigen specific receptor-encoding constructs into human expBregs.
4. Evaluation of antigen-specificity & function of genetically edited human Breg in vitro.

Introduction

Immunosuppression is a crucial component of solid organ transplantation mediating alloreactive responses in individuals facing end-stage organ failure [1, 2]. Potent immunosuppressive drugs have significantly impacted short-term graft and patient survival but show minimal benefit for long-term outcomes. Graft survival falls from 95 to 75% over 5 years with 40% of grafts from deceased kidney donors failing within 10 years [3]. Prolonged general immunosuppression has also been associated with enhanced morbidity, infection, risk of cancer and target organ damage [4-7]. To alleviate negative side effects, novel therapeutic strategies are required to improve long-term outcomes.

One method of alternative immunosuppression is the use of cellular therapy [2]. B cells possess immunosuppressive function, identified as early as the 1970s where depletion of B cells in splenocytes failed to suppress delayed-type hypersensitivity in guinea pigs [8]. Regulatory B cells (Bregs) role in improving transplantation was identified following antibody mediated depletion of CD20+ B cells, resulting in enhanced T cell mediated rejection in both human kidney and mouse skin transplant models [9, 10]. Bregs can suppress pro-inflammatory and alloimmune responses, reduce pro-inflammatory cytokine production and importantly induce the differentiation of regulatory T cells (Tregs), thus amplifying the immunoregulatory response [11-13]. Human Breg signatures are also enriched within peripheral blood of operationally tolerant kidney transplant recipients and can predict graft survival and function 5 years following transplantation [14]. Antigen-specific Tregs, often produced through introduction of a Chimeric Antigen Receptor (CAR), are more effective than polyclonal therapy in transplantation [15]. Genetically modified Bregs may amplify a multi-faceted, allospecific immunoregulatory response to minimise off-target responses. We aim to introduce into human Bregs a novel CAR construct targeting HLA-A2 as proof of concept, and to generate a new class of B cell-based personalised therapy. Secondary aims include the knockout of MHC expressed by human Bregs, to transition from an autologous to a potentially universal Breg cell therapy.

Summary of Activities

Development and Optimisation of Transduction Strategy

Preliminary data previously demonstrated we could efficiently transduce expanded regulatory B cell (expBreg) populations with EGFP while maintaining suppressive function. High efficiency was attributed to the use of transduction-assisting chemicals (polybrene). However, polybrene's use also conveyed a reduction in cell viability (Figure 1a); thus, refining transduction methods to optimise cell viability and transduction efficiency were prioritised for downstream applications.

To maximise transduction several options to replace and refine our technique were explored. Our first method attempted to replace polybrene by improving Breg viability and expansion, we hypothesised that greater proliferation would improve lentiviral uptake. One option is B cell receptor (BCR) signalling, a central pathway which governs B cell proliferation and differentiation [16], whose dysregulation is associated with multiple autoimmune conditions [17, 18]. Furthermore, Breg development and survival is associated with B cell receptor (BCR) stimulation in mice [19, 20]. Similarly, BCR stimulation is required to induce some regulatory B cell subsets in humans [21]. To test if BCR stimulation would improve transduction, polyclonal and IgM-specific antibody stimuli were included during Breg expansion. However, stimulation at early (Day 0) or late (Day 5) stage expansion conveyed no benefit to Breg transduction and was less effective than polybrene (Figure 1b). Our second method explored the use of alternative transduction enhancing techniques; spinfection is a method of improving efficiency by increasing cell-to-vector contact using centrifugal force instead of chemical interaction. We compared spinfection to polybrene as well as combining the two methods. While both showed improved efficiency relative to unassisted Breg transduction; polybrene alone was most effective (Figure 1c). Lastly, we sought to limit cytotoxic effects by minimising polybrene exposure; a timecourse comparing the length of polybrene assisted transduction demonstrated that exposure greater than 6 hours conveyed no increased transduction efficiency (Figure 1d). In contrast cell viability decreased with greater than 6-hour polybrene exposure (Figure 1e). Collectively our results [18] demonstrated that our expBregs could be transduced at high efficiency with minimal loss in viability by optimising exposure of polybrene, refining our method to more easily test our designed CAR constructs.

Design and Testing of HLA-A2⁺ CAR constructs in the expBreg population

Two CAR constructs were designed for generating lentiviral vectors. The constructs were based off either the TIM-1 receptor, identified as highly expressed on our expBreg population [22], or on the native BCR, both modified to possess an antigen binding region specific to human HLA-A2 and a GFP reporter gene. The constructs were designed to take advantage of the endogenous production of similar receptors to assist in CAR expression. Initial production of CAR vectors was successful, with both CARs conferring GFP expression following transduction of HEK293T cells (Figure 1f). However, GFP expression and therefore vector production was reduced in the CAR vectors compared to GFP vector control. Attempts to improve vector production by optimising plasmid ratios, growth conditions and purification techniques showed no improvement. To determine if integration or expression of the construct was the limiting factor; only the CAR-encoding plasmid was transfected into HEK293T cells, demonstrating that expression of GFP was limited in the CAR constructs independent of lentiviral integration or production (Figure 1g).

To compensate for the reduction in GFP expression, a fluorescently labelled HLA-A2 specific dextramer was used to directly identify CAR expression. HEK293T cells transduction showed CAR expression only with the TIM-1 CAR construct, prompting the BCR construct to be discarded (Figure 1h). Initial TIM-1 CAR transduction in human expBregs demonstrated no GFP but 48.9% of cells expresses the TIM-1 CAR (Figure 1i). Successive iterations of the CAR construct were then generated to improve expression; altering kozak sequences (TIM-1 V2) and removing unnecessary spacer regions (TIM-1 V3). Different kozak sequences alter how efficiently ribosomes initiate translation at the start codon, directly contributing to protein expression. In contrast the removal of extraneous spacer regions aids transduction by reducing the total size of the insert and by reducing the risk of protein misfolding, which may limit plasmid integration and protein production respectively. While neither version improved GFP expression both benefited CAR expression (Figure 1i), with average transduction efficiency improving from 39.7% with the GFP control vector to 71.5% with our CAR vector (Figure 1j). This suggests we have reliably transduced our novel CAR into human Bregs but that no or non-functional GFP is produced. One option is that the CAR and GFP proteins fail to separate following translation, resulting in a misfolded GFP molecule. As such, examining the expressed protein directly via Western blotting is our next priority, confirming CAR expression, while also determining the fate of the GFP protein. Should GFP prove non-function our intention is to replace it with Ab detection of a G4S-linker region embedded in our CAR via flow cytometry, allowing for CAR detection without risking antigen stimulation. We believe these results demonstrate we have achieved our primary goal of transducing a novel CAR into human Bregs, with further work aiming to reinforce our findings.

Antigen specific function of CAR⁺ expBreg

Functional evaluation indicated that CAR transduction maintained polyclonal expBreg suppressive function (Figure 1k). To initially assess how cells would respond to an antigen-specific challenge, we used an HLA-A2 dextramer to stimulate CAR⁺ Bregs and measure the suppressive effect on T cells. Following stimulation, CAR Breg populations demonstrated some improvement compared to unstimulated CAR Bregs. However non-regulatory, Activated B cell populations also demonstrated apparent enhanced suppression following CAR recognition (Figure 1l). This data was confounded by the overall low suppressive response of the donor prior to stimulation, making it difficult to determine what effect was derived from suppressive responses and which from co-culture limiting nutrient availability. Further assays testing a range of donors with proven responses is required to confirm our results. However, the improvement of both populations following stimulation suggests some underlying antigen specific response. Unfortunately, availability of dextramer stimulus had limited further assessment with this method. A potential replacement such as recombinant protein or antigens tetramers is currently being sought.

An alternative method of testing functional responses was to determine the impact of our CAR Bregs on alloreactivity. We invested in a mixed lymphocyte reaction model, identifying human PBMC donors to generate HLA-A2⁻ Responder and Breg populations and mismatched HLA-A2⁺ Stimulator populations. However, despite initial donors demonstrating proliferation of Responder populations when co-cultured, subsequent donors were found to be inconsistent; generating no or limited proliferation in response to HLA mismatch, preventing CAR specific responses from being robustly tested. To eliminate donor variability different HLA-A2⁺ expressing cell lines were tested to replace the stimulator population, as previously demonstrated in the interrogation of antigen-specific CAR T cell populations[23]. A modified K562 (HLA-A2⁺) and B-

Lymphoblastoid cell lines (B-LCL) (HLA-A2⁺, DR11⁺) were chosen due to their consistent HLA-A2 expression with several responder: stimulator ratios tested to optimise the response. Only the B-LCL cells at a 3:1 stimulator:responder ratio, demonstrated robust proliferation of responder cells above both autologous and allogenic PBMC co-culture and should allow for consistent antigen stimulation (Figure 1m). Recently, we applied the B-LCL cell line to our MLR model where CAR Bregs were co-cultured with responder CD4 T cells and B-LCL HLA-A2⁺ stimulators. The preliminary results demonstrated that T cell proliferation was reduced in CAR Breg co-cultures relative to polyclonal Bregs (Figure 1n) suggesting that antigen recognition via the CAR has directly enhanced suppressive ability. Further repeats will be required to confirm our findings, but this suggests that our secondary aim of enhancing Breg suppression is achievable, with the MLR model providing a basis for testing further iterations of CAR designs.

Future Project Plans

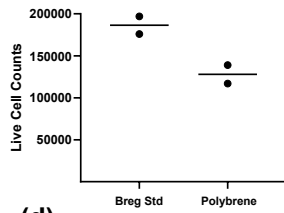
The next stage of the project will focus on a few key areas; the first being to verify existing data. The initial functional responses observed in the MLR and dextramer stimulation assays are limited by the number of donors. Repeat assays utilising multiple donors to validate the response is necessary. Furthermore, the assays should be expanded to assess the extent of CAR induced suppression. Examples include determining if suppression is dose-dependent, either by varying the ratio of Bregs to responder cells or titrating the level of antigen stimulation required to induce suppression.

After confirming antigen specific suppression, we will focus on how CAR activation has impacted Breg behaviour. The exact mechanisms of suppression can vary between cell subsets, therefore, to understand how our CAR impacts function, robust characterization of the CAR Breg phenotype is required. A spectral flow cytometry approach would determine first that Bregs are phenotypically unchanged following transduction and second, identify responses characteristic of CAR activation and enhanced suppression. Following from phenotype analysis we can assess CAR activation via Western Blotting; initially supporting flow data by confirming the expression of the CAR receptor, it can also assess the mechanism of activation. The CAR receptor is based on the endogenous TIM-1 receptor which upon activation undergoes tyrosine phosphorylation to signal downstream [24, 25]. As previous work has shown that TIM-1 expression is crucial for expBreg function, blotting techniques can determine if the CAR undergoes similar changes following antigen recognition [22]. Identifying the primary CAR signalling mechanism will provide insight into the underlying mechanisms of suppression, which subsequently may be used to inform future CAR iterations and modifications.

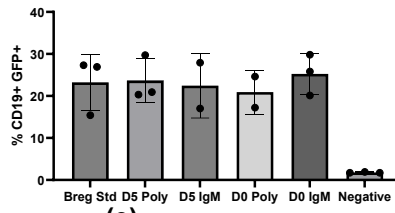
Lastly, once a CAR design is finalised, we will move to develop MHC-deficient CAR-Bregs as a precursor to universal Breg cell therapy. The main method will utilise a combination of precise CRISPR gene editing to introduce targeted knockouts in MHC class I and class II, as previously demonstrated [26], combined with our established lentiviral techniques to introduce our CAR constructs. Following MHC and CAR modifications, MLR assays will be used to verify function as a precursor to future ex vivo animal studies. Additionally, improving accessibility and scalability of Bregs is also a consideration. Peripheral blood is our primary source of B cells but is practically limited by extraction. Human spleen cells have the potential to be harvested in greater numbers due to their size and the potential acquisition as a byproduct of other transplantation procedures. Exploring the transduction potential of human spleen cells may provide a scalable option for developing a universal cell therapy.

In conclusion the project thus far has achieved its first primary aim, generating a human CAR Breg with preliminary evidence supporting our secondary aim of enhancing suppressive function. The results presented are promising and exciting but should be interpreted with caution. The extent of the CAR's impact on suppression and the underlying mechanisms is currently unknown. Further work is required to establish a robust CAR platform, capable enough for clinical application. Nonetheless, having developed a functional CAR design, future developments should proceed more swiftly, reinforcing our existing data and allowing us to begin our final group of aims.

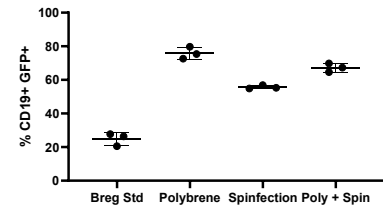
(a) Cell Viability post-transduction



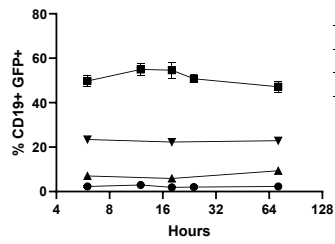
(b) Breg Transduction - BCR Stim



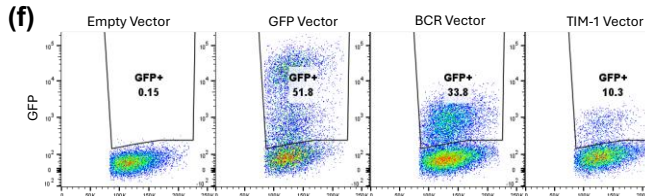
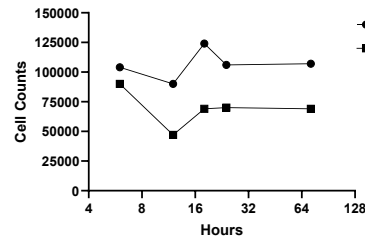
(c) Transduction Method Comparison



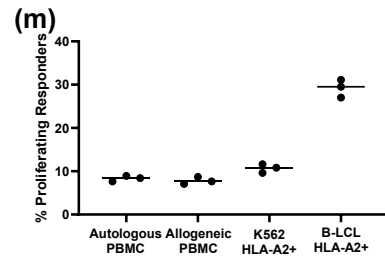
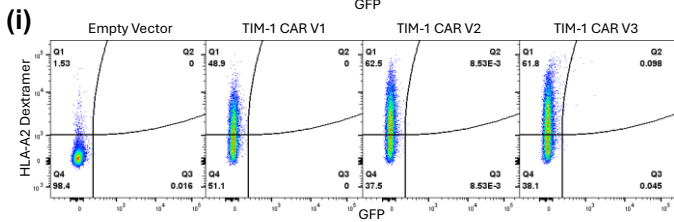
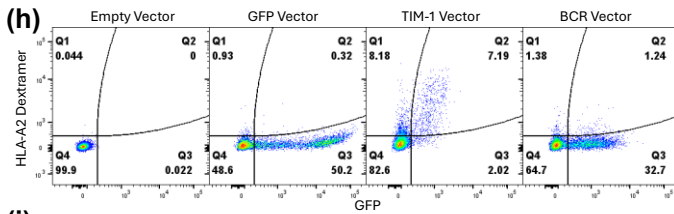
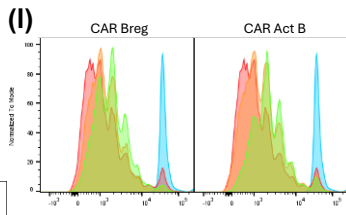
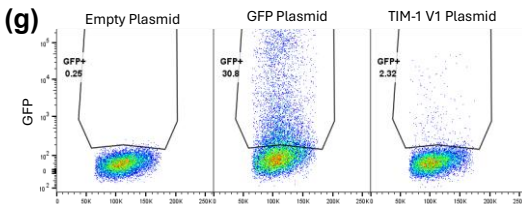
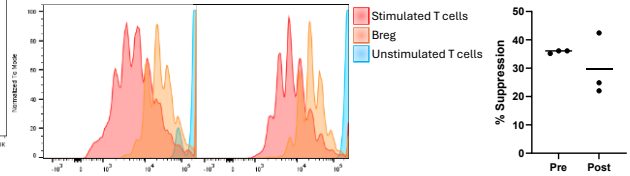
(d) Polybrene Timecourse Efficiency



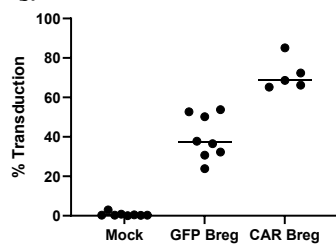
(e) Cell Viability



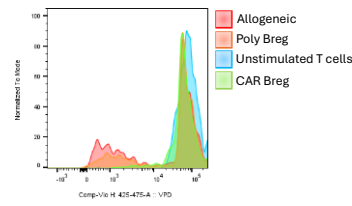
(k) Pre-Transduction Post-Transduction



(j) Transduction Efficiency



(n)



Breg MLR Suppression

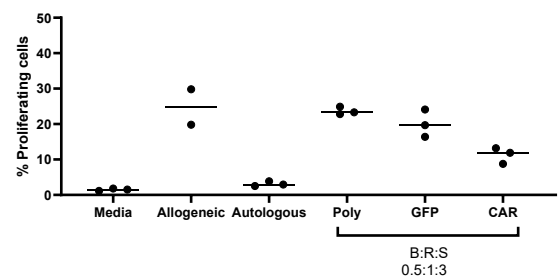


Figure 1. (a) Average viable cell counts following transduction protocol with or without 72hr polybrene incubation (n=2). (b) Percentage transduction efficiency of Bregs using detailed stimulation (n=2). Error bars represent standard deviation. (c) Percentage transduction efficiency of Bregs using detailed transduction methods (n=1), error bars represent StDev. (d) Average percentage transduction efficiency of expBregs over time for the detailed stimulation conditions (n=2). (e) Average viable cell counts following transduction protocol with or without polybrene stimulation over time (n=2). (f) Representative FACS plots of GFP expression following transduction of HEK293T cells at relative 1:250 dilution of different CAR or GFP encoding concentrated vectors. Gating set from empty vector control. (g) Representative FACS plots of GFP expression following HEK293T transfection of GFP or CAR TIM-1 encoding plasmid. Gating set on empty plasmid control. (h) Representative FACS plots of GFP expression and CAR surface expression following HEK293T cell transduction with the stated vector designs. Gating set on empty vector controls. (i) Representative FACS plots of GFP expression and CAR surface expression following Breg transduction with the stated vector designs. Gating set on empty vector control. (j) Summarised percentage transduction efficiency of expBreg using GFP or CAR vectors. Each dot represents an individual donor. (k) Representative histograms of live CD4⁺, VPD⁺ T cells and summarised data of % inhibition of CD4⁺ T-cell proliferation from suppression assay co-culture of pre or post CAR transduced expBreg (n=1). Groups are: stimulated T cells (red), stimulated T cell and Breg co-culture (orange), unstimulated T cells (blue). (l) Representative histograms of live CD4⁺, VPD⁺ T cells and summarised data of % inhibition of CD4⁺ T-cell proliferation from suppression assay co-culture of CAR Breg or CAR Act B cells with or without HLA-A2 dextramer stimulation (n=1). Groups are: stimulated T cells (red), stimulated T cell and B cell co-culture (orange), unstimulated T cells (blue), stimulated T cell and B cell co-culture with dextramer stimulation (green). (m) Summarised data of % proliferating live CD4⁺, VPD⁺ T cell responders following MLR co-culture (n=1). (n) Representative histograms of live CD4⁺, VPD⁺ T cells and summarised data of % proliferating T cells following MLR co-culture as described (n=1). Groups are: Allogeneic PBMC co-culture (red), B-LCL co-culture with T cells and polyclonal Breg (orange), unstimulated T cells (blue), B-LCL co-culture with T cell and CAR Breg (green).

Materials and Methods

Cell culture and Human Donors

Blood samples for PBMC isolation we sourced from healthy human donors provided by NHS Blood & Transplant; demographic data was not available for these donors. Cell culture of primary human cells used complete RPMI (cRPMI) (RPMI-1640 (Sigma-Aldrich) supplemented with 100 U/μg/ml penicillin/streptomycin (Life Technologies), L-glutamine and 10% FCS (Gibco). Cell lines were cultured with the following:

CHO-CD154⁺; DMEM containing 4500 μg/L glucose, 110 μg/L sodium pyruvate and L-glutamine (Sigma Aldrich) with 100 U/ml penicillin and streptomycin (Life Technologies) and 5% foetal calf serum (FCS) (Gibco). CD154 expression was routinely checked by flow cytometry analysis after staining with CD154-PE mAb. All CHO cell lines were cultured in T75 flasks and were passaged using standard operating procedure with Trypsin from porcine pancreas (2.5 g/L, Sigma, UK). Before being used in expBreg cell expansion cultures, CHO cells were γ-ray irradiated (65 Gy, 6500 rad) in 50 ml corning tubes for 30min using a Caesium 137 irradiator.

K562 HLA-A2⁺; RPMI-1640 supplemented with 100 U/μg/ml penicillin/streptomycin, L-glutamine and 10% FCS and were passaged by suspension dilution.

HEK293T; DMEM containing 4500 μg/L glucose, 110 μg/L sodium pyruvate and L-glutamine with 100 U/ml penicillin and streptomycin and 10% foetal calf serum (FCS). Cells and were passaged using standard operating procedure with Trypsin from porcine pancreas.

B-LCL HLA-A2⁺; RPMI- 1640 supplemented with 10% heat-inactivated FBS, 100 μg/mL streptomycin, 100 IU/mL penicillin, 2 mM L-glutamine, and 1 mM sodium pyruvate and were passaged by suspension dilution.

Generation of Human expBreg

Human PBMC were isolated from leucocyte cones, and CD19⁺ B cells were subsequently isolated 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. 6×10^4 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 or 1:10 CHO cells to CD19⁺ B cells in a total volume of 200 μl per well. Unless otherwise stated, CD19⁺ B-cell and irradiated CHO cells were co-cultured with a cytokine combination of 50 U/ml of IL-2, 100U/ml of IL-4 and 25U/ml of IL-10 (all PeproTech, Rocky Hill, NJ). Cultures were fed by replacing culture medium and cytokines every 2–3 days, while irradiated CHO-CD154 cells were renewed on day-3 ± expansion. Ratios of CHO-CD154 cells to CD19 were kept constant throughout the culture period. Viable CD19⁺ B cells were harvested on day-7, Some experiments additionally included anti-BCR Ab stimulus using polyclonal or anti-IgM Ab at day 0 or day 5 of expansion.

DNA Plasmids

Multiple DNA plasmids were generated to carry the constructs used to generate the various Breg types. Plasmid types consisted of: GFP Plasmid – containing the EGFP gene. TIM-1 CAR Plasmid – CAR construct consisting of either the human TIM-1 sequence or human IgG BCR, minus the Ig-like binding domain, which was replaced with a anti-HLA-A2 scFv previously described [23]. CAR constructs possessed a P2A-linker region and an EGFP gene. Initial

plasmids were designed and then synthesized externally (Genewiz). Subsequent modifications to CAR plasmid (TIM-1 V1 and V2) used common restriction enzyme digest protocols replacing desired elements with synthesised replacements (Genewiz).

Lentiviral Vector Production

Lentiviral particles were generated using standard production protocols. HEK293T cells were seeded at 4×10^6 cells per 10cm plate and allowed to adhere. Lentiviral DNA mastermix was prepared at the following ratio; Genome 4.5: GagPol 1.4, Env 0.7: Rev 1.1. DNA mastermix was combined at a 1:1 ratio with 1x Lipofectamine (ThermoFisher) diluted in OptiPro Media (Gibco). DNA/Lipofectamine mix was incubated at room temperature for 20 minutes before being added dropwise to HEK293T cells. After 24 hours incubation 1M sodium butyrate is added at 10 μ l/ml. 72 hours post transfection supernatant is harvested, filtered through a 0.45 μ m filter and concentrated via ultracentrifugation through a 20% sucrose buffer overlay. Lentiviral particles were resuspended in OptiPro media and stored at -80°C until needed. Lentivirus batches were titrated against known numbers of HEK293T cells, measure % GFP expressing transduced cells. Titre was calculated as Infectious Units (IU)/ml with the following formula ((%GFP⁺ as decimal x No. of cells at transduction)/Volume of inoculum (ml)).

Transduction Methods

Transduction procedures were used to generate all modified B cells. Transduction occurred in 96-well plates at a final volume of 200 μ l/well. 1×10^5 B cells/well were seeded along with 1×10^4 γ -irradiated CHO-CD154 cells, 8 μ g/ml polybrene, 50 U/ml of IL-2, 100U/ml of IL-4 and 25U/ml of IL-10 and lentiviral particles as follows; GFP Vector (MOI 20), TIM-1CAR Vector (MOI2). Cells were incubated at 37°C with polybrene for variable lengths of time before cells were transferred to a new 96-well plate and washed by centrifugation (500xg, 5 minutes). Cells were resuspended and transferred back to their original plate with and an additional, identical dose of cytokine was added. Cells were further incubated for 72 hours prior to harvest. Transduction efficiency is determined by flow cytometry measuring % GFP⁺ cells or by staining with HLA-A2 Dextramer-APC. Mock transduced cells receiving no Lentiviral vector were used as gating controls.

In vitro suppression assay

To assess the effect of expBreg on proliferation of CD4⁺ T cells 5×10^4 expBreg were co-cultured with autologous CD4⁺ T cells for 5 days in 96-well U-bottom plates in a total volume of 200 μ l. CD4⁺ T cells (responder cells) were stained with 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 cultured in the absence of expBreg, or instead with nCD19⁺ Activated B cells, served as control conditions. On day 5 of culture, cells were harvested, stained for cell-surface markers and analysed by flow cytometry. In some experiments, Human HLA-A2 dextramer-APC (1.3nM) were added to suppression assays at day 0 for 30-minute stimulation before being washed off. % Inhibition of proliferation is an expression of Proliferation Index (PI) of live CD4⁺ VPD⁺ T cells at day 5 relative to that of the stimulated CD4⁺ T-cell control, such that % Inhibition of proliferation = $(1 - (\text{PI of CD4}^+ \text{ T cells experimental condition} / \text{PI of stimulated CD4}^+ \text{ T cells control})) \times 100$.

Mixed Lymphocyte Reaction

The optimum conditions to induce T cell proliferation were first determined by co-culture of CD4 T cells responders with different stimulator cell types and ratios. CD4 T cells were labeled with VPD before being resuspended at a concentration of 2×10^6 cells/ml in cRPMI and kept on ice until needed. 1×10^5 T cells will be seeded per well as a constant. Stimulator populations include PBMC, K562 HLA-A2 and B-LCL HLA-A2 cell lines. Stimulator cells were harvested and resuspended at an appropriate concentration before being labelled with CFSE and γ -irradiated (40cGy). Cultures were activated with γ -irradiated PBMC (1:1 PBMC: T cell ratio) K562 (3:1 K562: T cell ratio) or B-LCLs (3:1 B-LCL:T cell ratio) and incubated at 37° C for 6 days. The suppressive capacity of Bregs was assessed by co-culturing autologous CD4 T cells with varying concentrations of Bregs (ranging from 1:1 to 0.5:1). Breg preparations were collected post transduction and resuspended at a concentration of 2×10^6 cells/ml. Bregs and T cells were seeded (with Bregs in a serially diluted manner to achieve Breg/T cell ratios as indicated above) into a 96-well round-bottom plate and made to a total volume of 200 μ L per well with cRPMI. T cell proliferation was measured through VPD label dilution by flow cytometry after 6 days. Breg suppression was determined as a percentage defined by inverse of T cell proliferation as identified when gating for VPD, relative to stimulated T cells alone.

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