


# CD40L-stimulated B cells for *ex-vivo* expansion of polyspecific non-human primate regulatory T cells for translational studies

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## Introduction

Since the introduction of calcineurin inhibitors, considerable improvement has been made in patient survival after organ transplantation [1]. Currently, the major reasons for graft loss are sequelae of immunosuppression, such as infection and cancer [2,3]. The induction of donor-specific tolerance would eliminate the need for immunosuppression and be expected to improve quality of life and patient/graft survival. However, given the good outcomes of transplants performed with the current standard of care, it is critical that tolerance-inducing strategies are shown to be safe and effective in non-human primate (NHP) models prior to clinical trials,

## Summary

The therapeutic applications of regulatory T cells ( $T_{\text{regs}}$ ) include treating autoimmune diseases, graft-versus-host disease and induction of transplantation tolerance. For *ex-vivo* expanded  $T_{\text{regs}}$  to be used in deceased donor transplantation, they must be able to suppress T cell responses to a broad range of human leukocyte antigen (HLA). Here, we present a novel approach for the expansion of polyspecific  $T_{\text{regs}}$  in cynomolgus macaques that was adapted from a good manufacturing practice-compliant protocol.  $T_{\text{regs}}$  were isolated by fluorescence-activated cell sorting (FACS) and expanded in the presence of a panel of CD40L-stimulated B cells (CD40L-sBc). Prior to  $T_{\text{reg}}$  culture, CD40L-sBc were expanded *in vitro* from multiple major histocompatibility complex (MHC)-disparate macaques. Expanded  $T_{\text{regs}}$  expressed high levels of forkhead box protein 3 (FoxP3) and Helios, a high percentage of  $T_{\text{reg}}$ -specific demethylated region (TSDR) demethylation and strong suppression of naïve T cell responses *in vitro*. In addition, these  $T_{\text{regs}}$  produced low levels of inflammatory cytokines and were able to expand post-cryopreservation. Specificity assays confirmed that these  $T_{\text{regs}}$  were suppressive upon activation by any antigen-presenting cells (APCs) whose MHC was shared by CD40L-sBc used during expansion, proving that they are polyspecific. We developed an approach for the expansion of highly suppressive cynomolgus macaque polyspecific  $T_{\text{regs}}$  through the use of a combination of CD40L-engineered B cells with the potential to be translated to clinical studies. To our knowledge, this is the first report that uses a pool of MHC-mismatched CD40L-sBc to create polyspecific  $T_{\text{regs}}$  suitable for use in deceased-donor transplants.

**Keywords:** CD40L-stimulated B cells, non-human primates, regulatory T cells, tolerance

as they represent the closest approximation to human immunology. Specifically, the results in the Mauritian origin cynomolgus macaque (MCM) model quite accurately reflect the rate of tolerance induction in subsequent human studies [4].

Regulatory T cells ( $T_{\text{regs}}$ ) represent a lymphocyte subpopulation with immunomodulatory properties characterized by cell surface expression of CD4 and CD25 and expression of the transcription factor forkhead box protein 3 (FoxP3).  $T_{\text{regs}}$  have shown promising results for the treatment of autoimmune diseases [5–9], graft-versus-host disease (GVHD) prophylaxis [10–17] and tolerance induction in transplantation [18,19]. Several methods for

expanding T<sub>regs</sub> *in vitro* have been reported [4,20]. Donor-specific T<sub>regs</sub> have been described to be more suppressive than polyclonal T<sub>regs</sub> [20]. However, in the transplantation setting, they cannot be produced in time for early infusion into deceased-donor transplant recipients, leaving 'off-the-shelf' polyclonal T<sub>regs</sub> as the only alternative for peritransplant infusion. Therefore, protocols for the production of highly suppressive polyclonal T<sub>regs</sub> that can be easily translated into the clinic must be optimized.

CD40L-stimulated B cells (CD40L-sBc) have been used as immunostimulatory antigen-presenting cells (APCs) for *in-vitro* expansion of donor-specific T<sub>regs</sub> in patients with autoimmune diseases [8]. As this protocol meets good manufacturing practice (GMP) standards, we sought to translate it to the MCM model to create polyspecific T<sub>regs</sub> (psT<sub>regs</sub>) and study their *in-vitro* potency prior to testing their efficacy *in vivo*. We adapted this approach [20] and combined CD40L-sBc isolated and expanded from donors with different MHC to produce T<sub>regs</sub> with a broad reactivity that could be used in transplantation to prevent graft rejection or as GVHD prophylaxis. In addition, we tested the potency of these cells after cryopreservation as an 'off-the-shelf' product and after re-expansion to demonstrate that they are high suppressors post-thaw and can become more potent suppressors after re-expansion.

Here, we present three approaches for the culture of MCM CD40L-sBc in addition to a novel approach for the expansion of MCM psT<sub>regs</sub> stimulated with a pool of MHC-mismatched CD40L-sBc.

## Materials and methods

### Sample collection and peripheral blood mononuclear cell (PBMC) isolation

Heparinized blood was drawn from MCM (Charles River Primates, Wilmington, MA, USA; Sanofi-Synthelabo, Bridgewater, NJ, USA; Bioculture Group, Glenmoore, PA, USA). All animals were negative for B virus, simian T lymphotropic virus, simian retrovirus, simian immunodeficiency virus, simian varicella virus and malaria. All macaques were housed at the Institute of Comparative Medicine (Columbia University Medical Center, New York, NY, USA). This facility holds a current United States Department of Agriculture (USDA) registration, public health service (PHS) assurance and is American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited. All experimental procedures were approved by the Columbia University Institutional Animal Care and Use Committee.

Diluted (1 : 2) blood with phosphate-buffered saline (PBS) (Corning, Corning, NY, USA) was overlaid on 60% Percoll (Sigma-Aldrich, St Louis, MO, USA) and centrifuged. The buffy coat was collected and contaminating red blood cells were lysed [4].

## B cell culture

Irradiated (46 Gy) human erythroleukemia cells (K562) expressing CD40L, human leukocyte antigen-D related (HLA)-DR and CD64 were used to stimulate B cells [20]. CD40L-expressing K562 cell growth medium consisted of 89% Iscove's modified Dulbecco's medium (IMDM) (Life Technologies, Grand Island, NY, USA), 10% fetal bovine serum (FBS) (Gemini Bio-Products, Sacramento, CA, USA) and 1% penicillin/streptomycin (Life Technologies).

B cell growth medium consisted of 90% X-Vivo 15 (Lonza, Basel, Switzerland) or AIM-V medium (Life Technologies), 10% heat-inactivated human AB serum (Gemini Bio-Products) and 100 U/ml of insulin (Eli Lilly, Indianapolis, IN, USA). Cryopreserved or freshly isolated MCM PBMCs or splenocytes were plated with CD40L-expressing K562 cells at a ratio of 2 : 1. B cell donors were selected in order to obtain a pool of disparate MHC to cover a broad range of alloantigens. Three approaches were investigated for B cell expansion that ranged from 10 to 14 days of culture (Table 1). Interleukin (IL)-4 (R&D Systems, Minneapolis, MN, USA), ganciclovir (GCV) (Genentech, South San Francisco, CA, USA) and cyclosporin A (CyA) (Sigma-Aldrich) were added to all cultures. Cells were phenotypically assessed for expression of CD3 (to assess T cell contamination), CD40, CD20, CD80 and MHC class II prior to cryopreservation.

## T<sub>reg</sub> culture

T<sub>regs</sub> were stained with CD4 (L200, BD Biosciences, San Jose, CA, USA), CD25 (BC96; BioLegend, San Diego, CA, USA), CD127 (HIL-7R-M21; BD Pharmingen, San Diego, CA, USA) and CD8 (RPA-T8, BD or BW135/80; Miltenyi Biotec, Auburn, CA, USA) monoclonal antibodies. The top 1% of CD25<sup>+</sup> cells within the CD4<sup>+</sup>CD8<sup>-</sup>CD127<sup>-</sup> gate were sorted. Fluorescence activated cell sorting (FACS) was performed on the Influx Cell Sorter (BD Biosciences).

T<sub>regs</sub> were expanded for a minimum of 14 days for up to 28 days, depending on the rate of cell growth. T<sub>reg</sub>-growth medium consisted of 90% X-Vivo 15 (Lonza) or AIM-V medium (Life Technologies) with 10% heat-inactivated human AB serum (Gemini Bio-Products). Irradiated (30 Gy) CD40L-sBc from three to four animals representative of diverse MHCs were pooled and plated at a 1 : 4 ratio of T<sub>reg</sub> : CD40L-sBc; 300 IU/ml of IL-2 (PeproTech, Rocky Hill, NJ, USA) [20] were added to the culture on day 0 and at time of restimulation (Table 2). Cells were split when overgrowth was observed, with the addition of T<sub>reg</sub> medium and 300 IU/ml of IL-2. Phenotype and suppressive capacity were assessed prior to cryopreservation.

## Suppression assays

PBMC responders were plated in triplicate. T<sub>regs</sub> were plated with PBMCs starting at a 1:1 T<sub>reg</sub> : PBMC ratio and

**Table 1.** MCM B cell expansion and activation protocols

	Protocol 1	Protocol 2	Protocol 3
Culture duration	10 days	14 days	14 days
Restimulation with CD40L-expressing K562 cells	Day 7	Day 10	Day 7
Medium feeding	Day 2, 4	Day 2, 4, 7	Days 2, 4, 10
CyA dose	2 µg/ml (days 0), 1 µg/ml (days 2, 4)	2 µg/ml (days 0, 2, 4), 1 µg/ml (days 7, 10)	2 µg/ml (days 0, 2, 4), 1 µg/ml (days 7, 10)
IL-4 dose	0.8 ng/ml (days 0), 0.4 ng/ml (days 2, 4, 7)	0.8 ng/ml (days 0), 0.4 ng/ml (days 2, 4, 7, 10)	0.8 ng/ml (day 0), 0.4 ng/ml (days 2, 4, 7, 10)
GCV dose	10 µg/ml (days 0), 5 µg/ml (days 2, 4, 7)	10 µg/ml (days 0), 5 µg/ml (days 2, 4, 7, 10)	10 µg/ml (day 0), 5 µg/ml (days 2, 4, 7, 10)

MCM = Mauritian cynomolgus macaque; IL = interleukin; GCV = ganciclovir; CyA = cyclosporin A.

**Table 2.** CD40L-sBc regulatory T cell (T<sub>reg</sub>) expansion protocol

	Day 0	Day 7	Day 14	Day 21	Day 28
CD40L-sBc	✓	✓	✓	✓	Harvest
Interleukin (IL)-2	✓	✓	✓	✓	

serially diluted up to 1 : 32 T<sub>regs</sub> : PBMCs. PBMCs were stimulated with anti-CD2 CD3 CD28 beads (Miltenyi Biotec); 1 µCi of thymidine (Perkin Elmer, Waltham, MA, USA) was added per well 4 days after plating. Cells were harvested (Tomtec, Chicago, IL, USA) 24 h thereafter. A 1450 MicroBeta plate reader (Perkin Elmer) was used to measure the thymidine uptake [4]. Percentage suppression was calculated with the formula:  $\{(1 - \text{counts per min (cpm) of T}_{reg}\text{-treated T cells})/\text{cpm of control T cells}\} \times 100$ .

### Cryopreservation

Cells were cryopreserved in 95% FBS (Gemini) and 5% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) at 10 million cells/ml in a step-down cell freezer (CryoMed Freezer; Thermo Fisher Scientific, Fremont, CA, USA) at 1°C/min and stored in a liquid-nitrogen freezer.

### Re-expansion post-cryopreservation

Cells were thawed in a 37°C water bath. Group 1 was stimulated with anti-CD2CD3CD28 beads (Miltenyi Biotec); group 2 with irradiated artificial APCs (aAPCs) [21] consisting of mouse fibroblast cells (L929) transduced with human CD32, CD58 and CD80 [22,23] preloaded with 100 ng/ml of anti-CD3; and group 3 with a pool of MHC-disparate CD40L-sBc at a 1 : 4 T<sub>reg</sub> : CD40L-sBc ratio. The three conditions received T<sub>reg</sub> medium and 300 IU/ml of IL-2 and were expanded for 9 days. T<sub>reg</sub> lines were expanded for 14–21 days before cryopreservation. Absolute numbers, phenotype and suppressive capacity were assessed throughout the culture.

### Flow cytometric analysis

The cell surface antibodies used for flow cytometric (FCM) analysis included anti-CD20 (Miltenyi Biotec), CD40 (BioLegend), CD80 (BD Biosciences), HLA-DR (BD Biosciences), CD3 (SP34-2, BD Biosciences), CD4 (L200, BD Biosciences), CD8 (BW135/80, Miltenyi Biotec), CD25 (BC96, BioLegend), CD45RA (T6D11, Miltenyi Biotec) and CD127 (HIL-7R-M21, BD Pharmingen). FoxP3 (236A/E7 and PCH101, eBioscience), Helios (BioLegend) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) (BD Biosciences) antibodies were used intracellularly for T<sub>reg</sub> assessment. CTLA-4 was assessed before and after stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 4 h. Gates were drawn based on isotype controls (IS11-3B2.2.3; Miltenyi Biotec). Cells were permeabilized with the BioLegend FoxP3 fixation/permeabilization buffer set. Data were collected on

LSRFortessa (BD Biosciences) and analyzed using FCS Express (De Novo Software, Glendale, CA, USA).

### Specificity assay

Responders (T cells) and stimulators (PBMCs) were plated at a 1 : 2 T cell : PBMC ratio. T<sub>regs</sub> were plated at a 1 : 1 T<sub>reg</sub> : T cell ratio and then serially diluted to 1 : 128. Each condition was plated in triplicate. T cells were isolated through magnetic-activated cell sorting (MACS) (Miltenyi Biotec) with CD3-positive selection; 1 µCi/well of thymidine (Perkin Elmer) was added 4 days after plating. Cells were harvested (Tomtec) 24 h after adding thymidine. A 1450 MicroBeta plate reader (Perkin Elmer) was used to measure thymidine uptake.

### T<sub>reg</sub>-specific demethylated region (TSDR) analysis of MCM T<sub>regs</sub>

Quantitative polymerase chain reaction (qPCR) for the FoxP3 region was performed by Epiontis (Berlin, Germany). Using a standard dilution curve, the number of demethylated and methylated genomic regions was determined, referred as TpG and CpG plasmid units, respectively. The T<sub>reg</sub> content was calculated by dividing the number of TpG plasmid units by the sum of all FoxP3 regions in the sample: TpG/(TpG+CpG) [4].

### Assessment of intracellular cytokine production

T<sub>regs</sub> and PBMCs (positive control) were stimulated for 4 h with PMA/ionomycin in the presence of brefeldin A (leukocyte activation cocktail; BD Biosciences). Prior to fixation and permeabilization, cells were stained with anti-CD3 (BD Biosciences), CD4 (BD Horizon), and CD8 (Miltenyi Biotec). Cells were stained intracellularly with antibodies against interferon (IFN)-γ (BioLegend), IL-17A (Miltenyi Biotec) and IL-13 (BD Bioscience). Cells were read using the Aurora spectral analyzer (Cytek Biosciences, Fremont, CA, USA).

### Statistics

Data were analyzed using unpaired Student's *t*-test with GraphPad Prism version 7.05. *P*-values of ≤ 0.05 were considered statistically significant (\**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001).

## Results

### Expansion and activation of MCM B cells

We studied three approaches for the expansion of MCM B cells from PBMCs or splenocytes (Table 1). An average of  $6.2 \times 10^6 \pm 3.5 \times 10^6$  B cells (based on the percentage of CD20<sup>+</sup> cells in PBMCs/splenocytes) was plated with CD40L-expressing K562 cells for up to 14 days (Fig. 1a) with IL-4 for B cell stimulation, CyA

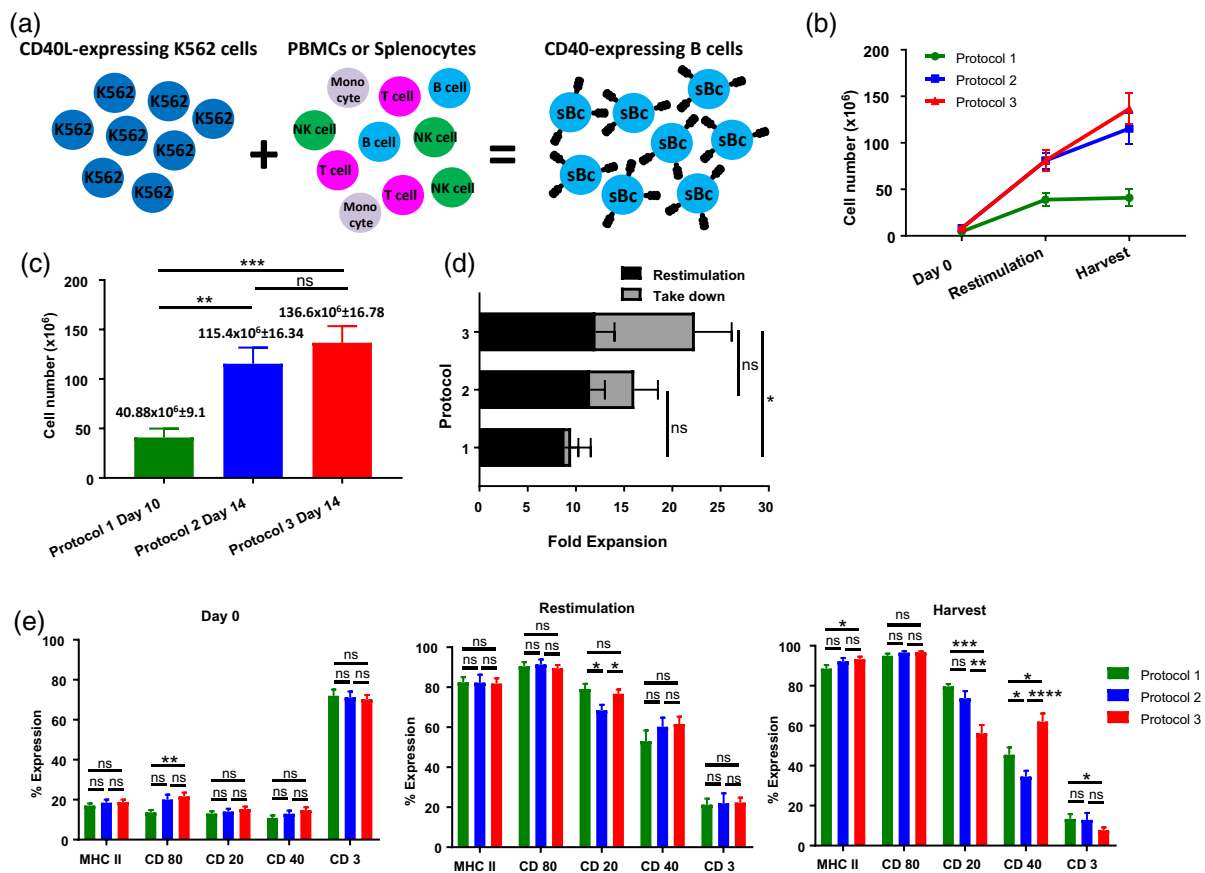
to avoid T cell growth and GCV for the control of Epstein–Barr virus (Table 1). Protocol 1 was based on previous human studies [20]. Cells were cultured for 10 days. Slower cell growth was observed with this approach compared to protocols 2 and 3 (Fig. 1b). At the end of the culture, an average of  $40.88 \times 10^6 \pm 9.1 \times 10^6$  cells was obtained (Fig. 1c). In order to improve the cell yield, culture time was increased to 14 days for protocols 2 and 3, achieving  $115.4 \times 10^6 \pm 16.34 \times 10^6$  and  $136.6 \times 10^6 \pm 16.78 \times 10^6$  cells, respectively, with significant increase in B cell numbers compared to protocol 1 [Fig. 1c; *P* = 0.0017 (protocol 1 versus 2), *P* = 0.0004 (protocol 1 versus 3)]. We calculated the fold expansion in order to normalize the cell growth to the number of B cells plated on day 0. The highest average fold expansion was achieved with protocol 3 (22.3-fold), compared to protocol 2 (16.1-fold) and protocol 1 (9.5-fold) (Fig. 1d), with a significant difference between protocols 1 and 3 (*P* = 0.02).

We analyzed the number of B cells and concurrent T cell contamination in culture over time (Fig. 1e). As expected on day 0, few cells expressed B cell markers and there was a high percentage of T cells (Fig. 1e). Restimulation with fresh CD40L-K562 cells was performed on day 10 for protocols 1 and 2 and on day 7 for protocol 3. Over time, the expression of B cell markers increased and the T cell counts decreased with all protocols (Fig. 1e). The concentration of CyA was increased in protocols 2 and 3 in an attempt to decrease the number of contaminating T cells. The three approaches produced cells with comparable MHC II and CD80 levels. Cells from protocols 1 and 2 had higher percentages of CD20<sup>+</sup> cells (*P* = 0.0003, protocol 1 versus 3; *P* = 0.0043, protocol 2 versus 3), but protocol 3 produced cells with the highest CD40 expression (*P* = 0.0118, protocol 1 versus 3; *P* < 0.0001, protocol 2 versus 3), and with the lowest T cell contamination [significant difference between protocols 1 and 3 (*P* = 0.0348)] (Fig. 1e).

Due to the higher yield and the lower T cell contamination observed in CD40L-sBc cultured under protocol 3, we utilized this approach in the experiments to follow.

### Expansion of CD40L-B cell-stimulated MCM T<sub>regs</sub> (sBc-T<sub>regs</sub>)

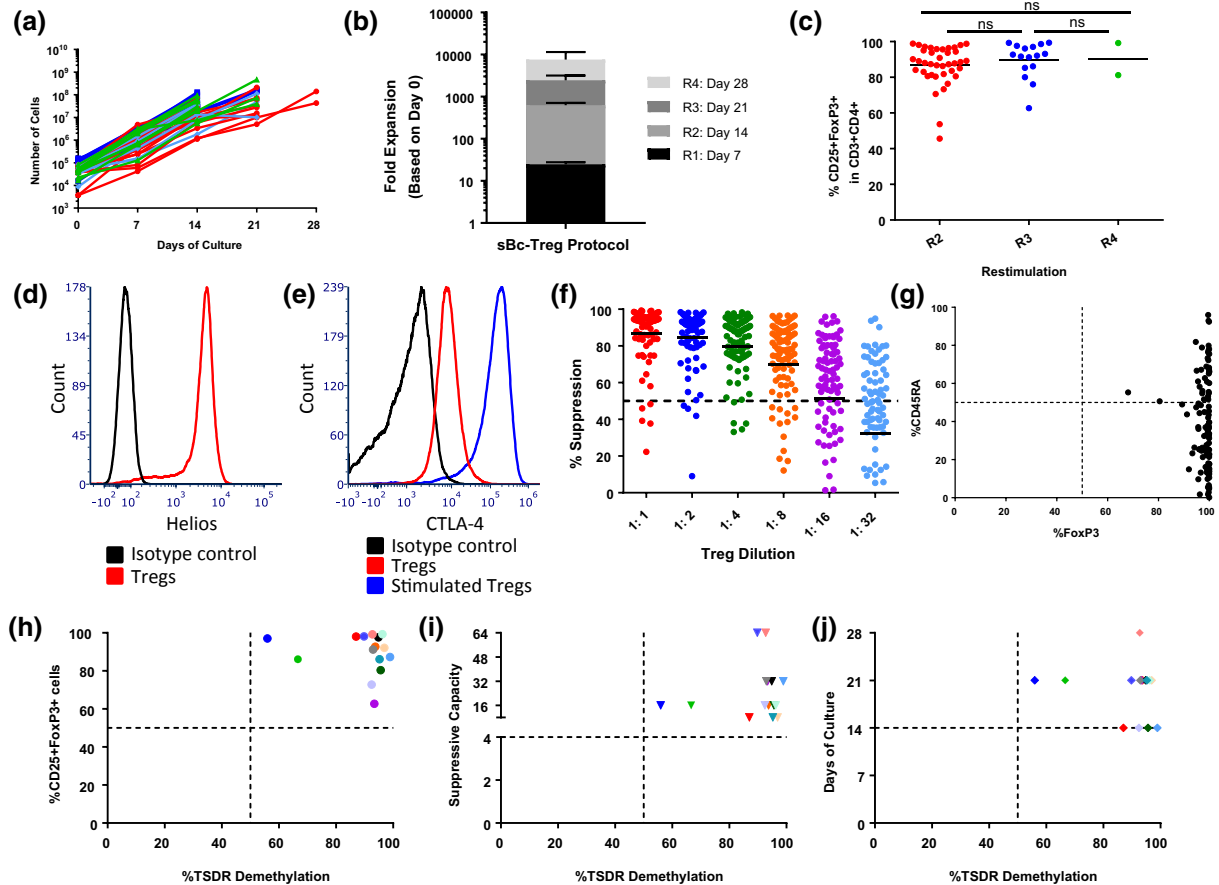
T<sub>regs</sub> were cultured with MHC-disparate CD40L-sBc from multiple donors at a ratio of 1 : 4 T<sub>reg</sub> : CD40L-sBc. T<sub>regs</sub> were FACS sorted based on the CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>high</sup>CD127<sup>-</sup> phenotype [4]. An average of 62 609 (± 34 668) T<sub>regs</sub> were plated on day 0 and cultured for a minimum of 14 days, but up to 28 days depending on cell growth (Fig. 2a), reaching an average of  $60.6 \times 10^6 \pm 9.2 \times 10^6$  T<sub>regs</sub> at the end of the culture.



**Fig. 1.** Expansion and activation of cynomolgus macaque (MCM) B cells for regulatory T cell ( $T_{reg}$ ) expansion. B cell expansion from fresh or cryopreserved peripheral blood mononuclear cells (PBMCs) or splenocytes. Protocol 1 includes 10 lines, protocol 2 includes 14 lines and protocol 3 includes 18 lines from nine animals. (a) Schema for the expansion and activation of MCM B cells. (b) B cell growth (mean and standard error of the mean (s.e.m.) from day 0 until the harvest for each of the B cell expansion protocols. (c) Average cell number at the end of the B cell culture for each protocol. Average and s.e.m. are detailed at the top of each bar. (d) Fold expansion for each B cell protocol. (e) Expression of B cell characteristic markers [major histocompatibility complex (MHC) II, CD80, CD20, CD40] and assessment of T cell contamination for each B cell expansion protocol at different time-points (day 0, restimulation and harvest).

The largest fold expansion was observed between days 7 and 14, at which time the majority of the  $T_{reg}$  lines were cryopreserved (Fig. 2b). On average, greater than 80% FoxP3 expression was detected, with no significant difference between the three harvest time-points. Therefore, the duration of the cell culture did not affect FoxP3 expression (Fig. 2c). Helios expression on  $T_{regs}$  has been associated with a thymic origin, in addition to a more activated phenotype and a higher suppressive capacity *in vitro* [24]. We observed that  $T_{regs}$  cultured with CD40L-sBc expressed high levels of Helios, suggestive of a stable  $T_{reg}$  phenotype (Fig. 2d, representative figure). CTLA-4 is used by  $T_{regs}$  to elicit suppression and it has been reported as an indicator of homeostasis in stable  $T_{regs}$  [25]. We found that CD40L-sBc  $T_{regs}$  homogeneously expressed CTLA-4, which was further increased upon additional stimulation (Fig. 2e, representative figure).  $T_{reg}$  lines exhibited potent suppressive capacity as,

on average, they mediated 50% suppression compared to the positive control (bead-stimulated PBMCs) at a 1 : 16 ratio of  $T_{reg}$  : PBMCs (Fig. 2f). Human CD45RA<sup>+</sup>  $T_{regs}$  have been reported to be more homogeneous compared to CD45RA<sup>-</sup> memory  $T_{regs}$  [26]. We have previously reported no correlation between the level of FoxP3 and CD45RA in MCM  $T_{regs}$  expanded with aAPCs and donor PBMCs [4]. Using CD40L-sBc as stimulators during  $T_{reg}$  expansion also resulted in no correlation between the expression of these two markers (Fig. 2g). To assess their stability, we determined the percentage of demethylation in the TSDR.  $T_{regs}$  expressing high levels of FoxP3 that were potent suppressors showed high levels of  $T_{reg}$ -specific demethylated region (TSDR) demethylation (Fig. 2h,i). In addition, we found no correlation between the level of TSDR demethylation and the duration of the cell culture, with similar levels in  $T_{reg}$  lines cultured for 14, 21 or 28 days (Fig. 2j).

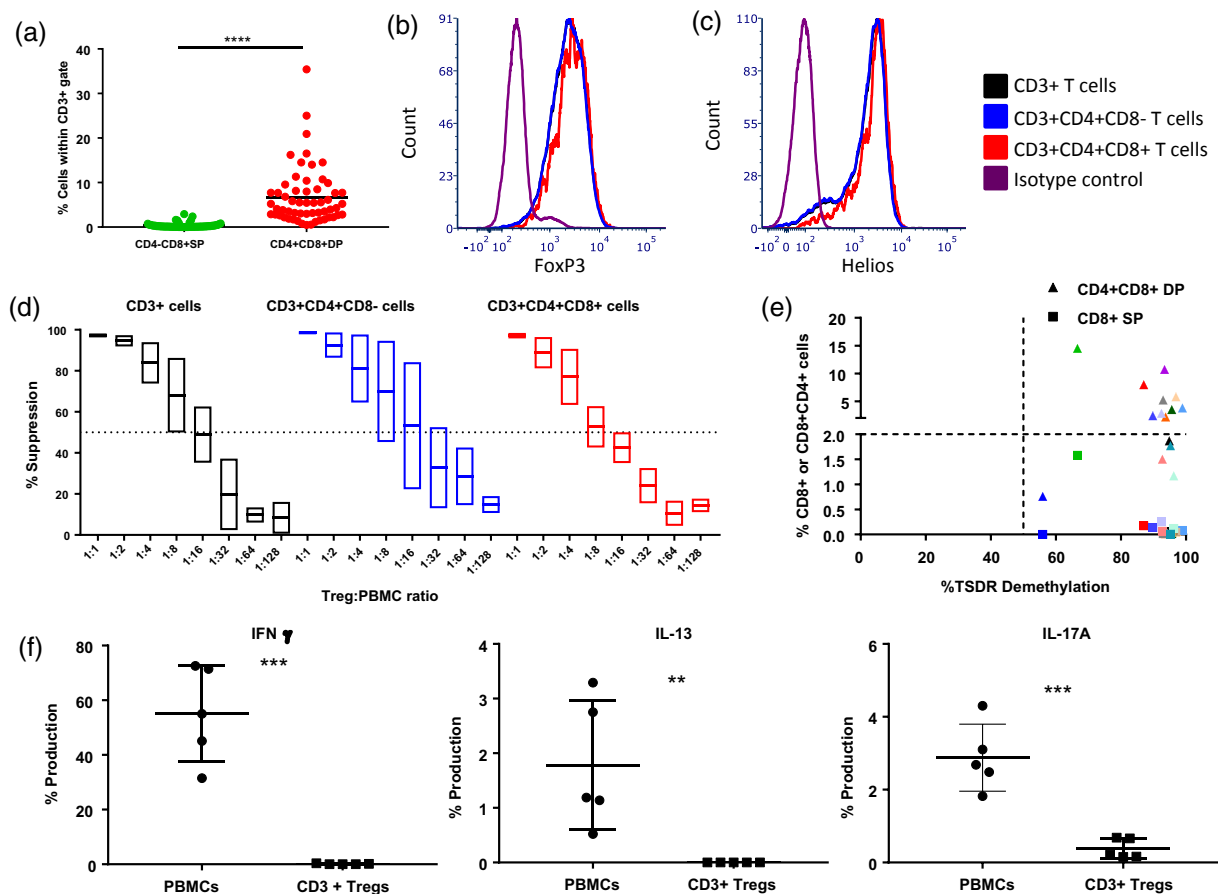


**Fig. 2.** Expansion of cynomolgus macaque (MCM) regulatory T cells (T<sub>regs</sub>) with CD40L-sBc. Expansion of MCM T<sub>regs</sub> with CD40L-sBc stimulation. (a) T<sub>reg</sub> numbers at each restimulation and take-down ( $n = 57$ ). (b) Mean and standard error of the mean (s.e.m.) of the fold expansion of the T<sub>reg</sub> lines at each restimulation until the take-down. 'R' represents restimulation number. (c) Percentage of CD25<sup>+</sup>FoxP3<sup>+</sup> cells within CD3<sup>+</sup>CD4<sup>+</sup> cells at the take-down. (d) Representative Helios expression on cultured T<sub>regs</sub> (red) ( $n = 5$ ). (e) Representative cytotoxic T lymphocyte antigen-4 (CTLA-4) expression on cultured T<sub>regs</sub> (red) and cultured T<sub>regs</sub> activated with phorbol myristate acetate (PMA)/ionomycin for 4 h (blue) ( $n = 5$ ). (f) Percentage of suppressive capacity of the T<sub>reg</sub> lines ( $n = 57$ ). The dotted line represents 50% suppression as reference. Mean of the suppressive capacity is represented with a black line for each ratio of T<sub>reg</sub> : peripheral blood mononuclear cells (PBMCs). (g) Correlation between CD45RA and forkhead box protein 3 (FoxP3) expression. Correlation between the percentage of T<sub>reg</sub>-specific demethylated region (TSDR) demethylation and the (h) expression of FoxP3, (i) suppressive capacity (the T<sub>reg</sub> : responder ratio that achieves 50% suppression of the positive control) and (j) days of culture. Each color represents a different T<sub>reg</sub> line that is the same throughout Fig. 2h–j ( $n = 15$ ).

### CD4<sup>+</sup>CD8<sup>+</sup> T cells in T<sub>reg</sub> cultures show T<sub>reg</sub>-like characteristics

In our previous studies, T<sub>regs</sub> cultured with aAPCs and/or donor PBMCs as stimulators variably showed outgrowth of CD8<sup>+</sup> single-positive (SP) T cells [4]. In this protocol, we observed overgrowth of CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) T cells and very few CD8<sup>+</sup> SP T cells (Fig. 3a,  $P < 0.0001$ ). FoxP3 and Helios expression was similar in CD4<sup>+</sup> SP, CD4<sup>+</sup>CD8<sup>+</sup> DP and total CD3<sup>+</sup> T cell populations after culture (Fig. 3b,c). Using FACS sorting, we assessed the suppressive capacity of the CD4<sup>+</sup>CD8<sup>+</sup> DP cell population and noted comparable

suppressive potency as the purified T<sub>reg</sub> from the same population (Fig. 3d). In addition, we did not observe an inverse correlation between the level of CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> T cells and the level of TSDR demethylation (Fig. 3e). The only T<sub>reg</sub> line that had higher CD4<sup>+</sup>CD8<sup>+</sup> T cell counts (green triangle) with a lower TSDR demethylation also had a higher percentage of CD8<sup>+</sup> T cells (green square), which have previously been shown to cause higher TSDR methylation when contaminating T<sub>reg</sub> populations [4]. Therefore, CD4<sup>+</sup>CD8<sup>+</sup> DP T cells presented T<sub>reg</sub>-like characteristics based on their phenotypical and functional data, and may not be detrimental if administered *in vivo*.



**Fig. 3.** Forkhead box protein 3 (FoxP3) expression and suppressive capacity of CD4<sup>+</sup>CD8<sup>+</sup> DP T cells. (a) Comparison between the percentage of CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>+</sup> SP and CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) T cells present in the regulatory T cell ( $T_{reg}$ ) cultures at the harvest time-point (days 14, 21 or 28) ( $n = 57$ ). Study of the (b) FoxP3, (c) Helios expression and (d) suppressive capacity [mean and standard error of the mean (s.e.m.)] of total CD3<sup>+</sup> T cells (black), CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> SP T cells (blue) and CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> DP T cells (red) ( $n = 2$ ). (e) Correlation between the percentage of  $T_{reg}$ -specific demethylated region (TSDR) demethylation in total CD3<sup>+</sup> cells and the percentage of CD8<sup>+</sup> single-positive (SP) and CD4<sup>+</sup>CD8<sup>+</sup> (DP) T cells present in culture. Each color represents a different  $T_{reg}$  line ( $n = 15$ ). (f) Assessment of the secretion of interferon (IFN)- $\gamma$ , interleukin (IL)-13 and IL-17A in peripheral blood mononuclear cells (PBMCs) and cultured sBc- $T_{regs}$  ( $n = 5$ ).

### CD40L-sBc $T_{regs}$ lack production of inflammatory cytokines upon *in-vitro* stimulation

To understand the function and stability of CD40L-sBc  $T_{regs}$ , we assessed their ability to secrete inflammatory cytokines upon activation. We compared the production of IFN- $\gamma$ , IL-13 and IL-17A between PBMCs (positive control) and CD40L-sBc  $T_{regs}$  at different time-points (days 7, 14 and 21). While PBMCs produced these three cytokines, as expected, there was negligible production from CD40L-sBc  $T_{regs}$  (Fig. 3f).

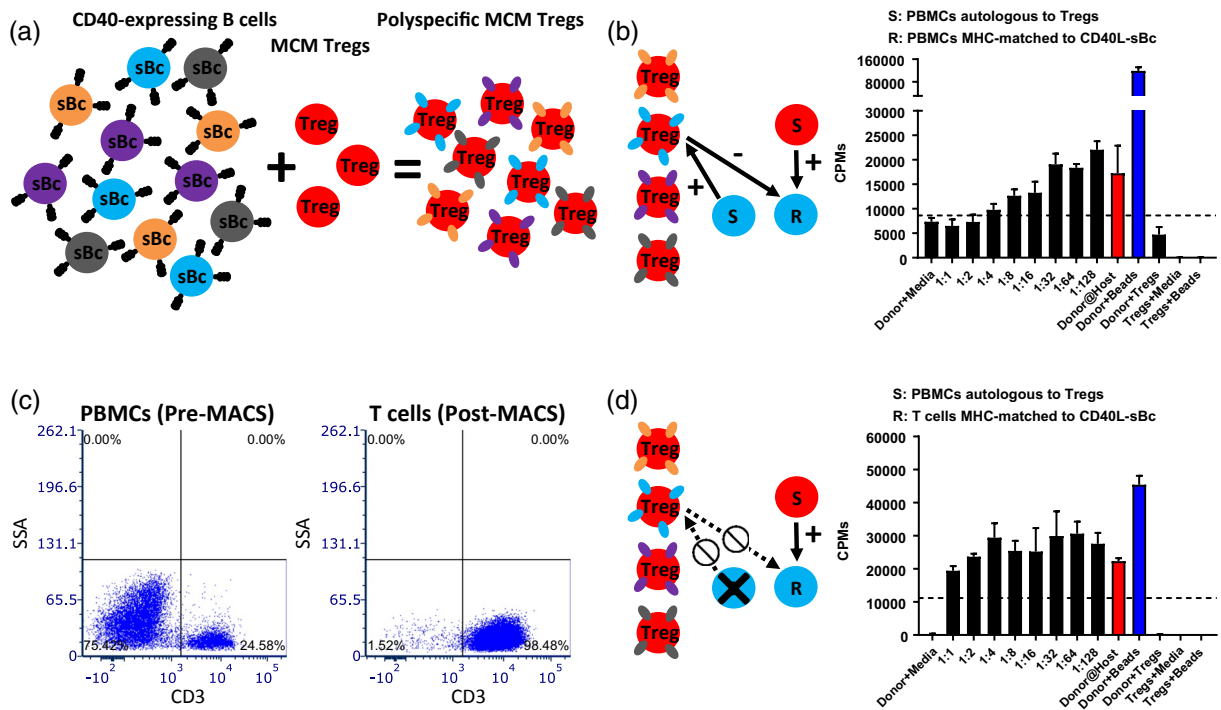
These data support the safety of CD4<sup>+</sup>CD8<sup>+</sup> DP T cells, as  $T_{reg}$  lines with a high percentage of CD4<sup>+</sup>CD8<sup>+</sup> DP T cells (14%) also lacked production of IFN- $\gamma$ , IL-13 and IL-17A (Fig. 3f).

### Study of the specificity of MCM sBc- $T_{regs}$

We used a combination of CD40L-sBc from multiple MCM donors with diverse MHC as stimulators to expose the

$T_{regs}$  to a variety of alloantigens (Fig. 4a). To study the polyspecificity of the  $T_{regs}$ , we cultured them with different stimulators and responders and quantified the suppressive effect of the  $T_{regs}$ . We defined cells as MHC-matched or mismatched to CD40L-sBc if the MHC was similar or different, respectively, from the CD40L-sBc used for  $T_{reg}$  expansion.

We first assessed if  $T_{regs}$  would suppress responses of PBMCs that were MHC-matched to CD40L-sBc when stimulated by PBMCs that were autologous to the  $T_{regs}$ . In this condition,  $T_{regs}$  could be stimulated by APCs from the 'responder' PBMCs or the stimulators and efficient suppression was observed (Fig. 4b). Then, to eliminate the potential for  $T_{reg}$  activation by 'responder' APCs leading to bystander suppression, responder T cells were MACS-enriched, which allowed for the isolation of highly purified T cells (98.48%) (Fig. 4c). The  $T_{reg}$  suppression



**Fig. 4.** Difference in suppression based on the responder-type cells. (a) Schema for the expansion protocol of cynomolgus macaque (MCM) regulatory T cells (T<sub>regs</sub>) with CD40L-sBc. (b) Representative figure of a specificity assay with peripheral blood mononuclear cells (PBMCs) as responders. (c) Representative figure of before (left panel) and after (right panel) magnetic-activated cell sorted (MACS)-isolated T cells. (d) Representative figure of a specificity assay with MACS-isolated T cells as responders.

was eliminated when purified T cells were used as responders (Fig. 4d), suggesting that T<sub>regs</sub> had been activated by the APCs from the 'responder' PBMCs from the same donors as those used for T<sub>reg</sub> expansion (Fig. 4b). Therefore, we performed the future specificity studies with MACS-isolated T cells as responders (Fig. 5).

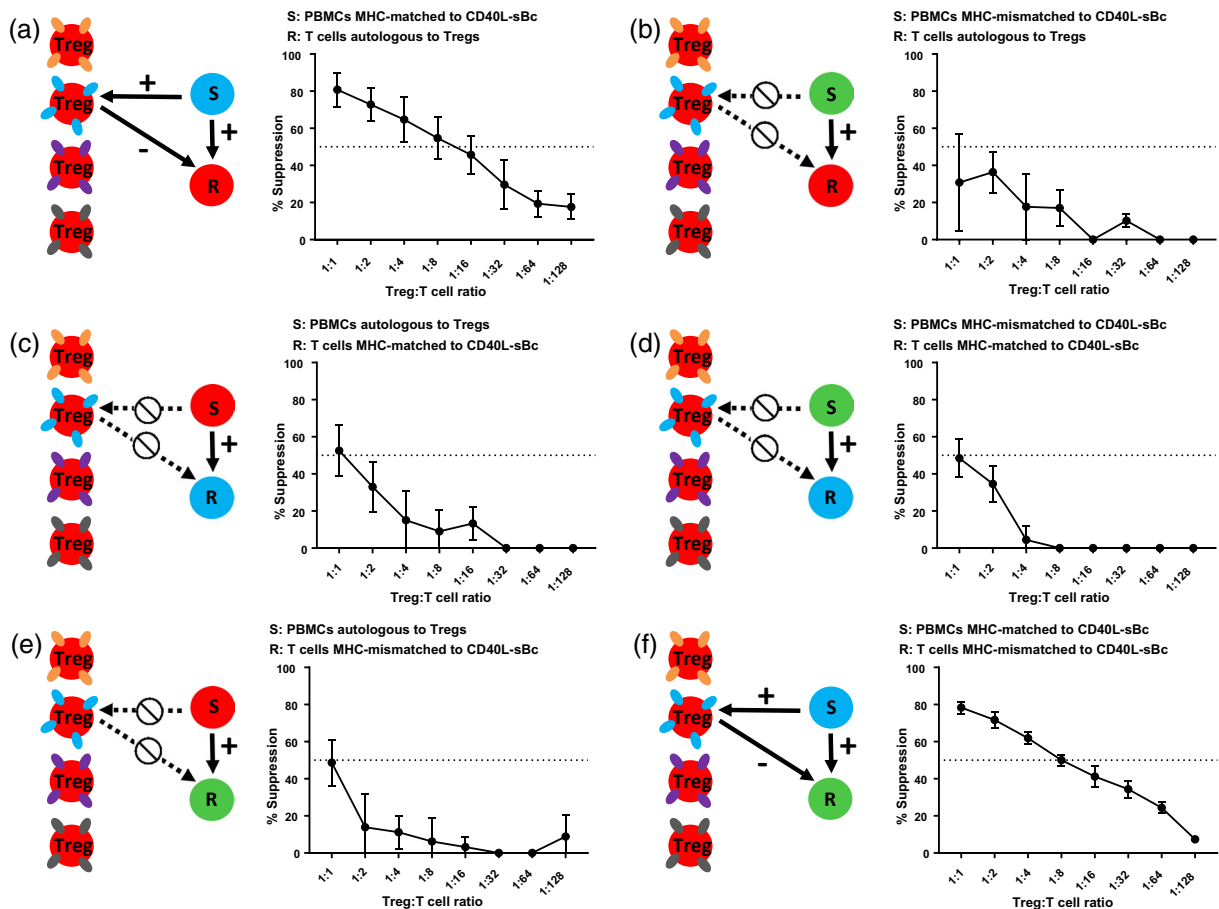
We then examined the T<sub>regs</sub>' ability to suppress autologous T cell responses stimulated by APCs that were either MHC-matched or mismatched to the CD40L-sBc used during T<sub>reg</sub> expansion (Fig. 5a,b). We found increased suppression by the T<sub>regs</sub> when stimulated by APCs that were MHC-matched to CD40L-sBc than when stimulated by APCs expressing MHC not present in the T<sub>reg</sub> expansion phase (Fig. 5a,b). If the stimulator did not express any MHC to which the T<sub>regs</sub> were exposed to during expansion, suppression was poor and typically did not exceed 50% suppression at a T<sub>reg</sub> : responder ratio of 1 : 1 (Fig. 5b). When responders that were MHC-matched to CD40L-sBc were stimulated by either APCs autologous to the T<sub>regs</sub> or MHC-mismatched to CD40L-sBc, T<sub>regs</sub> exhibited poor suppressive capacity (Fig. 5c,d). To confirm these findings, we stimulated T cells that were MHC-mismatched to CD40L-sBc with PBMCs autologous to the T<sub>regs</sub> (Fig. 5e) or MHC-matched to CD40L-sBc (Fig. 5f). We again found that the T<sub>regs</sub> exhibited good suppression (> 50% at 1 : 8 T<sub>reg</sub> : responder)

only when the stimulator's MHC was present during T<sub>reg</sub> expansion (Fig. 5f). Collectively, our data demonstrate that multiple CD40L-sBc drive the expansion of T<sub>regs</sub> specific for alloantigens presented by the CD40L-sBc irrespective of the responder MHC (Fig. 5a,f). After activation, T<sub>regs</sub> could suppress any naive T cell responder.

#### Expansion after cryopreservation of CD40L-sBc T<sub>regs</sub>

To ensure that CD40L-sBc T<sub>regs</sub> could be banked prior to *in-vivo* infusion, we studied the effect of cryopreservation on viability, phenotype and function with and without post-thaw restimulation. Three approaches were studied: stimulation with anti-CD2 CD3 CD28 beads, anti-CD3 preloaded aAPCs [21] or CD40L-sBc from four donors (with MHC matched to that of the CD40L-sBc used for prior T<sub>reg</sub> expansion), all in the presence of IL-2. There was no T<sub>reg</sub> expansion with anti-CD2 CD3 CD28 bead stimulation (Fig. 6a). Restimulation with aAPCs or CD40L-sBc led to a 10-fold expansion in T<sub>reg</sub> numbers after 7 days (Fig. 6b). FoxP3 expression was comparable in T<sub>regs</sub> regardless of stimulation approach (Fig. 6c). We determined the percentage of CD4<sup>+</sup>CD8<sup>+</sup> DP T cell contamination to determine if the method of restimulation affected this population. The protocol using aAPCs promoted the CD4<sup>+</sup>CD8<sup>+</sup> DP cell growth more than the other two





**Fig. 5.** Specificity data for cynomolgus macaque (MCM) CD40L-sBc-stimulated regulatory T cells ( $T_{regs}$ ). Mean and standard error of the mean (SEM) of the suppressive capacity of CD40L-sBc  $T_{regs}$  to responders that were (a,b) autologous to  $T_{regs}$  ( $n = 5, n = 3$ ), (c,d) major histocompatibility complex (MHC)-matched ( $n = 4, n = 2$ ) or (e,f) MHC-mismatched to CD40L-sBc used for  $T_{reg}$  expansion ( $n = 3, n = 4$ ) with a variety of stimulators. Responders were MACS-isolated T cells from peripheral blood mononuclear cells (PBMCs) or splenocytes and stimulators were PBMCs.

approaches, but the difference did not reach statistical significance (Fig. 6d). We also studied the functionality of the  $T_{regs}$  after re-expansion (Fig. 6e). The suppressive capacity of  $T_{regs}$  restimulated with aAPCs was comparable to that of sBc-restimulated  $T_{regs}$ . Optimal suppression was noted on day 3, followed by day 4 of restimulation. Given these results, we propose to restimulate psT  $T_{regs}$  for 4 days with aAPCs, anti-CD3 and IL-2, as this method provides optimal suppressive capacity and a degree of expansion of psT  $T_{regs}$ , while remaining relatively easy to perform.

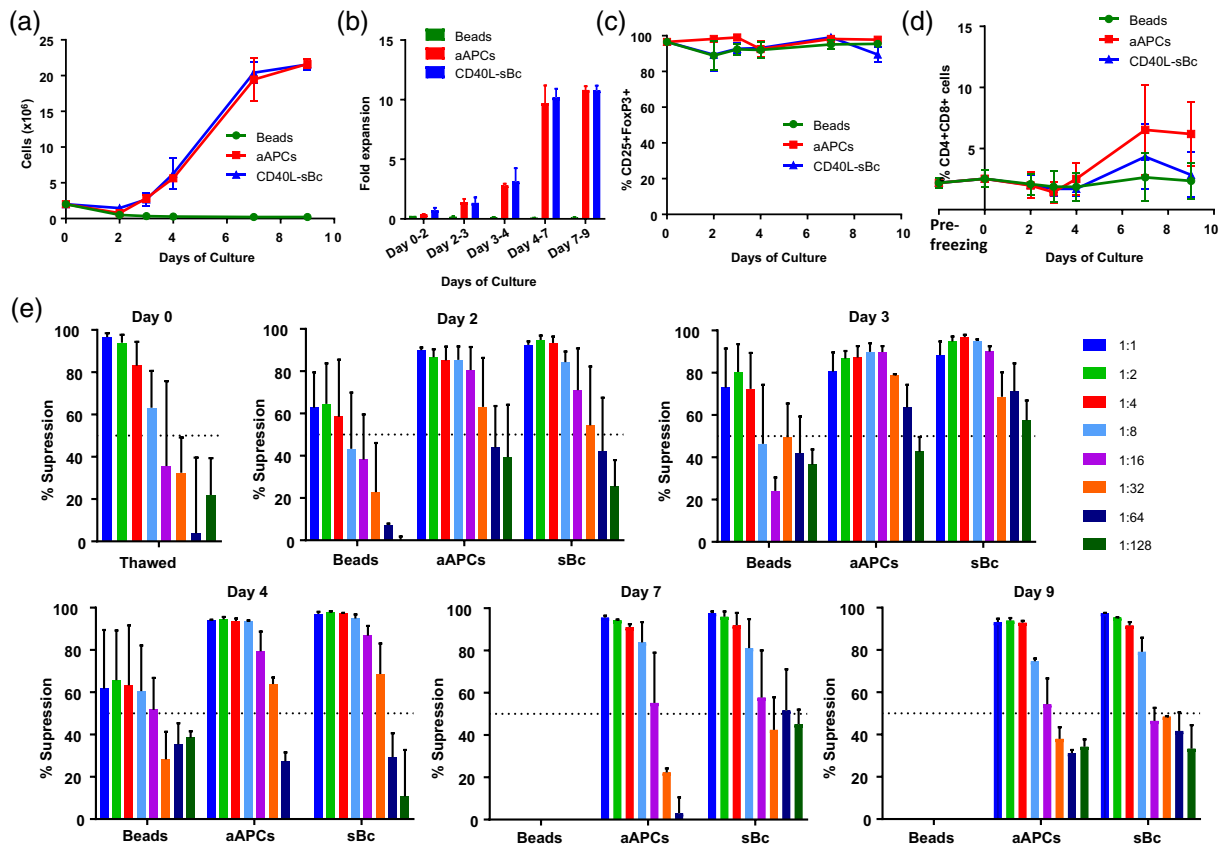
The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Discussion

$T_{reg}$  infusion has been reported safe in patients enrolled in clinical trials for the treatment of autoimmune diseases [27], graft-versus-host disease [14] and induction of

transplantation tolerance for kidney [28] or liver allografts [19] [Regulatory T Cell Therapy in Liver Transplant Patients (ThRIL) study].  $T_{reg}$  expansion protocols are therefore necessary to obtain sufficient numbers so that an *in-vivo* effect may be observed. We have previously reported four approaches for the expansion of polyclonal MCM  $T_{regs}$  for preclinical studies through the stimulation provided by aAPCs and/or donor PBMCs [4]. Here, we report a novel approach using CD40L-sBc to obtain MCM psT  $T_{regs}$ . Adapted from clinical studies where CD40L-sBc were used to grow donor-specific  $T_{regs}$  [20], our protocol combines CD40L-sBc from multiple donors with diverse MHC to produce  $T_{regs}$  that react to a broad range of alloantigens. Using this approach, we aimed to obtain  $T_{regs}$  that could suppress HVG as well as GVH responses, and therefore may be useful in combining solid organ and bone marrow transplantation for tolerance induction.

The expansion of NHP B cells has been reported for different purposes, such as the study of B cells, diagnostic



**Fig. 6.** Restimulation of sBc-regulatory T cells (T<sub>regs</sub>) after cryopreservation. Study of cryopreserved and re-expanded CD40L-sBc T<sub>regs</sub>. Representation of two T<sub>reg</sub> lines from two different animals recultured under three different conditions. (a) T<sub>reg</sub> growth after thawing. (b) Fold expansion after 9 days of culture. (c) CD25<sup>+</sup>forkhead box protein 3 (FoxP3)<sup>+</sup> expression in CD3<sup>+</sup>CD4<sup>+</sup> cells. (d) Percentage of CD4<sup>+</sup>CD8<sup>+</sup> T cells. (e) Suppressive capacity of the T<sub>reg</sub> lines cultured under each condition at the thawing day and days 2, 3, 4, 7 and 9 after reculture.

tools [i.e. enzyme-linked immunospot (ELISPOT)], therapeutic cell expansion (i.e. T<sub>regs</sub>) or cell therapy (i.e. B<sub>regs</sub>) [29]. We report for the first time, to our knowledge, the use of a combination of MHC-disparate CD40L-sBc for the expansion of MCM psT<sub>regs</sub>. We first studied three approaches to engineer B cells through co-culture with CD40L-expressing K562 cells. Protocol 3, which had an extended culture time and higher CyA doses compared to the original clinical protocol [20], produced the largest number of CD40L-sBcs with the lowest T cell contamination. Therefore, this was the optimal protocol for B cell expansion.

We developed a novel approach to obtain psT<sub>regs</sub> by stimulation with a pool of MHC-mismatched CD40L-sBc. Using this approach, we obtained > 600-fold expansion of T<sub>regs</sub> in 14 days. We have previously reported higher T<sub>reg</sub> yields through the use of aAPCs [4], but the culture time was 26 days instead of 14–21 days for our current approach. If higher yields are desired with the CD40L-sBc approach, T<sub>regs</sub> can also be expanded for longer while maintaining the FoxP3 expression and suppression,

although higher CD40L-sBc numbers would be also required for expansion to maintain the ratio of stimulators with T<sub>regs</sub> at 1 : 4 T<sub>regs</sub> : CD40L-sB.

FoxP3 and Helios levels remained high for up to 28 days with minimal secretion of inflammatory cytokines. CTLA-4 expression was homogeneously expressed in the T<sub>reg</sub> populations, which demonstrated increased levels upon stimulation. T<sub>regs</sub> showed potent suppressive capacity and had high TSDR demethylation levels. These data suggest that T<sub>regs</sub> expanded under this approach are a potentially good product for *in-vivo* use.

We have previously reported the outgrowth of CD8<sup>+</sup> SP T cells with other approaches for MCM T<sub>reg</sub> expansion [4], raising the concern that we were generating donor-specific effectors along with T<sub>regs</sub>. Based on our previous experience with this CD8<sup>+</sup> SP T cell population, we did not find that these CD8<sup>+</sup> SP T cells share T<sub>reg</sub> characteristics [4]. With the CD40L-sBc T<sub>reg</sub> expansion protocol, we report minimal CD8<sup>+</sup> SP T cell growth that could be easily removed by sorting, if desired. In contrast, we observed the development of CD4<sup>+</sup>CD8<sup>+</sup> DP T cells. FACS-sorted

CD4<sup>+</sup>CD8<sup>+</sup> T cells were able to suppress responders at the same T<sub>reg</sub> : PBMC ratios as traditional CD4<sup>+</sup>CD8<sup>-</sup> T<sub>regs</sub>. FoxP3 and Helios expression was comparable to that in CD4<sup>+</sup>CD25<sup>+</sup> cells and minimal secretion of inflammatory cytokines was observed. In humans, CD4<sup>+</sup>CD8<sup>+</sup> DP T cells are a small subpopulation of lymphocytes that have been reported to be activated T cells with memory phenotype, while in animal models they have been reported to have regulatory properties [30]. The results in our model suggest that, in MCM, the CD4<sup>+</sup>CD8<sup>+</sup> DP T cells might be a subpopulation of T<sub>regs</sub> that up-regulate CD8 in addition to CD4. Another explanation could be that a T<sub>reg</sub> phenotype has been induced in CD4<sup>+</sup>CD8<sup>+</sup> cells isolated from circulation in this model. Regardless, these findings would need further assessment in human T<sub>regs</sub> expanded with this approach.

In order to translate this psT<sub>reg</sub>-expansion approach to deceased donation protocols, T<sub>regs</sub> need to suppress immune responses to a broad array of HLA with high prevalence in the organ donor pool. We therefore assessed the specificity capabilities of T<sub>regs</sub> expanded under this approach. We showed that for T<sub>regs</sub> to be potent suppressors of the recipient's response to any given donor, they must be exposed to donor-HLA-matched CD40L-sBc during the cell expansion. This T<sub>reg</sub>-expansion strategy would, therefore, be a promising option in deceased donor transplantation where the donor HLA is known only immediately before the transplant.

Cryopreservation is a necessary step in generating T<sub>regs</sub> suitable for use with deceased-donor organs as they would be readily accessible when an organ is accepted. For T<sub>regs</sub> to become an 'off-the-shelf' product, they need to maintain their suppressive properties through the cryopreservation process. While thawed T<sub>regs</sub> were able to preserve 50% suppressive capacity at an average of a 1 : 8 T<sub>reg</sub> : PBMC ratio, after reculturing with aAPCs or CD40L-sBc, T<sub>regs</sub> become more activated and showed higher suppressive potency. We therefore opted to restimulate the T<sub>regs</sub> for 4 days with aAPCs for optimal combination of T<sub>reg</sub> function and expansion for ongoing *in-vivo* studies given the ease of using aAPCs, specifically when high T<sub>reg</sub> numbers are desired.

Other approaches that include the use of aAPCs have been reported for the expansion of NHP and human T<sub>regs</sub> that led to polyclonal T<sub>regs</sub> [4,31]. In addition, as in human studies, anti-CD3CD28 beads have been reported for the expansion of T<sub>regs</sub> in NHP models [32]. Although T<sub>regs</sub> cultured under these approaches have potential to be used as 'off-the-shelf' products, antigen-primed T<sub>regs</sub> have been reported to be more suppressive than polyclonal T<sub>regs</sub>. Therefore, higher yields would be needed to achieve similar *in-vivo* effects. Another approach that has been reported is the use of donor PBMCs, alone or in combination with

aAPCs [4]. These T<sub>regs</sub> had increased suppression towards the donor, and an overall lower suppression to other allogeneic not-previously encountered stimulators. Approaches for the expansion of donor-specific T<sub>regs</sub> have included the use of CD40L-stimulated B cells isolated from the donor that demonstrated superior potency than those stimulated partially with beads [20]. In our protocol, we modified this last approach using a combination of different donors to obtain B cells with disparate MHC in order to create polyspecific T<sub>regs</sub>. Although we obtained lower yields than previously reported in this MCM model [4], cells were overall more suppressive, and therefore the total number of T<sub>regs</sub> needed for *in-vivo* infusion may actually be less. In addition, these cells can be further expanded after thawing since their potency was further improved in this process.

Several clinical trials have reported the infusion of T<sub>regs</sub> for the induction of kidney or liver transplantation tolerance [18,19], with the majority of these studies involving living-donor transplants and infusion of donor-specific T<sub>regs</sub>. Even though donor-specific T<sub>regs</sub> have been reported to be more potent than polyclonal T<sub>regs</sub> [20], they cannot be produced in a timely manner for early infusion into deceased-donor transplant recipients, leaving polyclonal T<sub>regs</sub> as the only alternative for peritransplant infusion. Therefore, protocols for the production of highly suppressive polyclonal T<sub>regs</sub> that can be easily translated into the clinic must be optimized. Previous studies performed by our group in a MCM model reported the use of polyclonal T<sub>regs</sub> to promote renal allograft tolerance through mixed hematopoietic chimerism across MHC barriers, but success was achieved in only a few recipients [33]. The T<sub>regs</sub> generated with the current protocol suppress naive T cell responses at a lower T<sub>reg</sub> : responder ratio than those in our previous report. Additionally, this protocol does not generate substantial numbers of CD3<sup>+</sup>CD4-CD8<sup>+</sup> T cells in the culture, decreasing the likelihood of expanding alloreactive effector T cells. Finally, this protocol has the advantage of being adapted from a clinical protocol that meets GMP standards, which could facilitate translation of studies from primates to the clinic.

## Conclusions

We have developed an approach for the expansion of MCM psT<sub>regs</sub> through the use of CD40L-engineered B cells that may be translated to clinical studies. Significant numbers of highly suppressive T<sub>regs</sub> with stable levels of FoxP3, Helios and demethylated TSDR were obtained, which could be further expanded post-cryopreservation. This is the first report, our knowledge, that uses a pool of MHC-mismatched CD40L-sBc to create psT<sub>regs</sub> suitable for use in deceased-donor transplantation.

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## Disclosures

Q. T. is a co-inventor on two patents on regulatory T cell therapy and a cofounder of Sonoma Biotherapeutics. The rest of the authors declare that they have no competing interests.

## Author contributions

P. A. G., M. S. and A. G. performed research, analyzed data and wrote the manuscript. N. L., E. L., S. K., S. H. H., J. S., G. P. and K. B. performed research, collected samples and analyzed data. Q. T. provided the CD40L-K562 cell line.

## Data Availability Statement

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

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