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ORIGINAL ARTICLE

Developing in vitro expanded CD45RA$^+$ regulatory T cells as an adoptive cell therapy for Crohn's disease

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ABSTRACT

Background and aim Thymus-derived regulatory T cells (T_{regs}) mediate dominant peripheral tolerance and treat experimental colitis. T_{regs} can be expanded from patient blood and were safely used in recent phase 1 studies in graft versus host disease and type 1 diabetes. T_{reg} cell therapy is also conceptually attractive for Crohn's disease (CD). However, barriers exist to this approach. The stability of T_{regs} expanded from Crohn's blood is unknown. The potential for adoptively transferred T_{regs} to express interleukin-17 and exacerbate Crohn's lesions is of concern. Mucosal T cells are resistant to T_{reg}-mediated suppression in active CD. The capacity for expanded T_{regs} to home to gut and lymphoid tissue is unknown.

Methods To define the optimum population for T_{reg} cell therapy in CD, $CD4^+CD25^+CD127^{lo}CD45RA^+$ and $CD4^+CD25^+CD127^{lo}CD45RA^-$ T_{reg} subsets were isolated from patients' blood and expanded in vitro using a workflow that can be readily transferred to a good manufacturing practice background.

Results T_{regs} can be expanded from the blood of patients with CD to potential target dose within 22–24 days. Expanded CD45RA$^+$ T_{regs} have an epigenetically stable *FOXP3* locus and do not convert to a Th17 phenotype in vitro, in contrast to CD45RA$^-$ T_{regs}. CD45RA$^+$ T_{regs} highly express $\alpha_4\beta_7$ integrin, CD62L and CC motif receptor 7 (CCR7). CD45RA$^+$ T_{regs} also home to human small bowel in a C.B-17 severe combined immune deficiency (SCID) xenotransplant model. Importantly, in vitro expansion enhances the suppressive ability of CD45RA$^+$ T_{regs}. These cells also suppress activation of lamina propria and mesenteric lymph node lymphocytes isolated from inflamed Crohn's mucosa.

Conclusions $CD4^+CD25^+CD127^{lo}CD45RA^+$ T_{regs} may be the most appropriate population from which to expand T_{regs} for autologous T_{reg} therapy for CD, paving the way for future clinical trials.

Significance of this study

What is already known on this subject?

- Thymically derived regulatory T cells (T_{regs}) can modulate effector immune responses and, when expanded in vitro, have recently shown promise for graft versus host disease and type 1 diabetes in humans, leading to interest in this therapeutic approach for Crohn's disease.
- Barriers to autologous T_{reg} therapy in Crohn's include the requirement for in vitro expansion to a target dose, potential T_{reg} plasticity to pathogenic interleukin-17$^+$ cells, uncertain homing to mucosal tissue and effector T cell resistance to T_{reg}-mediated suppression in inflamed Crohn's mucosa.
- Initial enrichment on the basis of CD45RA$^+$ expression can improve the phenotypic stability of an expanded T_{reg} population obtained from healthy control blood, but the value of this approach in Crohn's disease is unknown.

What are the new findings?

- We show that it is technically feasible to expand functional T_{regs} to numbers consistent with a target dose from the blood of patients with Crohn's disease.
- In vitro expansion enhances the in vitro suppressive activity of these cells. Expanded T_{regs} suppress activation of lamina propria and mesenteric lymph node lymphocytes isolated from inflamed Crohn's mucosa.
- In contrast to T_{regs} expanded from CD45RA$^-$ precursors, expanded CD45RA$^+$ T_{regs} have epigenetically stable FOXP3 expression and are resistant to Th17 conversion.
- Expanded CD45RA$^+$ T_{regs} also express $\alpha_4\beta_7$ integrin, CD62L and CCR7, and home to human small bowel in a SCID mouse bearing subcutaneously implanted human intestine.

INTRODUCTION

Thymically derived FOXP3$^+$ regulatory T cells (T_{regs}) are key mediators of peripheral tolerance and are likely to have a role in preventing inappropriate mucosal inflammation in response to bacterial, and other, luminal antigens. In mice, T_{reg} depletion impairs oral tolerance.[1] Adoptively transferred T_{regs} prevent the onset of colitis or treat established colitis in a number of murine models.[2–7]

Significance of this study

How might it impact on clinical practice in the foreseeable future?

- These results demonstrate that initial T_{reg} enrichment on the basis of $CD45RA^+$ expression is required to produce a phenotypically stable and suppressive T_{reg} population following in vitro expansion and that these in vitro expanded cells have the capacity to home to mucosal tissue, paving the way for autologous T_{reg} therapy in this therapeutically challenging disease.

FOXP3 mutations lead to multisystem autoimmunity with enteropathy in mice and humans.[8 9] Disruption of other key molecules implicated in T_{reg} function, such as transforming growth factor (TGF)-β, Cytotoxic T Lymphocyte-Associated (CTLA)-4, interleukin (IL)-10R subunits, IL-2 or its receptor subunits, is associated with autoimmunity and intestinal inflammation.[10]

Human peripheral blood (PB) or umbilical cord blood T_{regs} can be expanded in vitro through T cell receptor (TCR) stimulation in the presence of IL-2.[11–26] In vitro expanded human T_{regs} prevent transplant rejection,[27 28] transplant arteriosclerosis[29] and graft versus host disease (GvHD)[21 30] in humanised mice. Promisingly, recent phase 1 clinical trials have shown T_{reg} cell therapy to be safe in patients with GvHD[12 24] and type 1 diabetes.[18] Additional phase 1 studies have started in renal (the ONE study) and liver transplantation (ThRIL study).[19 31]

Lamina propria (LP) T_{regs} are increased in the mucosa of patients with active Crohn's disease (CD) and decreased in blood, compared with healthy controls.[32–34] LP T_{regs} obtained from inflamed CD mucosa suppress proliferation of conventional $CD4^+CD25^{lo/int}$ T cells (T_{con}) obtained from blood but not LP T_{cons},[35] suggesting that mucosal T_{cons} in active CD may be resistant to T_{reg}-mediated suppression. LP T_{cons} from CD mucosa overexpress Smad7, an inhibitor of TGF-β signalling, which confers resistance to T_{reg}-mediated suppression.[35 36] Activated T_{cons} also have an effector-memory phenotype, conferring relative resistance to T_{reg}-mediated suppression.[37] However, T_{regs} expanded in vitro in the presence of rapamycin from the PB of patients with end-stage renal failure (ESRF), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS) and asthma are more suppressive than freshly isolated T_{regs} obtained from the same donor.[26 38] If it can be shown that in vitro expansion enhances the suppressive ability of CD PB T_{regs} and that these expanded cells suppress mucosal inflammation, parenteral therapy with autologous in vitro expanded T_{regs} generated from CD PB would become a conceptually attractive approach to induce remission in active CD.

IL-17 contributes to mucosal homoeostasis but has also been implicated in the pathogenesis of CD. T_{regs} isolated from healthy donor PB or tonsils can be induced to express IL-17 and the Th17 transcription factor RORC when activated in vitro in the presence of IL-1, IL-2, IL-21 and IL-23.[39–42] Although major sources of IL-17 in the gut include T_{cons} and γδ T cells, a proportion of T_{regs} obtained from inflamed CD mucosa co-express FOXP3 and IL-17.[43] Th1 T_{reg} plasticity has also been described in vitro and in vivo.[44 45] In humans, phenotypically distinct T_{reg} populations can be delineated on the basis of CD45RA expression.[17 46] 'Resting' $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ T_{regs} (rT_{regs}) are resistant to induction of IL-17 and interferon (IFN)-γ in vitro.[46] In contrast, 'activated' $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ T_{regs} (aT_{regs}) can be induced to express IL-17 and IFN-γ in vitro.[46] Similarly, T_{regs} expanded from healthy donor $CD45RA^+$ T_{regs} (in the absence of rapamycin) do not contain cytokine producers and are highly suppressive, while T_{regs} expanded from $CD45RA^-$ T_{regs} express proinflammatory cytokines and lose *FOXP3* expression with repetitive stimulation in vitro.[17 47] T_{regs} expanded from healthy control $CD45RA^-$ precursors (but not $CD45RA^+$ precursors) also have stimulation-induced demethylation of *RORC*, which may be permissive for IL-17 expression.[48] Furthermore, imprinting $\alpha_4\beta_7$ integrin expression on in vitro expanded T_{regs} by supplementing culture with all-trans retinoic acid (ATRA) results in high IL-17 expression.[21] Even though $IL\text{-}17^+$ T_{regs} isolated from human blood and tonsil retain their suppressive ability in vitro,[39–41] the potential for adoptively transferred T_{regs} to exacerbate inflammation in CD lesions through the production of proinflammatory cytokines is of significant concern.

Using cell enrichment strategies achievable with currently available good manufacturing practice (GMP) technologies, we show that initial enrichment on the basis of $CD45RA^+$ expression is required to generate a homogenous and epigenetically stable T_{reg} population following expansion, in the presence of rapamycin, from the PB of patients with CD. These cells are resistant to Th17 plasticity, express lymphoid and gut homing markers, and home to human gut following adoptive transfer to a SCID mouse bearing subcutaneously implanted human small bowel (SB). In vitro expansion also enhances the suppressive ability of these cells, licensing them to suppress activation of LP and mesenteric lymph node (MLN) T_{cons} obtained from inflamed CD resection specimens. These data suggest that CD PB $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ cells may be the most appropriate population from which to expand T_{regs} in vitro for forthcoming clinical trials of autologous T_{reg} cell therapy in CD.

MATERIALS AND METHODS

Patient samples

Following Institutional Review Board (IRB) approval (SE London REC 2; 10/H0804/65 and East London REC 2 (10/H0704/74)), patients with CD attending Guy's & St Thomas' National Health Service (NHS) Foundation Trust and Bart's Health NHS Trust were invited to donate blood and/or resected tissue. Prospective written consent was obtained. Demographic details are shown in table 1.

T_{reg} enrichment and sorting

Online supplementary figure S1 illustrates the experimental design. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation over lymphocyte separation medium (LSM) 1077 and $CD4^+$ lymphocytes enriched to >95% by positive magnetic activated cell separation (MACS) selection (Miltenyi, Bergisch-Gladbach, Germany). Lymphocytes were labelled using the 'Human Regulatory T Cell Sorting Kit' (BD Biosciences, San Diego, California, USA), as described previously,[25] and sorted to $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ and $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ T_{reg} subsets, and autologous $CD4^+CD25^-$ T_{cons} on a FACSAria (BD; see online supplementary figure S2A–D). Median (IQR) postsort purity was 86.5% (80.8–91.6%; n=13) for $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ T_{regs} ($CD45RA^+$ T_{regs}) and 92.7% (87.7–94.9%; n=13) for $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ T_{regs} ($CD45RA^-$ T_{regs}). Autologous T_{cons} were stored at −80°C.

Table 1 Demographic details of study patients

Female sex	6	(46.1%)
Age (mean±SD)	42.6	(±13.0)
Disease duration	15.4	(±10.4)
Age at diagnosis (mean±SD)	27.7	(±13.1)
Diagnosis <16 years old (A1)	2	(15.4%)
Diagnosis 17–40 years old (A2)	9	(69.2%)
Diagnosis >40 years old (A3)	2	(15.4%)
Location		
Ileal only (L1)	1	(7.7%)
Colonic only (L2)	4	(30.8%)
Ileo-colonic (L3)	8	(61.5%)
Concomitant upper GI disease (L4)	2	(15.4%)
Perianal disease (p)	2	(15.4%)
Behaviour		
Inflammatory (B1)	9	(69.2%)
Stricturing (B2)	2	(15.4%)
Penetrating (B3)	2	(15.4%)
HBI (median, range)	0	(0–7)
Active disease HBI ≥5	4	(30.8%)
Previous surgery	8	(61.5%)
Medications		
Thiopurines	7	(53.8%)
Biologics	4	(30.8%)
Others	3	(23.1%)

GI, gastrointestinal; HBI, Harvey Bradshaw Index.

In vitro generation of T_{reg} lines

Precursor T_{reg} populations were expanded in vitro as described previously[21 25] and described in detail in online supplemental methods.

Cell surface and intracellular stains

Fluorochrome-conjugated antibodies, buffers and experimental technique are listed in online supplemental methods.

Assessment of the in vitro suppressive ability of putative T_{regs}

Assays to determine T_{reg} function in vitro were performed as described previously,[25 49] and described in detail in the online supplemental methods.

rtPCR

Following total RNA extraction from Trisure (Bioline, London, UK), cDNA was synthesised using the RevertAid First Strand cDNA Synthesis Kit and multiplex rtPCR performed in duplicate using the Maxima Probe/ROX qPCR Master Mix (both Thermo Fischer Scientific) on a BioRad C1000 Thermal Cycler. Primers are listed in online supplemental methods.

Estimation of cytokine concentrations

Cytokine concentrations were estimated in culture supernatants using the Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit (BD) or sandwich ELISAs (R&D), as indicated.

Assessment of IL-17 production under proinflammatory conditions

In vitro generated T_{regs} were activated with anti-CD3/anti-CD28 beads at a 1:1 ratio and cultured at 10^6 cells/mL in complete Roswell Park Memorial Institute (RPMI) for 5 days at 37°C/5% CO_2, supplemented with the following cytokine cocktails, as previously described:[21 23 39] (A) IL-2 (10 IU/mL, Proleukin); (B) IL-2, IL-1 (10 ng/mL), IL-6 (4 ng/mL) and TGF-β (5 ng/mL); (C) IL-2, IL-21 (25 ng/mL), IL-23 (25 ng/mL) and TGF-β (all R&D Systems). Supernatant IL-17 concentrations were measured by ELISA.

Assessment of FOXP3 promoter demethylation

Genomic DNA was isolated using a 'DNeasy kit' (Qiagen, Manchester, UK). Bisulfite conversion and assessment of the methylation status of the *FOXP3* T_{reg}-specific demethylated region (TSDR) was performed by Epiontis.[50 51] The genomic locations of *FOXP3* and *GAPDH* CpG-rich regions probed have been reported.[51]

Isolation of LP mononuclear cells and MLN mononuclear cells

LP mononuclear cells (LPMCs) and MLN mononuclear cells (MLNMCs) were isolated as described previously[52] and listed in online supplemental methods.

C.B-17 SCID mouse human intestinal xenotransplant model

Experimental design is illustrated in figure 3C. The C.B-17 SCID mouse human intestinal xenotransplant model has been described previously[53 54] and is described in detail in online supplemental methods. IRB and IACUC approvals were obtained prospectively (Ethics Committee for Animal Experimentation, Hebrew University of Jerusalem; MD-11-12692-4 and the Helsinki Committee of the Hadassah University Hospital; 81-23/04/04). Techniques for the detection of adoptively transferred T_{regs} are also described in detail in online supplemental methods.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5 (GraphPad Software Inc, La Jolla, California, USA) and the methods used are described in detail in the online supplemental methods.

RESULTS

T_{regs} can be expanded from the blood of patient with CD using GMP-compatible protocols

Hoffmann *et al*[17] showed that initial T_{reg} enrichment on the basis of $CD45RA^+$ expression was required to expand homogenous and stable T_{reg} lines from healthy donors in the absence of supplemental rapamycin. Rapamycin prevents the outgrowth of contaminating T_{cons} in T_{reg} cultures, and may make the requirements for the starting population less stringent.[11 13 15 21 23 55] However, the optimum precursor population from which to expand a homogenous, suppressive and epigenetically stable T_{reg} population from CD PB is currently unknown. In previous studies, we accomplished in vitro expansion of in vitro suppressive T_{regs} from healthy controls[21] and renal transplant candidates.[26] We sought to determine if T_{regs} could be expanded in vitro from the blood of patients with CD.

Freshly isolated $CD4^+$ lymphocytes from 13 patients with CD were fluorescence-activated cell sorting (FACS)-sorted into $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ (median (IQR) of 2200 cells/mL PB (860–4400)) and $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ subsets (3700 cells/mL (2000–4500)), then expanded in vitro in the presence of high-dose IL-2, rapamycin and anti-CD3/anti-CD28 beads. Active disease, evidenced by a Harvey Bradshaw Index >5 (n=4) *or* elevated C reactive protein (n=1), was not associated with a significantly reduced yield (see online supplementary figure S2E). Donor clinical characteristics are given in table 1.

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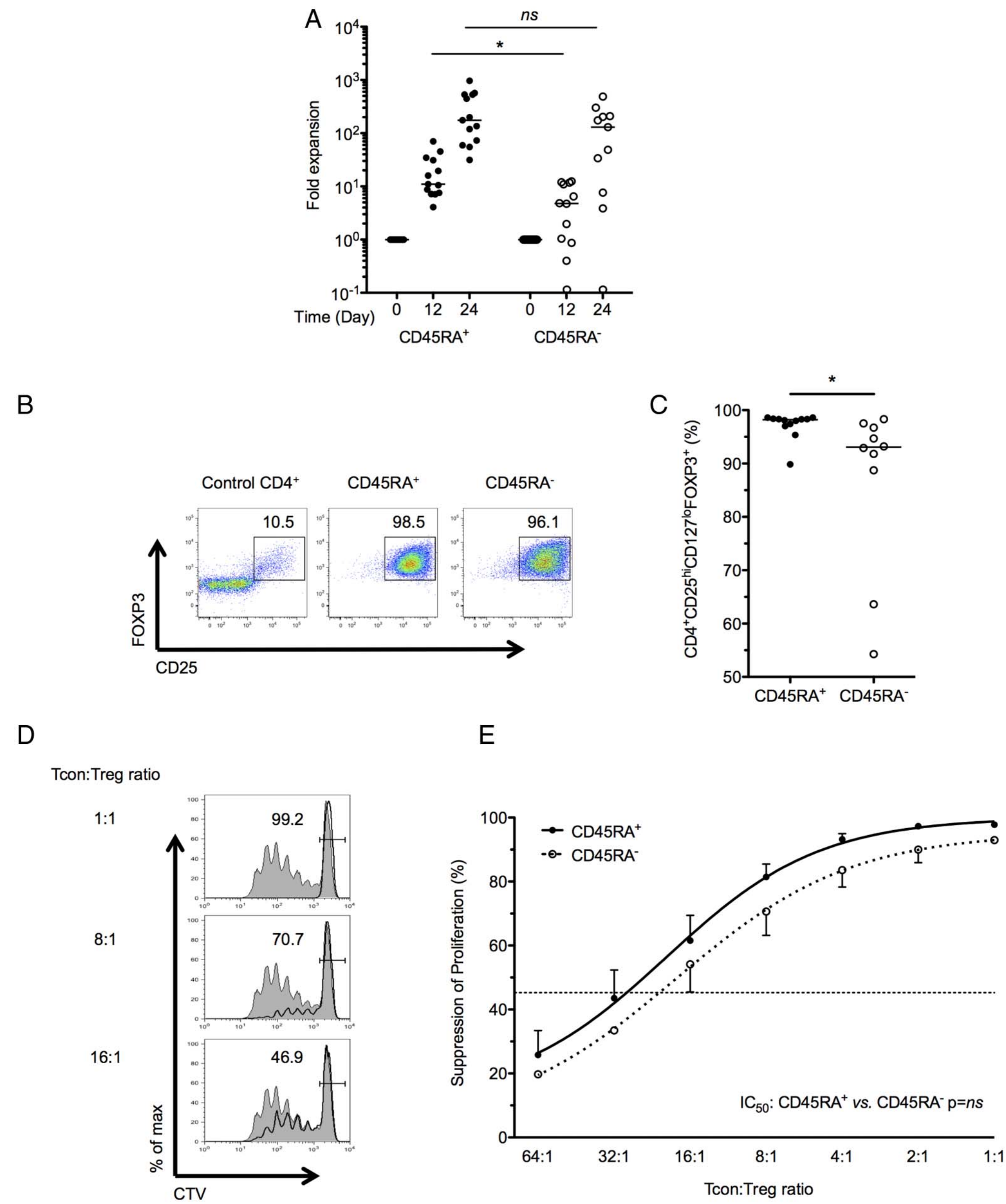

Figure 1 Expansion, phenotype and potency of in vitro expanded T_{regs}. (A) Cumulative fold expansion of T_{reg} lines at days 12 and 24 of culture, grouped according to $CD4^{+}CD25^{hi}CD127^{lo}CD45RA^{+}$ or $CD4^{+}CD25^{hi}CD127^{lo}CD45RA^{-}$ precursors; n=13 each, bar: median. (B) Representative FACS plots gated on live events showing CD25 and FOXP3 expression at D24. (C) Proportion of T_{regs} with a $CD4^{+}CD25^{hi}CD127^{lo}$ T_{reg} phenotype at D24. (D) Representative plots from a proliferation assay, illustrating dose-dependent suppression of T_{con} proliferation by $CD45RA^{+}$ T_{regs}. Proliferation CTV-labelled autologous $CD4^{+}CD25^{-}$ T_{cons} alone (filled) or with T_{regs} at various T_{con}:T_{reg} ratios (bold line) is shown. (E) D24 T_{reg}-mediated suppression of T_{con} proliferation. Cumulative data showing mean±SEM suppression seen at each T_{con}:T_{reg} ratio. Pooled data from 29 T_{reg} lines. Comparisons between suppression seen in study conditions and mean non-specific suppression seen in '2X' control condition (dotted line) are shown. *$p<0.05$,***$p<0.001$ and ****$p<0.0001$. T_{regs}, thymus-derived regulatory T cells; FACS, fluorescence-activated cell sorting; T_{cons}, conventional $CD4^{+}CD25^{lo/int}$ T cells; CTV, Cell Trace Violet; NS, not significant.

Every $CD45RA^{+}$ T_{reg} line proliferated, to a median (IQR) of 175-fold (66–531; n=13) at D24 (figure 1A). In contrast, 3 of 13 (23%) $CD45RA^{-}$ T_{reg} lines did not proliferate and were discontinued. $CD45RA^{-}$ T_{regs} expanded 130-fold (8–209; n=10). Expanded T_{regs} were exclusively $CD4^{+}$ lymphocytes. Expression of CD25 and FOXP3 was comparable in D24 $CD45RA^{+}$ and $CD45RA^{-}$ T_{regs} (figure 1B), but a greater proportion of $CD45RA^{+}$ T_{regs} maintained a $CD4^{+}CD25^{hi}CD127^{lo}FOXP3^{+}$ phenotype (p=0.037; figure 1C).

Proliferation assays were performed to determine if in vitro expanded T_{regs} retained the ability to suppress proliferation of autologous $CD4^{+}CD25^{-}$ T_{cons}. $CD45RA^{+}$ and $CD45RA^{-}$ T_{regs} suppressed T_{con} proliferation to an equivalent degree (figure 1D–E), demonstrating specific suppression (vs the 2X cell density control) above an 8:1 T_{con}:T_{reg} ratio. $CD45RA^{+}$ and $CD45RA^{-}$ T_{regs} reduced IL-2 expression in 96 h co-culture supernatants (see online supplementary figure S3A). $CD45RA^{+}$ T_{regs} also suppressed IFN-γ expression in 96 h co-culture supernatants (see online supplementary figure S3B).

In vitro expanded $CD45RA^{+}$ T_{regs} are resistant to IL-17 induction and stably express FOXP3

The 'inflammatory potential' of in vitro expanded T_{regs} from patients with CD was examined. Genes important in

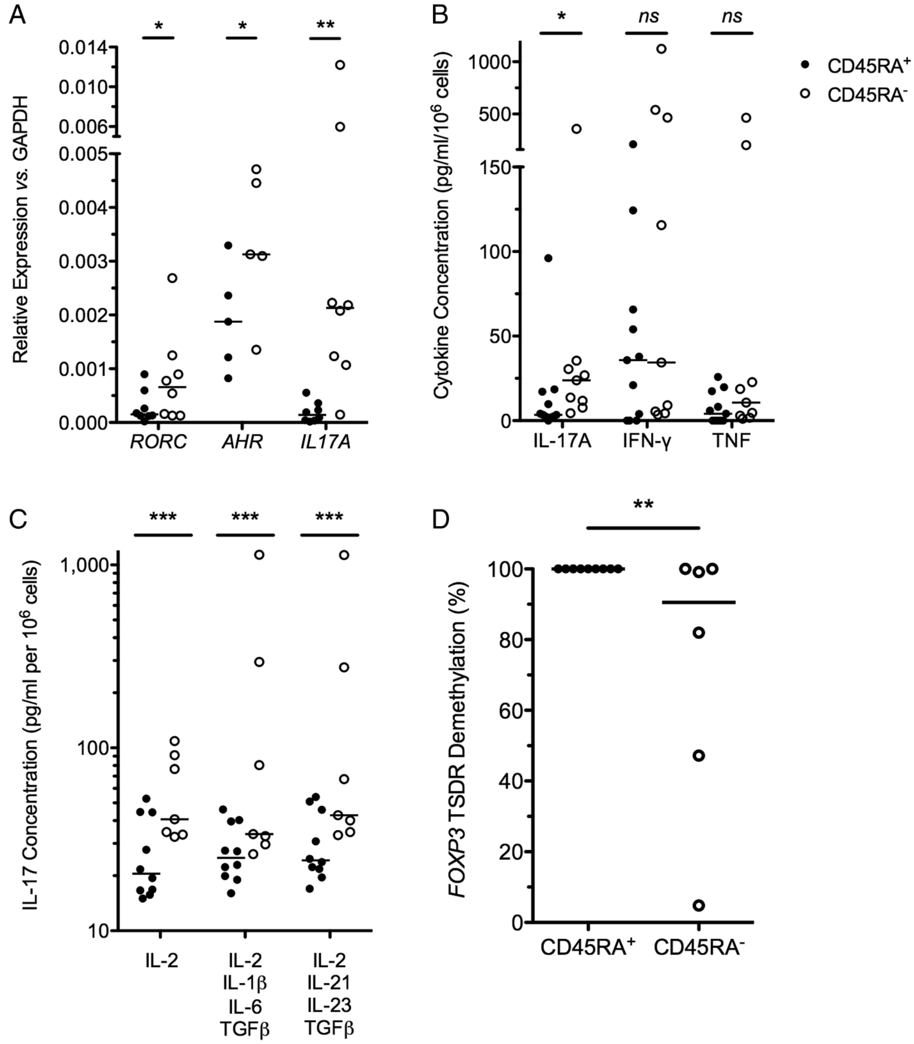

Figure 2 D24 CD45RA$^+$ T$_{regs}$ are resistant to IL-17 induction. (A) Relative expression of *IL17A*, *RORC* and *AHR* in D24 CD45RA$^+$ and CD45RA$^-$ T$_{regs}$, relative to *GAPDH*; n=16, bar at median. (B) D24 T$_{reg}$ IL-17, IFN-γ and TNF secretion in 24 h culture supernatants; n=20, bar at median. (C) IL-17 detected by ELISA from 5-day culture supernatants of D24 T$_{regs}$ cultured in the absence of rapamycin but with supplemental IL-2 alone, a cocktail of IL-2, IL-1, IL-6 and TGF-β or a cocktail of IL-2, IL-21, IL-23 and TGF-β. n=17, bar at median. (D) % FOXP3 TSDR demethylation; n=15, bar at median. *p<0.05,**p<0.01, ***p<0.001. T$_{regs}$, thymus-derived regulatory T cells; IL, interleukin; IFN, interferon; TNF, tumour necrosis factor; TGF, transforming growth factor; TSDR, T$_{reg}$-specific demethylated region; NS, not significant.

Th17 biology, including *RORC*, *AHR* and *IL-17*, were significantly overexpressed in CD45RA$^-$ T$_{regs}$, in comparison with expression in paired CD45RA$^+$ T$_{regs}$ ($p<0.05$ for each comparison, figure 2A). IL-17 secretion was also significantly different in these T$_{reg}$ subsets. IL-17 expression was below the limit of detection in 10/11 (91%) CD45RA$^+$ T$_{regs}$ and significantly higher in CD45RA$^-$ T$_{regs}$ (p=0.02; figure 2B).

The potential of in vitro expanded T$_{regs}$ to turn on an inflammatory programme following exposure to Th17-inducing cytokines, as occurs in vitro in T$_{regs}$ isolated from blood,[39–41] was examined. D24 T$_{regs}$ were washed and cultured for a further 5 days in the presence of IL-2 alone, or Th17-inducing cytokines (IL-2, IL-1, IL-6 and TGF-β or IL-2, IL-21, IL-23 and TGF-β; figure 2C). These proinflammatory cytokines failed to induce IL-17 production by CD45RA$^+$ T$_{regs}$. In contrast, IL-17 production by CD45RA$^-$ T$_{regs}$ was 3-fold higher than CD45RA$^+$ T$_{regs}$ in neutral conditions (IL-2 alone) and 10-fold higher in skewing conditions ($p<0.001$ each comparison).

To ensure that phenotypic stability of CD45RA$^+$ T$_{regs}$ correlated with an epigenetically stable *FOXP3* locus, we determined the methylation status of the *FOXP3* 'TSDR' (figure 2D). We found the TSDR to be completely demethylated in all CD45RA$^+$ T$_{reg}$ lines tested (100%; n=9), suggesting an epigenetically stable FOXP3 locus in CD45RA$^+$ T$_{regs}$ even after 24d of in vitro expansion. In contrast, variable degrees of TSDR demethylation were seen in CD45RA$^-$ T$_{reg}$ lines (median (IQR) of 90.6% (36.6%–100%); n=6; p=0.008).

In vitro expanded CD45RA$^+$ T$_{regs}$ express homing receptors for gut and lymphoid tissue

The ability of in vitro expanded T$_{regs}$ to home to relevant immune niches, where they may suppress inflammation, is thought to be critical for cell therapy. Consequently, the expression of gut homing receptors on in vitro expanded T$_{regs}$ was examined by FACS (figure 3A, B). We found that D24 CD45RA$^+$ T$_{regs}$ modestly expressed $\alpha_4\beta_7$ integrin and CCR6 (20.8%±7.8% and 12.2%±7.9%, respectively) and did not express CCR9. Both CD62L (84.8%±20.6%; p=0.04 vs CD45RA$^-$) and CCR7 (92.1%±12.8%; p=0.03) required for lymph node homing were more highly expressed in CD45RA$^+$ T$_{regs}$ than CD45RA$^-$ T$_{regs}$. CCR4 (95.4%±4.2%) was also highly expressed.

Adoptively transferred CD45RA$^+$ T$_{regs}$ home to inflamed human small intestine in a C.B-17 SCID human SB xenotransplant model

In view of the favourable phenotype of CD45RA$^+$ T$_{regs}$ as a candidate cell therapy, we next sought to determine whether these cells could home to inflamed human SB in vivo. D24 CD45RA$^+$ T$_{regs}$ were administered to a C.B-17 SCID mouse bearing human small intestinal xenotransplants and homing assessed

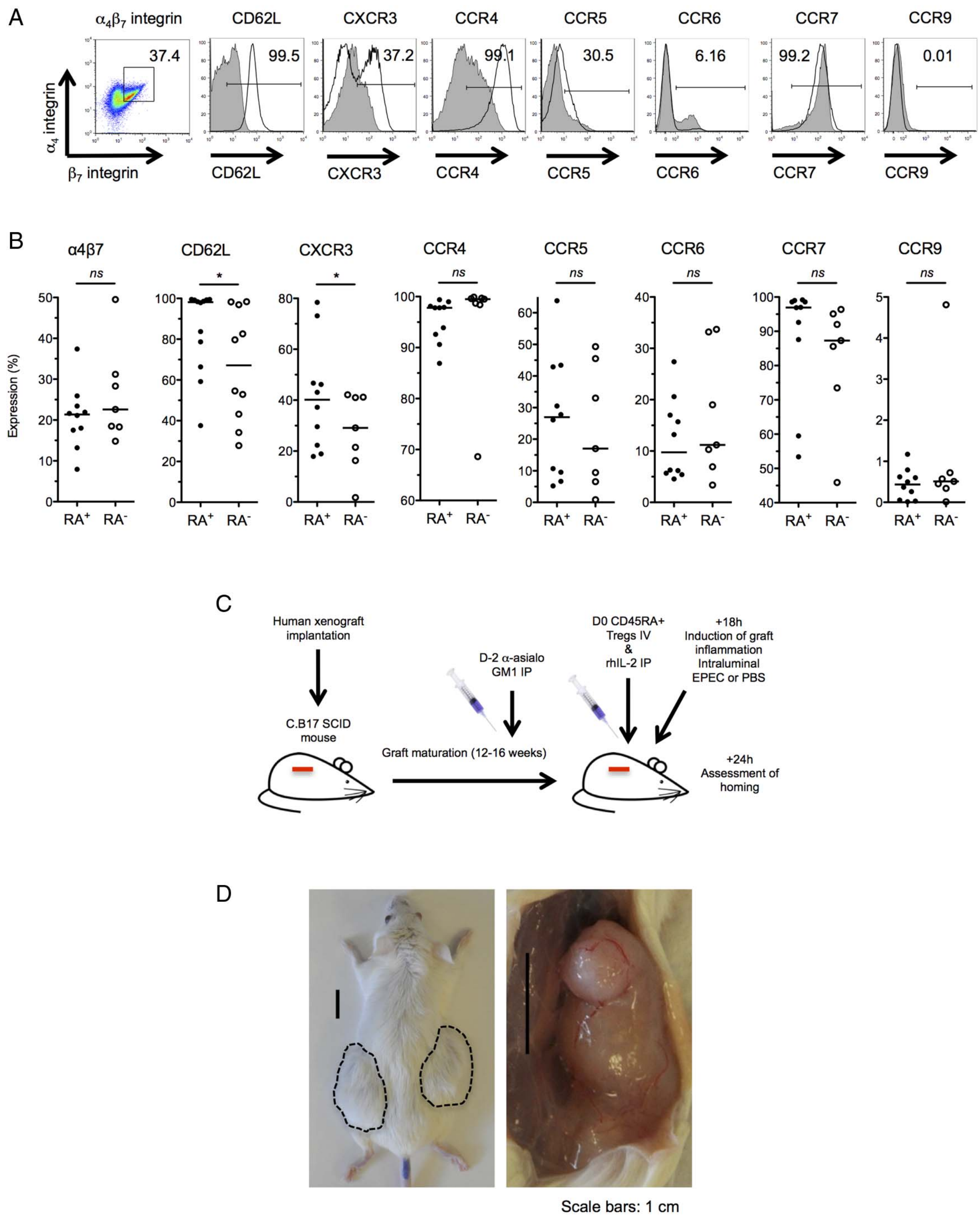

Figure 3 D24 CD45RA$^+$ T$_{regs}$ express gut and lymphoid homing receptors and home to inflamed human LP in a C.B-17 severe combined immunodeficiency (SCID) mouse human intestinal XG model. (A) Representative FACS plots illustrating gut and lymphoid homing receptor expression on D24 CD45RA$^+$ T$_{regs}$ (bold line). Gates were drawn on the basis of fully stained CD4$^+$ lymphocytes (filled) and fluorochrome minus one (FMO) controls. (B) Dot plots showing expression of intestinal and lymphoid homing receptors in D24 CD45RA$^+$ and CD45RA$^-$ T$_{regs}$. n=17; *p<0.05. (C) Design of the XG mouse experiment. (D) Left panel: mature XGs (circled) are visible subcutaneously on the dorsum of the mouse. Right panel: dorsal skin has been removed in an anaesthetised mouse to reveal the mucus-filled XG in situ (right panel). Microscopic images of the XG are shown in online supplementary figure S4A. (E) FACS plots showing live human CD45$^+$CD3$^+$CD4$^+$ events in single cell suspensions prepared from murine spleen, non-inflamed and inflamed XGs, 24 h after intravenous phosphate buffered saline (PBS) (left panels) or adoptive transfer of T$_{regs}$ (right panels). The absolute numbers of CD3$^+$CD4$^+$ events in the XG human CD45$^+$ gates are highlighted. The gating strategy is illustrated in online supplementary figure S4B. (F) Immunofluorescence staining of XG cryosections with antihuman CD3 (red), antihuman CD45 (green) and 4′,6-diamidino-2-phenylindole (DAPI) (blue). (E and F) Representative of two independent experiments. T$_{regs}$, thymus-derived regulatory T cells; LP, lamina propria; XG, xenograft; FACS, fluorescence-activated cell sorting; EPEC, enteropathogenic *Escherichia coli*; NS, not significant.

24 h later (figure 3C, D). Intraluminal injection with enteropathogenic *Escherichia coli* was used to induce mucosal inflammation (see online supplementary figure S4A). Following adoptive transfer, human CD45$^+$CD3$^+$CD4$^+$ cells were detected in mouse spleen and inflamed human SB LP by FACS (see figure 3E; gating strategy online supplementary figure S4B),

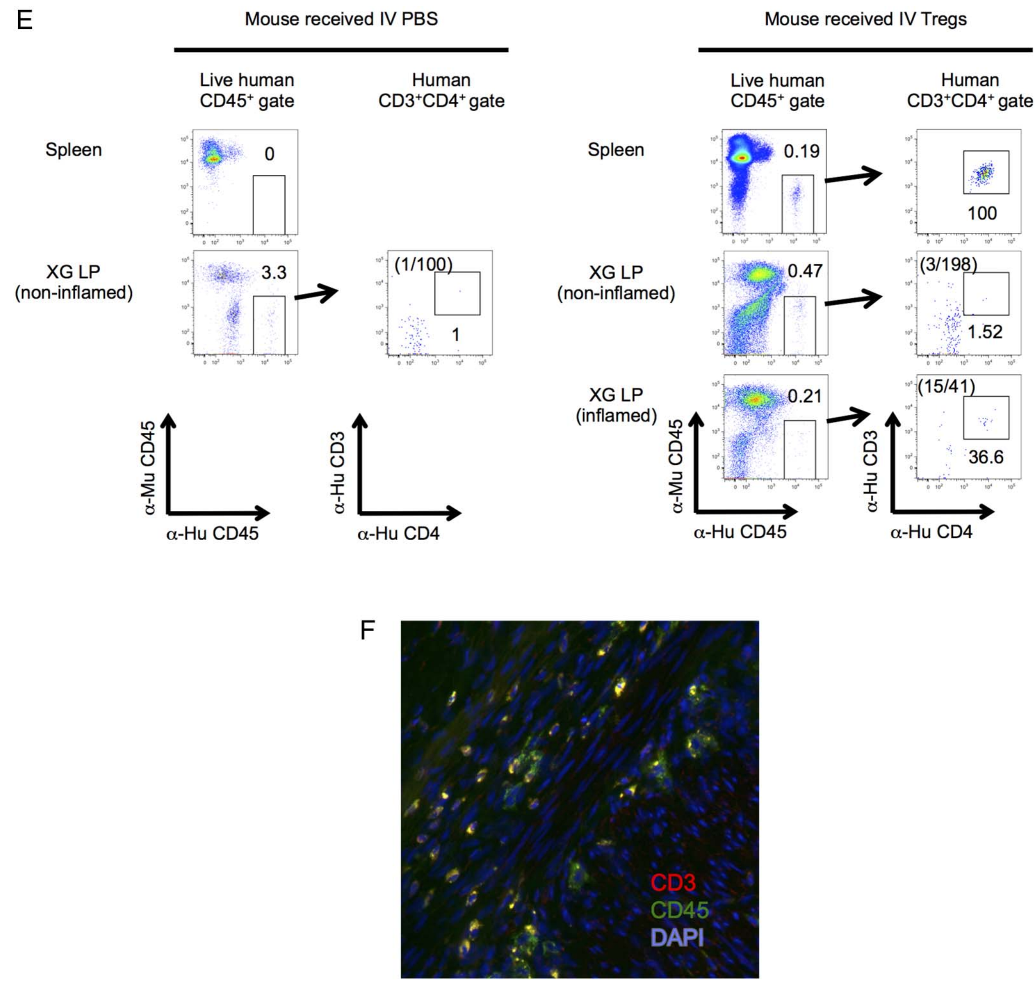

Figure 3 Continued

indicating that adoptively transferred CD45RA$^+$ T$_{regs}$ homed to inflamed human SB LP in this model. This was confirmed by the detection of human CD45$^+$CD3$^+$ cells in inflamed human SB LP by immunofluorescence (figure 3F). We previously showed that human fetal SB contains a population of CD3$^-$CD7$^+$ cells that persist following xenotransplantation.[53] Human CD45$^+$CD3$^-$ events were also detected in non-inflamed human SB LP in both mice that received intravenous PBS and intravenous T$_{regs}$ (figure 3E), suggesting that a population of long-lived human immune cells was co-transferred with the human SB transplant.

In vitro expansion enhances the in vitro suppressive ability of CD45RA$^+$ T$_{regs}$

LP T$_{cons}$ from inflamed CD mucosa are resistant to in vitro suppression by autologous LP T$_{regs}$.[35 36] Consequently, it is possible that in vitro expanded T$_{regs}$ will need an enhanced suppressive function in order to be successful as a future cell-based therapy. Expansion with supplemental rapamycin enhances the in vitro suppressive ability of T$_{regs}$ from patients with ESRF, SLE, RA, MS and asthma.[26 38] In order to determine if in vitro expansion enhanced T$_{reg}$ function in patients with CD, freshly isolated CD4$^+$CD25^{hi}CD127^{lo}CD45RA$^+$ T$_{regs}$ or D24 CD45RA$^+$ T$_{regs}$ that were expanded in vitro from these FACS-sorted CD4$^+$CD25^{hi}CD127^{lo}CD45RA$^+$ precursors were co-cultured with allogeneic Carboxyfluorescein succinimidyl ester (CFSE)-labelled CD4$^+$CD25$^-$ T$_{cons}$ (n=3 independent experiments; cells from the same lot of single-donor, freeze-thawed T$_{cons}$ for each experiment). D24 CD45RA$^+$ T$_{regs}$ suppressed T$_{con}$ proliferation to a greater degree than the freshly isolated CD4$^+$CD25^{hi}CD127^{lo}CD45RA$^+$ T$_{regs}$ from which they were expanded, at both a 4:1 and 8:1 T$_{con}$:T$_{reg}$ ratio ($p<0.01$ and $p<0.001$, respectively; figure 4A). This suggests that in vitro expansion enhances the suppressive ability of D24 CD45RA$^+$ T$_{regs}$.

In vitro expanded CD45RA$^+$ T$_{regs}$ suppress proliferation and activation of MLN and LP T cells in active CD

We next wished to determine if D24 CD45RA$^+$ T$_{regs}$ could suppress activation and proliferation of T$_{cons}$ taken from the MLN and LP of patients with CD (figure 4B, C). MLNMCs were co-cultured with T$_{regs}$ and CD3$^+$ proliferation assessed at 96 h. Dose-dependent T$_{reg}$-mediated suppression of MLN CD3$^+$ proliferation was seen at each MLNMC:T$_{reg}$ ratio (figure 4D). We were unable to demonstrate in vitro suppression of LPMC CD3$^+$ proliferation with this technique, as both freshly isolated and freeze-thawed LPMCs obtained from inflamed CD mucosa died prior to acquisition at 96 h (n=4 independent experiments; see online supplementary figures S5 and S6).

We recently validated a novel co-culture assay for the assessment of in vitro expanded T$_{reg}$ function. This takes advantage of T$_{reg}$-mediated suppression of the early activation marker CD154 (CD40 L) on T$_{cons}$ at 7 h, which correlates with T$_{reg}$-mediated suppression of CFSE dilution and cytokine expression in T$_{cons}$

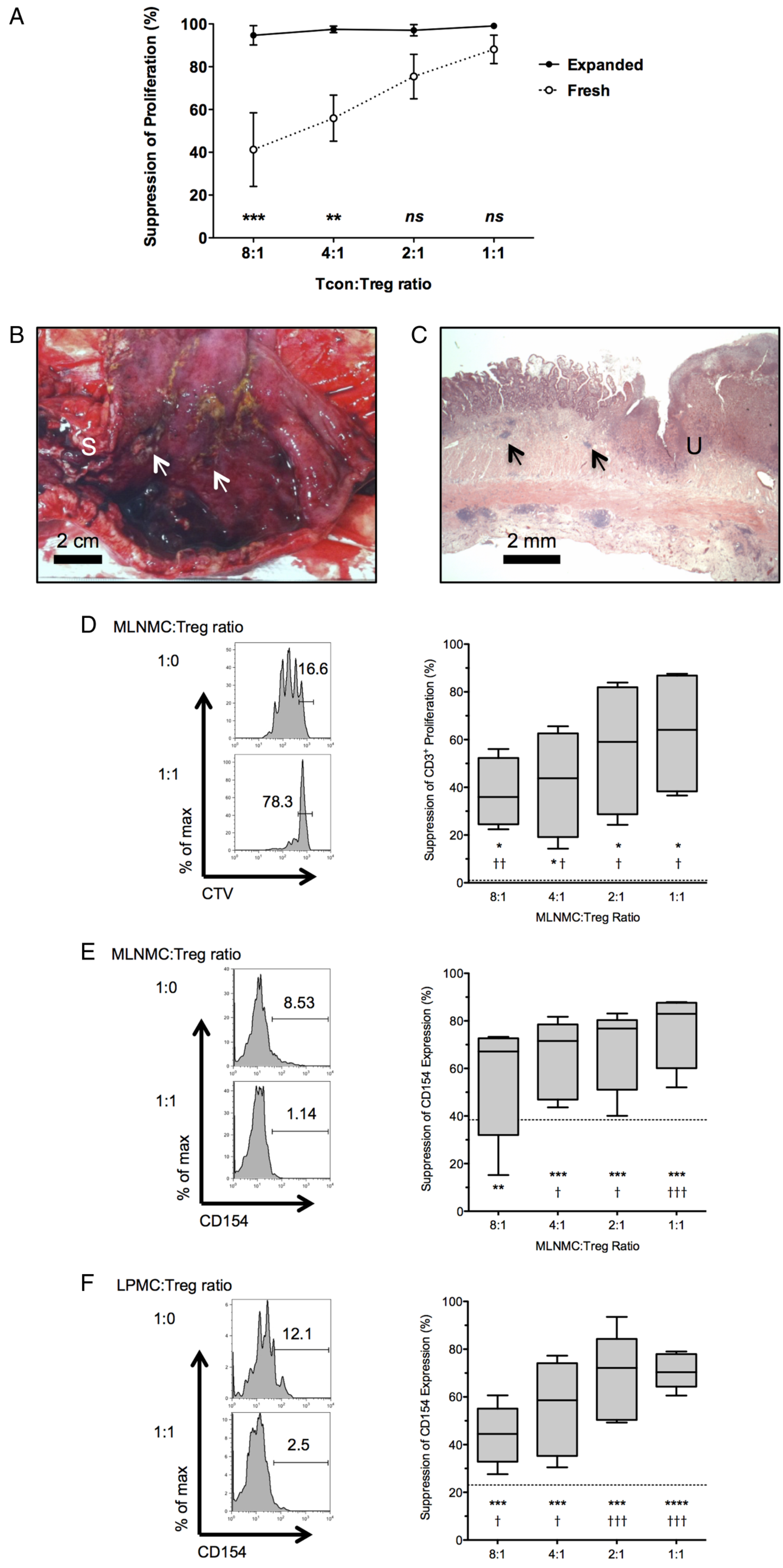

Figure 4 In vitro expanded CD45RA$^+$ T$_{regs}$ suppress CD3$^+$ T cell responses from inflamed Crohn's MLN and LP. (A) Suppression of proliferation of a single lot of freeze-thawed, allogeneic T$_{cons}$ by freshly isolated PB CD4$^+$CD25^{hi}CD127^{lo}CD45RA$^+$ T$_{regs}$, or D24 CD45RA$^+$ T$_{regs}$ that were expanded in vitro from these freshly isolated precursors. Pooled data from three sets of freshly isolated PB T$_{regs}$ and subsequently expanded T$_{reg}$ populations. Data points are mean ±SEM. (B) Fresh ileal resection specimen opened longitudinally to show ileal stricture (marked 'S') and proximal inflamed, haemorrhagic mucosa with deep ulceration (arrows). Scale bar: 2 cm. (C) Representative microscopic image from this resection showing mucosal distortion, ulceration (marked 'U') and transmural inflammation, including lymphoid aggregates (arrows). 12.5× H&E. Scale bar: 2 mm. (D) Representative FACS plots gated on live CD3$^+$ events, showing proliferation of MLN T$_{cons}$ cultured alone (top left panel) or with T$_{regs}$ at a 1:1 MLNMC:T$_{reg}$ ratio (bottom left panel). Pooled data showing T$_{reg}$-mediated suppression of MLN CD3$^+$ proliferation (right panel, n=5). Box and whisker plot shows median, IQR and range. (E) Representative FACS plots gated on live MLN CD3$^+$ events showing CD154 expression on MLN T$_{cons}$ cultured alone (top left panel) or with T$_{regs}$ at a 1:1 MLNMC:T$_{reg}$ ratio (bottom left panel). Pooled data showing T$_{reg}$-mediated suppression of CD154 expression in live MLN CD3$^+$ cells (right panel, n=5). (F) Representative FACS plots gated on live LP CD3$^+$ events showing CD154 expression on LP T$_{cons}$ cultured alone (top left panel) or with T$_{regs}$ at a 1:1 LPMC:T$_{reg}$ ratio (bottom left panel). Pooled data showing T$_{reg}$-mediated suppression of CD154 expression in live LP CD3$^+$ cells (right panel, n=5). (D–F) Dotted line shows non-specific suppression from '2X control'. Comparisons between observed suppression and non-specific suppression (†p<0.05, ††p<0.01, †††p<0.001, ††††p<0.0001) and observed suppression and no suppression (zero, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001) are shown. T$_{regs}$, thymus-derived regulatory T cells; MLN, Mesenteric lymph node; LP, lamina propria; T$_{cons}$, conventional CD4$^+$CD25$^{lo/int}$ T cells; PB, peripheral blood; FACS, fluorescence-activated cell sorting; MLNMC, MLN mononuclear cell; LPMC, LP mononuclear cell; CTV, Cell Trace Violet.

at 96h[25 49] Significant dose-dependent suppression of CD154 expression in MLN and LP T cells was observed (figure 4E, F), demonstrating that in vitro expanded D24 CD45RA$^+$ T$_{regs}$ suppress early activation of MLN and LP T$_{cons}$ in vitro.

DISCUSSION

There remains an unmet need to develop novel therapies for CD, as current drug treatments frequently fail to maintain long-term remission and may be complicated by significant side effects. Cellular therapies are emerging as potentially attractive therapeutic strategies. T$_{regs}$ are effective in preclinical models of colitis[2 6] and phase 1 clinical trials suggest that in vitro expanded T$_{regs}$ are safe in the prophylaxis and treatment of GvHD[12 24] and type 1 diabetes.[18] We built on recent work to describe a method for isolation and expansion of T$_{regs}$ from Crohn's blood that is readily transferable to a GMP background and addresses several barriers to the use of expanded T$_{regs}$ as an autologous cell-based therapy in this important disease.

T$_{regs}$ can be selected and expanded in vitro to clinically useful numbers under both R&D-grade,[11 13 16 21 23 26] and GMP conditions[12 18 24] retaining an in vitro suppressive function before infusion into humans. We showed that it is feasible to do the same using T$_{regs}$ obtained from Crohn's blood, including patients receiving thiopurines or anti-tumor necrosis factor (TNF) medications. Even after prolonged culture, these T$_{regs}$ maintained FOXP3 expression and suppressed activation of autologous T cells.

T cell lineage plasticity is well described. A major potential barrier to T$_{reg}$ therapy is the possibility that these cells might adopt an inflammatory phenotype and worsen inflammation on adoptive transfer. Freshly isolated thymus-derived T$_{regs}$ from both mice and humans can express proinflammatory cytokines and transcription factors (TF) canonical to effector CD4$^+$ lineages, including IL-17[39–41] and IFN-γ,[44] both of which are implicated in CD pathogenesis. Indeed, IL-17$^+$FOXP3$^+$ T$_{regs}$ have been identified in non-inflamed human blood and lymphoid tissue,[40] and inflamed Crohn's mucosa.[43] While there is some evidence that plastic cytokine and TF expression may license efficient T$_{reg}$ homing to, and suppression of, Th1-mediated and Th17-mediated inflammation,[44 56] this may also lead to the generation of T$_{regs}$ with an effector phenotype that contribute to inflammation.

We and others have demonstrated that in vitro expanded T$_{regs}$ cultured in the presence of rapamycin have enhanced phenotypic stability.[13 21] We show that as well as retaining their suppressive capacity, CD45RA$^+$ rT$_{regs}$ expanded from the blood of patients with CD in the presence of rapamycin do not express IL-17A or other Th17-related genes, even following exposure to proinflammatory cytokines that they would likely meet in inflamed intestinal mucosa. These data corroborate data from Hoffmann *et al*[17 47] in healthy controls, showing that expanded CD45RA$^+$ T$_{regs}$ are resistant to the induction of proinflammatory cytokines on stimulation and highly express CD62L and CCR7, which are associated with phenotypic stability.

Freshly isolated CD45RA$^+$ rT$_{regs}$ have an epigenetically stable *FOXP3* locus with extensive TSDR demethylation.[46] TSDR demethylation correlates with stable FOXP3 expression in vitro[50] and T$_{reg}$-mediated protection from autoimmunity in vivo[57] in humans. However, the significance of TSDR demethylation for in vitro expanded T$_{regs}$ is poorly understood. Barzaghi *et al*[57] recently described a cohort of patients with 'Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome (IPEX)-like syndrome', severe multisystem autoimmunity in the absence of identifiable mutations in molecules implicated in T$_{reg}$ function, with decreased TSDR demethylation despite normal T$_{reg}$ numbers and in vitro suppression. This suggests that ex vivo expanded CD45RA$^-$ T$_{regs}$, with incomplete TSDR demethylation, may have suboptimal biological activity in vivo, despite suppressive function in vitro. These data also suggest that CD45RA$^+$ T$_{regs}$ are more likely to retain phenotypic stability and are less likely to acquire an effector phenotype than CD45RA$^-$ T$_{regs}$, consistent with a more favourable safety profile of this T$_{reg}$ subset as a cell-based therapy for CD.

In order to be therapeutically effective, adoptively transferred T$_{regs}$ may need to traffic to intestinal lymphoid tissue or LP. Some groups have taken advantage of TCRs specific for luminal antigens to direct T$_{regs}$ to the intestinal mucosa, such as IL-10-producing T cell clones with ovalbumin-specific TCRs,[58] or T cells with transgenic Cbir1 flagellin-specific TCRs.[59] Alternatively, T$_{reg}$ expansion in the presence of ATRA induces $\alpha_4\beta_7$ integrin expression but also increases effector cytokine expression, such as IL-17 and IFN-γ, potentially limiting its use in GMP cell expansion.[13 21] We show that CD45RA$^+$ T$_{regs}$ expanded in the presence of IL-2 and rapamycin highly express CD62L and CCR7, allowing homing to, and anatomical orientation within lymphoid tissue.[60 61] T$_{reg}$ CD62L expression is also required for T$_{reg}$-mediated cure of GvHD.[30] CD45RA$^+$ T$_{regs}$ also expressed CCR4, required for T$_{reg}$-mediated prevention of CD45RB^{hi} colitis.[62] Interestingly, murine T$_{regs}$ do not need to home to intestinal LP to prevent CD45RB^{hi} adoptive transfer colitis. β_7 integrin-null T$_{regs}$ home to MLN and prevent colitis in this model, despite almost undetectable LP homing.[63] Consequently, the ability to home to MLN is highly desirable in potentially therapeutic cells.

CD45RA$^+$ T$_{regs}$ also express $\alpha_4\beta_7$ integrin and CXC motif receptor 3 (CXCR3), indicating an ability to home to LP and sites of inflammation, respectively. Moreover, we used a human small intestinal xenotransplant model to show, for the first time, that in vitro expanded CD45RA$^+$ T$_{regs}$ from patients with CD home to inflamed human gut in vivo. Xenotransplanted SB segments develop into tissue that is morphologically and functionally identical to normal gut and is capable of peristalsis and nutrient absorption.[53 54] The xenografts also possess a chimeric endothelium that expresses human MadCAM-1.[64] This is the first demonstration that this model can be used in the assessment of immune cell homing.

Xenograft-bearing mice received rhIL-2 in order to support survival of adoptively transferred human T$_{regs}$,[23] as murine IL-2 is less efficient at promoting proliferation of human T cells than rhIL-2, despite cross-reactivity.[65] As recent phase 1 trials of in vitro expanded T$_{regs}$ in GvHD and type 1 diabetes mellitus showed signs of clinical efficacy without supplemental rhIL-2, it is likely that this is a feature of the experimental system and will not be required in clinical trials in Inflammatory bowel disease (IBD).[12 18 24]

Future work will include 'humanising' xenograft-bearing mice and developing additional techniques to induce xenograft inflammation, thus allowing us to assess the functional impact of CD45RA$^+$ T$_{regs}$ on gut inflammation. The percentage of LP human T cells that could be recovered from human bowel transplants was relatively modest compared with the percentage of T cells recovered from the spleen. Given that the expression of the gut homing integrin $\alpha_4\beta_7$ was only expressed on ~20% of the purified T$_{regs}$, future work may need to address methods to increase $\alpha_4\beta_7$ expression, such as the use of retinoic acid, as we have previously shown.[21]

An additional barrier to T$_{reg}$ therapy in CD is that effector T cells from the diseased mucosa of patients with CD may be resistant to the suppressive action of T$_{regs}$. Indeed, we previously showed that T_{cons} isolated from inflamed Crohn's mucosa are relatively resistant to T_{reg}-mediated suppression, due to overexpression of Smad7, an inhibitor of TGF-β signalling.[35 36] In this study, we utilised T_{regs} cultured in the presence of rapamycin, which has been shown to enhance the suppressive ability of in vitro expanded T_{regs}, compared with T_{regs} freshly isolated from the same donor[26 38] and show that in vitro expansion enhances the suppressive ability of T_{regs} obtained from CD PB. Rapamycin-expanded CD45RA$^+$ T_{regs} effectively suppress both MLN and LP T cells obtained from inflamed Crohn's resection specimens. These data suggest that in vitro expanded CD45RA$^+$ T_{regs} may modulate immune responses in niches directly relevant to the pathogenesis of CD. T_{regs} use multiple mechanisms to suppress in vitro and in vivo, including contact-dependent mechanisms (CTLA-4, perforin-granzyme B) and contact-independent mechanisms (IL-10, TGF-β, extracellular ATPase activity via CD39/CD73,etc). Sakaguchi *et al*[10] has proposed a multistep model of in vitro suppression that initially requires cell-cell contact but is subsequently contact independent. The mechanism of suppression of erstwhile 'resistant' mucosal T_{cons} by in vitro expanded T_{regs} is currently unknown and will be the subject of further study. In addition, not all of the patients in this study had active disease, so it will be important to extend these data further to broaden the therapeutic relevance of these findings. However, a substantial proportion of the patients in this study did have evidence of disease activity (n=5/13), which did not affect either T_{reg} expansion or function.

In conclusion, we have shown that in vitro expanded CD45RA$^+$ T_{regs} are likely to be the most suitable T_{reg} subset for cellular therapeutics in CD. This subset is readily expandable to sufficiently high numbers under conditions that are readily transferable to GMP, for clinical use. They express an appropriate repertoire of homing receptors for MLN and gut, and effectively traffic to inflamed gut in vivo. As well as retaining powerful suppressive properties, these cells show little or no capacity for plasticity towards a potentially harmful effector phenotype, which correlates with an epigenetically stable *FOXP3* locus. This study addresses many of the perceived barriers to T_{reg} cell treatment for CD and paves the way for a clinical trial of in vitro expanded CD45RA$^+$ T_{regs} in this therapeutically challenging disease.

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Competing interests None.

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Supplemental Methods

In vitro generation of T_{reg} lines

T_{regs} were expanded *in vitro* in X-VIVO-15 (Lonza, Walkersville, MD), supplemented with 5% human AB serum (Biosera, Uckfield, UK) and rapamycin (100nM, Rapamune, Pfizer, Sandwich, UK). Sorted precursor T_{regs} were plated at 10^6/ml and stimulated with anti-CD3/anti-CD28-coated beads (Dynalbeads, Invitrogen, Paisley, UK) at a 1:1 ratio, then incubated at 37°C/5% CO_2. rhIL-2 (1,000IU/ml, Proleukin®, Novartis, Basel, Switzerland) was added at the start of culture and replenished every other day. Re-stimulation frequency was optimized by daily assessment of proliferation and Ki-67 expression. Cells were re-stimulated with new beads after 10-12 days of culture, with the addition of fresh rapamycin and IL-2. The phenotype and suppressive ability of T_{reg} lines was assessed after 22-24 days of culture.

Other experiments were performed in RPMI 1640 (PAA) supplemented with HEPES (10mM, Thermo Fisher Scientific, Loughborough, UK), L-glutamine (2mM), penicillin (100IU/ml), streptomycin (100g/ml), sodium pyruvate (1mM), MEM nonessential amino acids (0.1mM), and 10% fetal calf serum (all PAA).

Antibodies for Flow Cytometry

Live/Dead Aqua staining was performed for 20 minutes at room temperature. Cell surface staining was performed for 30 minutes at 4°C. Intracellular staining was performed using the “FoxP3/Transcription Factor Staining Buffer Set” (eBioscience, Hatfield, UK). Isotype and “fluorochrome minus one" controls were acquired, as appropriate. Experiments were acquired on LSRII and Fortessa SORP cytometers running FACSDiva 6.1.3 software (BD), and analysed with FlowJo v9.8 for Mac (FlowJo, Ashland OR, USA).

The following antibodies used for flow cytometry: CD3-APC-H7 (clone SK7), CD4-FITC (SK3), CD4-V500 (RPT-T4), CD8-V500 (RPA-T8), CD8-PE-Cy7 (RPA-T8), CD25-PE (2A3), CD45RO-PE-Cy7 (UCHL1), CD154-APC (89-76), CCR5-PE-CF594 (2D7/CCR5), CCR7-PE-CF594 (150503), CXCR3-PE-Cy7 (IC6/CXCR3), and IL-17-Pacific Blue (N49-653, all BD Biosciences); CD3-Pacific Blue (OKT3), CD4-eFlour450 (OKT4), anti-mouse CD45-PerCP-Cy5.5 (30-F11), CD127-PerCP-Cy5.5 (eBioRDR5), FOXP3-FITC (PCH101, all eBioscience); α_4 integrin/CD49d-PerCP-Cy5.5 (9F10), β_7 integrin-FITC (FIB504), CD45RA-AlexaFluor700 (HI100), CD62L-Brilliant Violet (BV) 421 (DREG-56), CD127-BV650 (A019D5), CD161-BV605 (HP-3G10), CCR4-PE-Cy7 (TG6/CCR4), CCR6-APC (G034E3), CCR9-Alexa Fluor 647 (BL/CCR9, all BioLegend). Dead cells were excluded using Live/Dead Blue (Invitrogen) or propidium iodide (Sigma).

Assessment of the in vitro suppressive ability of putative Tregs

FACS-sorted $CD4^+CD25^-$ PB T_{cons}, or LPMCs or MLN mononuclear cells were used as responder cells, as indicated in the text.

In order to assess the ability of putative T_{regs} to suppress proliferation of responder cells *in vitro*, $5x10^4$ responder cells were labelled with CellTrace Violet (CTV, 1μM, Invitrogen) and cultured alone (a 1:0 responder cell:T_{reg} ratio), or with T_{regs} at responder cell:T_{reg} ratios ranging from 1:1 to 32:1 for 96h. Responder cell numbers were kept constant. $CD4^+CD25^-$ T_{cons} were stimulated with Dynalbeads at a T_{con}:bead ratio of 40:1. LPMCs and MLNMCs were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies (R&D Systems, Abingdon, UK; coated with 2μg/ml of each in PBS at 4°C overnight). Controls included a "2X control" condition (2:0 responder cell:T_{reg} ratio; to exclude non-specific cell density-mediated inhibition of proliferation), and a stained-unstimulated control. The number of non-proliferating cells and the number of precursors of proliferating cells were calculated using standard formulas[1]. Percent suppression of proliferation (*S*) was calculated using the following formula:

$$S = 100 - (\left[\frac{a}{b}\right] x100)$$

where *a* is the percentage of proliferating precursors in the presence of T_{regs} and *b* is the percentage of proliferating precursors in the absence of T_{regs}.

The BD Fastimmune Human Regulatory T Cell Function Kit (BD Biosciences) was used according to manufacturer's instructions to assess the ability of putative T_{regs} to suppress activation of LPMCs and MLNMCs *in vitro*. Briefly, responder cells were cultured for 7h at the cell numbers and responder cell:T_{reg} ratios described above. Cells were stimulated with plate-bound anti-CD3 and anti-CD28, as described above. And anti-CD154 (CD40L)-APC (clone 89-76) was added at time 0. Controls included a "2X control", unstained-unstimulated, stained-unstimulated and stained-unstimulated responder cell conditions. Assessment of T_{reg}-mediated suppression of CD154 expression on lymphocytes was performed as described previously[2,3]. Percent suppression of proliferation (*S*) was calculated using the following formula:

$$S = 100 - (\left[\frac{c}{d}\right] x100)$$

where c is the percentage of $CD154^+$ events in live $CD3^+$ responder cells in the presence of Tregs, while d is the percentage of $CD154^+$ events in live $CD3^+$ responder cells in the absence of T_{regs}.

rtPCR

The following FAM-conjugated probes were used: AHR (Hs00169233), CCR9 (Hs01890924), IL-17A (Hs00174383) and RORC (Hs01076112). GAPDH-VIC (Applied Biosystems, Paisley, UK) was used as an endogenous housekeeping gene.

Isolation of LPMCs and MLN mononuclear cells

LPMCs were isolated from inflamed mucosa and peri-colic fat obtained from CD right hemicolectomy resection specimens. Mucosal epithelium was removed by agitation in pre-warmed HBSS (PAA) supplemented with EDTA (1 mM, Sigma) and gentamycin (30 mg/ml, PAA) for 30 minutes at 37°C. A single cell suspension was then prepared by agitation with pre-warmed RPMI supplemented with collagenase 1a (1 mg/ml, Sigma), DNase I (10 IU/ml, Roche) and gentamycin for 60 minutes at 37°C. LPMCs were then enriched by gradient density centrifugation.

MLNs were harvested from peri-colic fat of the same resections. MLNs were mechanically disrupted between mesh, then washed with cold RPMI, to yield a single layer suspension of MLN mononuclear cells.

C.B-17 SCID mouse human intestinal xeno-transplant model

IRB and IACUC approvals were obtained prospectively (Ethics Committee for Animal Experimentation, Hebrew University of Jerusalem; MD-11-12692-4 and the Helsinki Committee of the Hadassah University Hospital; 81-23/04/04). Women undergoing legal terminations of pregnancy gave written, informed consent for use of fetal tissue in this study.

C.B-17 SCID mice were purchased from Harlan, Israel and housed under SPF conditions. Human fetal small bowel up to 16 weeks gestational age was implanted subcutaneously on the dorsum of the mouse, as described previously[4,5]. Grafts developed *in situ* for 12-16 weeks prior to manipulation. Mice were treated IP with rabbit anti-asialo GM1 (15μl, Cedarlane Labs, Burlington NC) 24h prior to T_{reg} administration. As murine IL-2 is significantly less efficient at promoting the survival and proliferation of human T cells than human IL-2[6], mice were treated with rhIL-2 ($2x10^4$IU, Proleukin) directly prior to T_{reg} administration, as described by Tresoldi *et al.*[7]. $10x10^6$ T_{regs} that were expanded *in vitro* from $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ precursors were administered *via* tail vein injection. Homing was assessed 24h later.

Enteropathogenic *Escherichia coli* (EPEC) WT-GFP were grown in LB medium overnight at 27°C without agitation, as described previously[5]. In order to induce mucosal inflammation, up to 10^8 bacteria in 100μl PBS (or PBS alone negative control) was injected percutaneously into the lumen of the human small intestinal grafts. were injected intraluminally with either up to 10^8 bacteria in 100μl PBS or PBS alone. Because mice could accommodate 2 xenografts, each animal acted as their own control.

Twenty four hours after IV adoptive transfer of T_{regs}, animals were sacrificed and T_{regs} homing to the human small intestinal grafts was assessed by flow cytometry and immunofluorescence.

In order to detect adoptively transferred T_{regs} by FACS, single cell suspensions were prepared from murine spleen and xenograft LP, as described above, followed by staining with anti-mouse CD45-PerCP-Cy5.5 (30-F11), anti-human CD45-Pacific Blue (2D1), anti-human CD4-FITC (OKT4, all eBioscience), anti-human CD3-APC-H7 (SK7, BD) and live/dead blue, then FACS acquisition.

In order to detect adoptively transferred T_{regs} by immunofluorescence, fixed cryostat sections were blocked with 20% horse serum (PAA) and stained with anti-human CD45-FITC (2D1) and anti-human CD3-biotin (OKT3, both eBioscience), followed by streptavidin-AlexaFluor 594 (Invitrogen). To visualize EPEC, sections were stained with phalloidin-rhodamine (Sigma). Nuclei were stained with DAPI (1g/ml, Invitrogen). Negative controls were stained with isotype-matched antibodies. Images were acquired on an Olympus BX51 microscope using Micro-Manager software (Vale Lab, UCSF, San Francisco, CA).

Statistical analysis

The Kolmogorov-Smirnov test was used to determine if continuous variables were normally distributed. Normally distributed variables were then compared using unpaired or paired t tests. Non-normally distributed variables were compared using Mann-Whitney U or Wilcoxon matched-pairs signed rank tests, as appropriate. Paired tests were used with paired data. Categorical variables were compared using Chi-square tests. Grouped variables were compared using ANOVA. Post-hoc corrections were not performed. A p value of <0.05 was considered statistically significant.

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Supplemental Figures

Figure S1. Strategy to determine the optimum enrichment method for *in vitro* expansion of T_{regs} from PB of CD patients.

(A-E) Cartoon illustrating the strategy used to enrich starting populations for T_{reg} expansion. (A) PB was obtained from CD patients and (B) PBMCs enriched by density gradient centrifugation. (C) Cells for culture were then enriched with MACS technology using two strategies achievable with currently available reagents for the Miltenyi "CliniMACS" system. (D) PBMCs were enriched in a two-step selection involving $CD8^+$ depletion, followed by $CD25^+$ enrichment. (D') Alternatively, PBMCs were positively selected for $CD4^+$, then stained for CD4, CD25, CD127 and CD45RA, (D'') followed by sorting on a BD FACSAria II into $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$ and $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ subsets. (E) Each enriched population was stimulated with anti-CD3/anti-CD28 coated Dynabeads and cultured for 20-24 days in X-VIVO15 supplemented with 5% human AB serum, IL-2 and rapamycin. ILX2 was replenished every other day. Cells were re- stimulated and placed in fresh medium at day 12.

Figure S1

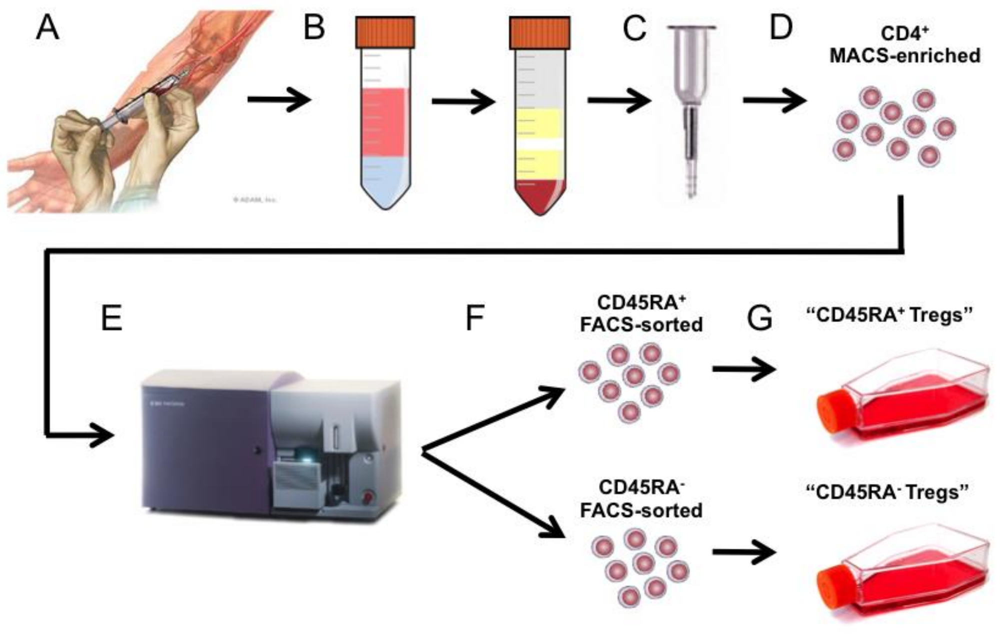

Figure S2. Gating strategy for FACS sorting of MACS-enriched $CD4^+$ lymphocytes into $CD4^+CD25^+CD127^{lo}CD45RA^+$ and $CD4^+CD25^+CD127^{lo}CD45RA^-$ subsets for subsequent expansion *in vitro*.

(A) Pre-sort $CD4^+$ lymphocytes. Following exclusion of doublets, lymphocytes are sorted on the basis of $CD4^+CD25^{hi}$, then $CD127^{lo}$, then CD45RA expression. (B) Post-sort enrichment for $CD4^+CD25^+CD127^{lo}CD45RA^+$ T_{regs}. (C) Post-sort enrichment for $CD4^+CD25^+CD127^{lo}CD45RA^-$ T_{regs}. (D) Post-sort enrichment for autologous $CD4^+CD25^-$ T_{cons}. Representative of 13 independent experiments. (E) Cell counts per ml of PB according to disease activity status and sorted T_{reg} subset.

Supplemental Figure 2

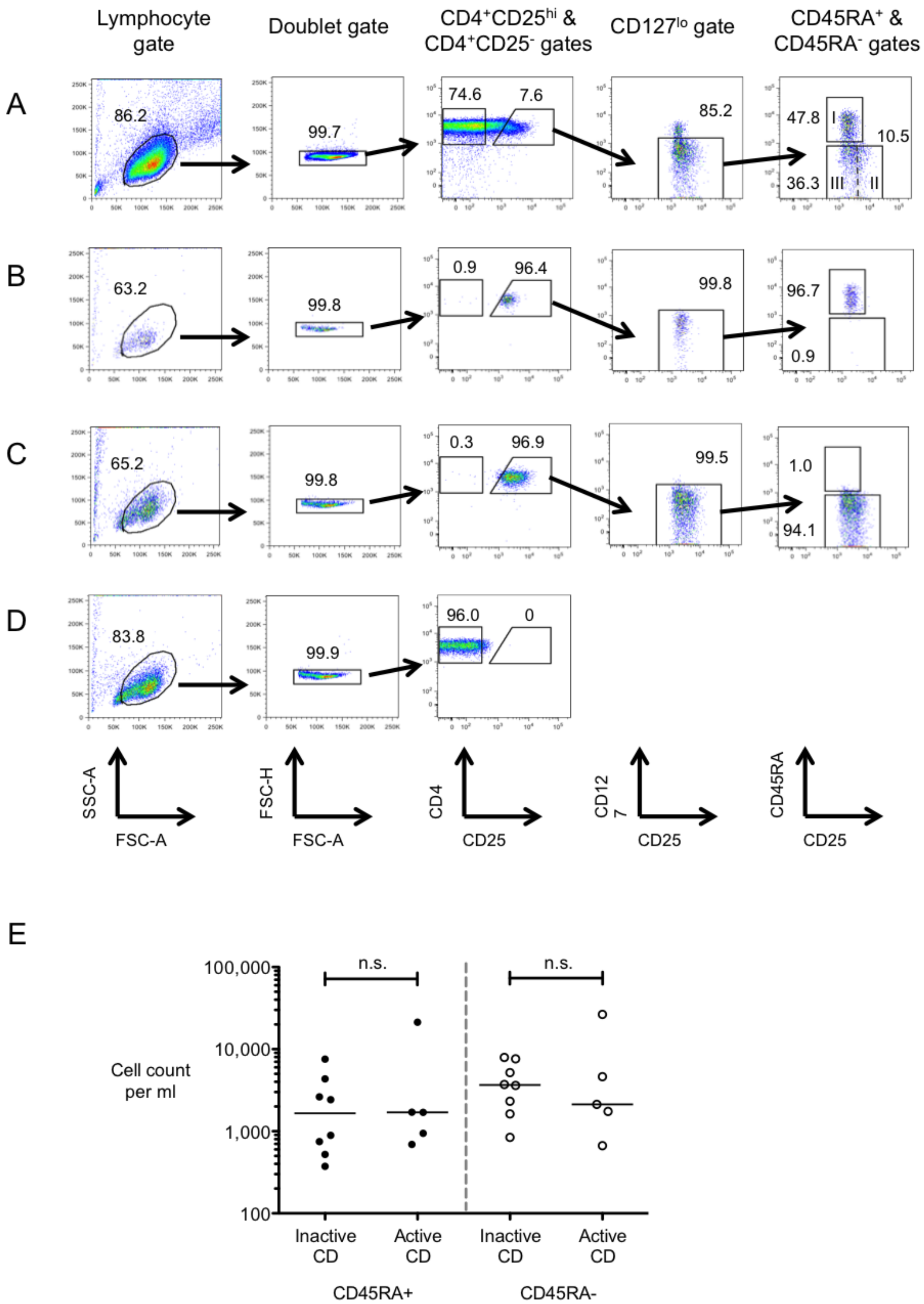

Figure S3. T_{reg}-mediated reduction of IL-2 and IFN-γ in 96h co-culture supernatants.

(A) T_{reg}-mediated reduction of IL-2 in supernatants correlated with T_{reg}-mediated suppression of proliferation for both CD45RA$^+$ (left panel) and CD45RA$^-$ T_{regs} (right panel). (B) T_{reg}-mediated reduction of IFN-γ in supernatants correlated with suppression of proliferation for CD45RA$^+$ T_{regs} (left panel) but not CD45RA$^-$ T_{regs} (right panel).

Supplemental Figure 3

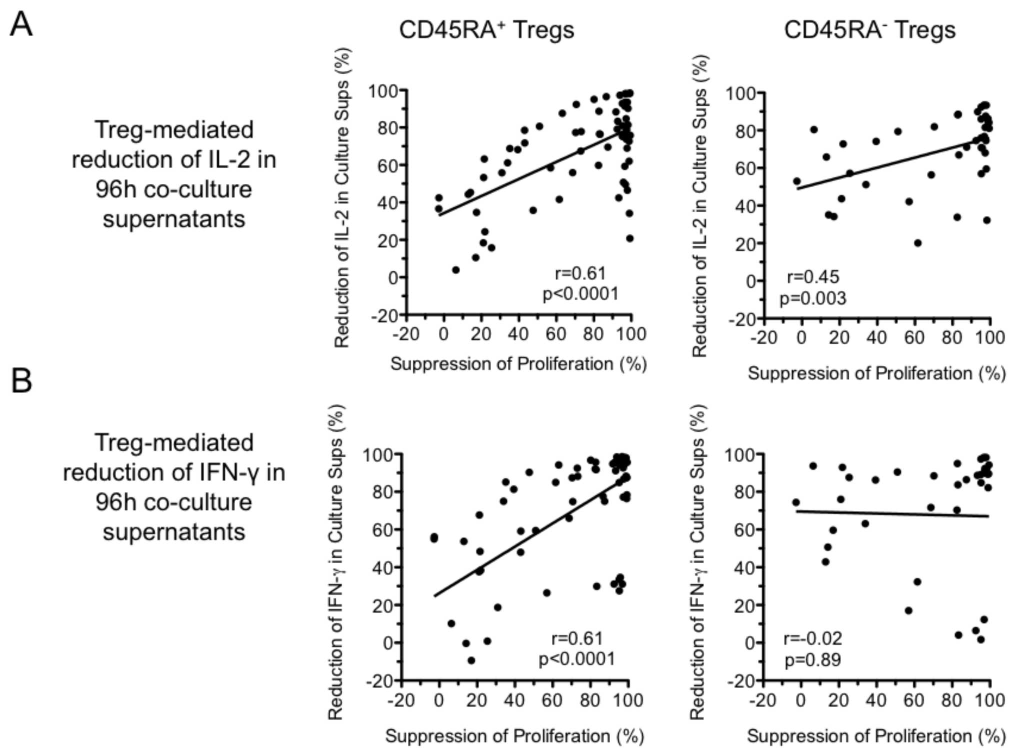

Figure S4. C.B-17 SCID intestinal xenograft model.

(A) EPEC induces mucosal inflammation in human fetal intestinal xeno-transplants. Top left panel: Representative microscopic image of a xenograft 8h after intraluminal injection with PBS, showing non-inflamed small bowel mucosa with normal villi (arrows) and intestinal crypts (arrowheads). H&E, 20X. Bottom left panel: In contrast, intraluminal injection with EPEC caused mucosal inflammation with destruction of villi and cytoplasmic vacuolization. Top right panel: Fluorescence staining of PBS-treated xenograft cryosection showing a normal small bowel villus. F-Actin is visualized with phalloidin-rhodamine (red). DAPI (blue). Bottom right panel: GFP-expressing EPEC (green, arrows) adhere to mucosal epithelial cells and induce formation of AE lesions. (B) FACS plots showing healthy control PBMCs and the gating strategy for identification of live human $CD45^{+}CD3^{+}CD4^{+}$ events in the C.B17 SCID human intestinal xeno-transplant model.

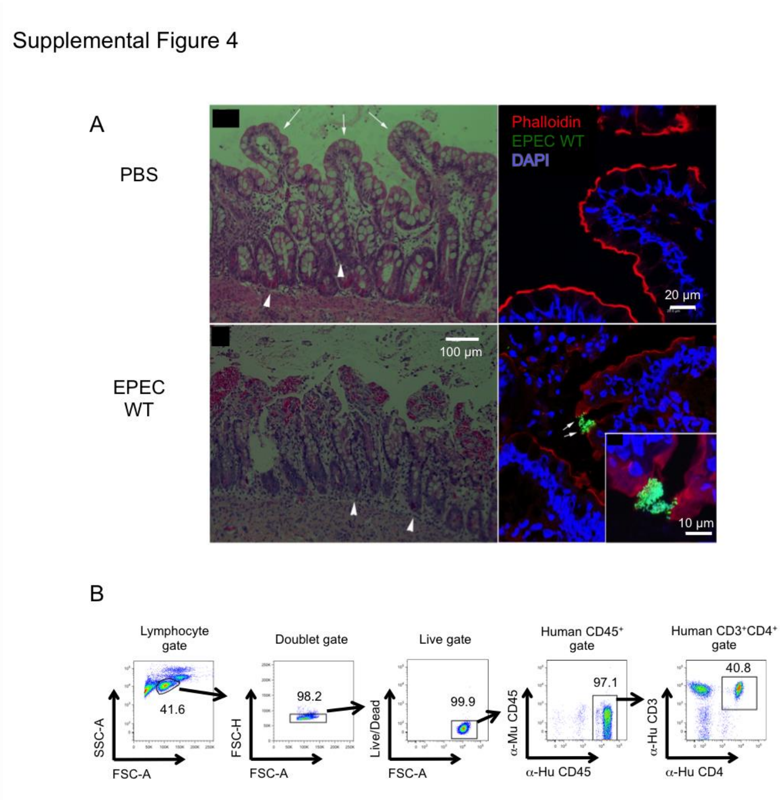

Figure S5. Viability and proliferation of healthy donor PBMCs following 5 days' *in vitro* proliferation.

(A) Representative microscopic image of thawed healthy donor PBMCs showing good viability (0.4% Trypan blue, 100X). (B) PBMCs were then labelled with CFSE or CellTrace Violet (CTV) and cultured for 5 days, stimulated with plate-bound anti-CD3/anti-CD28 antibodies. End-of-culture representative forward- *vs.* side-scatter plot is shown. (C) Representative plots illustrating live events in CFSE-labelled (left) and CTV-labelled (right) PBMCs, at 5 days. (D) Representative plots illustrating proliferation at 5 days.

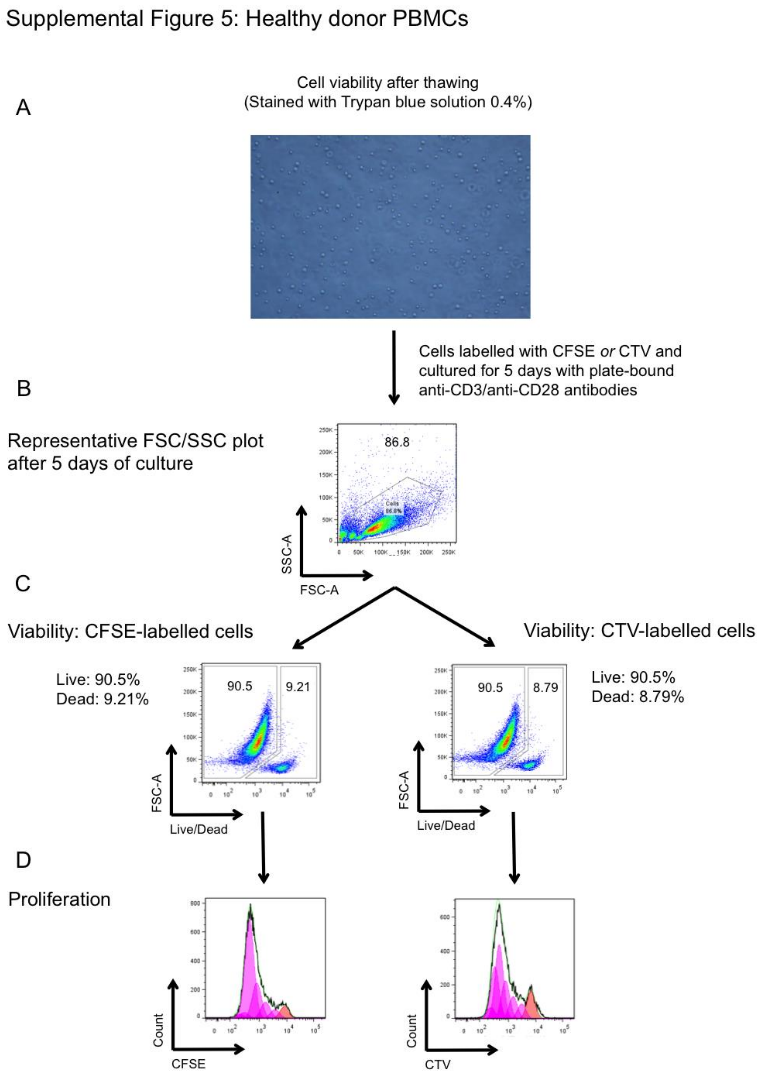

Figure S6. Viability and proliferation of CD LPMCs following 5 days' *in vitro* proliferation.

(A) Representative microscopic image of thawed CD LPMCs showing poor viability and clumping (0.4% Trypan blue, 100X). (B) LPMCs were then treated as described in Figure S4B. End-of-culture representative forward- *vs.* side-scatter plot is shown. (C) Representative plots illustrating live events in CFSE-labelled (left) and CTV-labelled (right) LPMCs, at 5 days. Similar results were obtained using fresh or thawed LPMCs. (D) Poor viability at 5 days meant that assessment of proliferation was not feasible.