# Correction of Defective T-regulatory Cells From Patients With Crohn's Disease by Ex Vivo Ligation of Retinoic Acid Receptor Alpha

**Manuscript Number:** GASTRO-D-18-01603R2

**Full Title:** Correction of Defective T-regulatory Cells From Patients With Crohn's Disease by Ex Vivo Ligation of Retinoic Acid Receptor Alpha

**Article Type:** Basic - Alimentary Tract

**Corresponding Author:** Graham Michael Lord  
Department of Experimental Immunobiology, Division of Transplantation Immunology and Mucosal Biology, King's College London, United Kingdom, SE1 9RT  
London, UNITED KINGDOM

**Corresponding Author's Institution:** Department of Experimental Immunobiology, Division of Transplantation Immunology and Mucosal Biology, King's College London, United Kingdom, SE1 9RT

**First Author:** Rimma Goldberg, MBBS, BMedSci

**First Author Secondary Information:** Rimma Goldberg, MBBS, BMedSci

**Order of Authors:**  
- Cristiano Scotta, MSc PhD  
- Dianne Cooper, MSc PhD  
- Einat Nissim-Eliraz, MSc PhD  
- Eilam Nir, MSc  
- Scott Tasker, BSc  
- Fowzia Ibrahim, MSc  
- Peter M Irving, MA MD FRCP  
- Jeremy Sanderson, MD FRCP  
- Paul Lavender, MSc PhD  
- Jonathan Corcoran, MSc PhD  
- Toby Prevost, PhD  
- Nahum Y Shpigel, BVSc PhD  
- Federica Marelli-Berg, MD PhD  
- Giovanna Lombardi, MSc PhD  
- Graham M Lord, MA PhD FRCP FRSB FMedSci

**Order of Authors Secondary Information:**

**Abstract:**  
**Background & Aims:** Crohn’s disease (CD) is characterized by an imbalance of effector and regulatory T cells in the intestinal mucosa. The efficacy of anti-adhesion therapies led us to investigate whether impaired trafficking of T-regulatory (Treg) cells contributes to the pathogenesis of CD. We also investigated whether proper function could be restored to Treg cells by ex vivo expansion in the presence of factors that activate their regulatory activities.
Methods: We measured levels of the integrin α4β7 on Treg cells isolated from peripheral blood or lamina propria of patients with CD and healthy individuals (controls). Treg cells were expanded ex vivo and incubated with rapamycin with or without agonists of the retinoic acid receptor alpha (RARA) and their gene expression profiles were analyzed. We also studied the cells in cytokine challenge, suppression, and flow chamber assays and in SCID mice with human intestinal xenografts.

Results: We found that Treg cells from patients with CD express lower levels of the integrin α4β7 than Treg cells from control patients. The pathway that regulates expression of integrin subunit alpha is induced by retinoic acid (RA). Treg cells from patients with CD incubated with rapamycin and an agonist of RARA (RAR568) expressed high levels of integrin α4β7, as well as CD62L and FOXP3, compared to cells incubated with rapamycin or rapamycin and ATRA. These Treg cells had increased suppressive activities in assays and migrated under conditions of shear flow; they did not produce inflammatory cytokines and RAR568 had no effect on cell stability or lineage commitment. Fluorescently labelled Treg cells incubated with RAR568 were significantly more likely to traffic to intestinal xenografts than Treg cells expanded in control medium.

Conclusions: Treg cells from patients with CD express lower levels of the integrin α4β7 than Treg cells from control patients. Incubation of patients’ ex vivo expanded Treg cells with rapamycin and an RARA agonist induced expression of α4β7 and had suppressive and migratory activities in culture and in intestinal xenografts in mice. These cells might be developed for treatment of CD. clinicaltrials.gov no: NCT03185000
Dear Drs Peek and Corley,

We wish to resubmit our manuscript entitled “A selective defect in regulatory T cell trafficking in Crohn’s disease is corrected by ex vivo ligation of retinoic acid receptor alpha” for further consideration by Gastroenterology.

We would like to thank the editors and the reviewers for taking the time to review the paper and provide a second round of comments. We have taken great care to edit the figures to ensure clarity for the reader. We have also amended the wording to reflect our findings that a higher proportion of RAR568 treated cells express integrin $\alpha 4\beta 7$ in line with reviewer 2 comments.

We hope that you will find the manuscript and associated figures to be of sufficient quality for publication in Gastroenterology.

Yours sincerely,

Prof Graham Lord , MD, PhD, FRCP, FMedSci (on behalf of the authors)
Comments from the Editors and Reviewers:

Your manuscript is improved. But there are still issues to correct.
First please see the comments from Rev 1. They are not agreeing with your response related to Figure 3b. Please address.
Please address Rev 2 comments about figures.

I have these additional concerns about the figures, that I suggest you improve.

Fig. 1a: The writing in the x and y axes from the flow software is even smaller than I am used to. It is not readable at all until one zooms to 200%, if that can be fixed it would be helpful. More concerning is that the text within the plots, demarcating the quadrant percentages and the types of cells gated are also extremely small. Please try to fix this with labeling that would be visible to the reader.

The x and y axes labels as well as the text within the plots have been adjusted to a larger font size for improved readability.

Fig 2a: upper right panel- the“Inactive” on the x axis is cut off. The x axis font size is very small. I suggest you consider not using the 45deg angle on the text and instead rotate horizontal and increase the font size and be sure its bolded.

The positioning of the panels has been fixed to ensure all axis labels are visible. The axis labels have been made horizontal and in bold type.

Fig 3a same issue with the flow plots as in 1a.

The x and y axes labels as well as the text within the plots have been adjusted to a larger font size for improved readability.

Fig 4a, as pointed out by rev 1, the entire proportion of the left upper panel is off. The fonts in the graph and on the axis labels are too small compared to the other volcano plots. Please standardize. Actually the fonts on the lower left panel seem slightly smaller than those in the upper right. So you have 3 plots with all different sizes of fonts in the fig and in the axes.

The plots have been re-sized so that they are uniform in dimension and text size.

Fig 6b- the labels on the flow plots are too small. Please correct.

The x and y axes labels as well as the text within the plots have been adjusted to a larger font size for improved readability.

Reviewer 1: This is a better version of the manuscript. This reviewer just have minor, mostly cosmetic, comments about the manuscript.

In page 3 line 34, in the Results summary, please define ATRA.

Definition for ATRA has been added

In page 3 line 36, the sentence following the first mention of ATRA, please add a ‘to’: “These Tregs cells showed…and the ability TO migrate under conditions of shear flow;”

The word “to” has been added to this sentence

In reference to the current Figure 3b (previously Figure 2a), the authors indicate in page 14 line 29 that cells cultured in rapa-rar568 expressed more a4b7 than the control group. Although this is correct, what the authors I believe mean is that there is an increase in the frequency of cells expressing a4b7 (no MFI data is shown to indicate more a4b7 molecules per cell); also, the authors added in the text in page 14 line 32 the word 'cumulatively' ("cells cultured in the presence of RAR568 cumulatively expressed more a4b7 than those cultured in the presence of ATRA"). This statement still indicates that each cell expresses more a4b7 molecules. As mentioned in the first revision, this is not the case. What Figure 3b indicates is that there are more cells expressing a4b7 in the RAPA+RAR568 group, and in terms of a4b7 expression, it is a homogenous population.

The two sentences have been amended to reflect that there is a higher proportion of a4b7 expressing cells when treated with RAR568. The word cumulatively has been removed.
This reviewer appreciates adding a Supplemental Figure 3. To maintain a visually pleasant and publishable figure for this journal, please do not stack the dot plots, try to align them and make them all of them of the same size. May be arrange the figure horizontally instead of vertically to have more space.

The plots in supplemental figure 3 have been aligned horizontally and resized for clarity.

Figure 2 is too crowded, difficult to read, the x axis legend in a graph in panel a) is cut off. It may be better to have less graphs and send the others as supplementary data.

CCR9 and inactive CD data has been moved to supplementary Figure 2. The plots have been resized for clarity and the x-axes re-labelled.

Page 14, line 8, please change the font to italics.

The font has been italicised

Page 15, line 22 change Fig 3f to Figure 3f

Fig 3f has been changed to Figure 3f

Figure 4, there is not mention in the general body of the text about the differential gene transcription between RAR568 and ATRA treated cells. Also, please standardize the axis fonts in the volcano plots. Fonts are different sizes.

A sentence has been added to the results regarding the comparison of differential gene transcripts between RAR568 and ATRA treated cells. The axis fonts in the volcano plots have been standardised.

Reviewer 2: The authors have addressed most of the reviewers comments. I congratulate them on this very innovative work.
Correction of Defective T-regulatory Cells From Patients With Crohn’s Disease by Ex Vivo Ligation of Retinoic Acid Receptor Alpha

Rimma Goldberg1, 2, 3, Cristiano Scotta2, Dianne Cooper4, Einan Nissim-Eliraz7, Eilam Nir7, Scott Tasker2,3, Peter M. Irving1, Jeremy Sanderson1, Paul Lavender5, Fowzia Ibrahim8, Jonathan Corcoran6, Toby Prevost6, Nahum Y. Shpigel 7, Federica Marelli-Berg4, Giovanna Lombardi2 & Graham M. Lord2,3,10

1 IBD Unit, Department of Gastroenterology, Guy’s and St Thomas’ NHS Foundation Trust
2 School of Immunology and Microbial Sciences, King’s College London
3 National Institute for Health Research Biomedical Research Centre, Guy’s and St Thomas’ NHS Trust and King’s College London
4 William Harvey Research Institute, Queen Mary University of London, London, United Kingdom,
5 Department of Respiratory Medicine and Allergy, King’s College London,
6 Wolfson Centre for Age Related Diseases, King’s College London,
7 Koret School of Veterinary Medicine, Hebrew University of Jerusalem, Rehovot, Israel
8 Department of Rheumatology, King’s College London School of Medicine, Weston Education Centre, King’s College London
9 Imperial Clinical Trials Unit, Imperial College London, London UK
10 Correspondence to graham.lord@kcl.ac.uk;

Lord Lab - 5th Floor, Tower Wing, Guy’s Hospital
London UK, SE1 9RT

Author Contributions

RG was responsible for study content and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, statistical analysis and obtaining funding. CS and DC contributed to acquisition of data and critical revision of manuscript for important intellectual content. EN-E, EN, ST contributed to acquisition of data. PI, JS, PL, JC contributed to acquisition of data, analysis and interpretation of data and critical revision of the manuscript for important intellectual content. FI and TP contributed a detailed review of the statistical methods. NS, FM-B and GL contributed to acquisition of data, critical revision of the manuscript for important intellectual content and study supervision. GML defined the study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, obtaining funding and overall study supervision.
Grant Support

The authors acknowledge financial support from the Department of Health via the National Institute for Health Research (NIHR) comprehensive Biomedical Research Centre award to Guy’s & St Thomas’ NHS Foundation Trust in partnership with King’s College London and King’s College Hospital NHS Foundation Trust. Additional support for some of the initial *in vitro* culture work and the *ex vivo* patient sample assays was provided by Litwin IBD Pioneers Funding Program at the Crohn’s and Colitis Foundation (grant number 504039), the Freemason’s Grand Charity (grant number SPG00268) and the Rosetree’s Trust (A1219).

Conflicts of Interest

JC holds a composition of matter patent for RAR568. RG, CS, EN-E, EN, ST, PMI, JS, PL, NYS, FM-B, GL and GML have no conflicts of interest to declare.
Abstract:

**Background & Aims:** Crohn's disease (CD) is characterized by an imbalance of effector and regulatory T cells in the intestinal mucosa. The efficacy of anti-adhesion therapies led us to investigate whether impaired trafficking of T-regulatory (Treg) cells contributes to the pathogenesis of CD. We also investigated whether proper function could be restored to Treg cells by ex vivo expansion in the presence of factors that activate their regulatory activities.

**Methods:** We measured levels of the integrin α4β7 on Treg cells isolated from peripheral blood or lamina propria of patients with CD and healthy individuals (controls). Treg cells were expanded ex vivo and incubated with rapamycin with or without agonists of the retinoic acid receptor alpha (RARA) and their gene expression profiles were analyzed. We also studied the cells in cytokine challenge, suppression, and flow chamber assays and in SCID mice with human intestinal xenografts.

**Results:** We found that Treg cells from patients with CD express lower levels of the integrin α4β7 than Treg cells from control patients. The pathway that regulates expression of integrin subunit alpha is induced by retinoic acid (RA). Treg cells from patients with CD incubated with rapamycin and an agonist of RARA (RAR568) expressed high levels of integrin α4β7, as well as CD62L and FOXP3, compared to cells incubated with rapamycin or rapamycin and ATRA. These Treg cells had increased suppressive activities in assays and migrated under conditions of shear flow; they did not produce inflammatory cytokines and RAR568 had no effect on cell stability or lineage commitment. Fluorescently labelled Treg cells incubated with RAR568 were significantly more likely to traffic to intestinal xenografts than Treg cells expanded in control medium.

**Conclusions:** Treg cells from patients with CD express lower levels of the integrin α4β7 than Treg cells from control patients. Incubation of patients’ ex vivo expanded Treg cells with rapamycin and an RARA agonist induced expression of α4β7 and had suppressive and migratory activities in culture and in intestinal xenografts in mice. These cells might be developed for treatment of CD. clinicaltrials.gov no: NCT03185000

**KEY WORDS:** IBD, tissue engineering, cell therapy, immune regulation
Background

CD is a chronic, immune-mediated inflammatory bowel disease (IBD) with no known cure, resulting in significant morbidity. Goals of therapy include resolution of symptoms and mucosal healing. However, many patients have sub-optimal responses to currently available therapies. This represents a significant unmet medical need. There is good evidence from both genetic and functional studies implicating defective Treg function in the pathogenesis of inflammatory bowel disease 1-4.

The maintenance, or indeed loss, of intestinal homeostasis hinges on the balance between inflammatory effector T-cells (Teff), which have been implicated in auto-immunity and transplant rejection, and a population of immunoregulatory T cells (Treg)5-7. Tregs are a unique subset of CD4+ T-cells with powerful immunosuppressive action. They are defined by expression of the master transcriptional regulator FOXP3 and a set of key surface markers 8-10. Tregs serve to limit immune mediated pathology and mice, or humans, lacking functional Tregs develop severe multisystem inflammatory disease, including chronic intestinal inflammation (IPEX syndrome) 11.

We have established a clinical protocol for GMP (Good Manufacturing Practice) compliant large scale expansion of regulatory T cells from patients on the waiting list for liver and kidney transplants 12-14. Similar efforts have been made by other groups in diseases such as graft vs host disease and Type 1 Diabetes 5, 7, 15, 16. Recent advances in therapy for IBD have focused on T cell trafficking and more specifically, the diversion of effector T cells from the inflamed gut by blocking the gut specific trafficking molecule integrin α4β717. The efficacy of this therapy would suggest that trafficking of lymphocytes to the inflamed gut is a key step in the pathogenesis of CD. Current reports suggest that there is no defect in Treg trafficking in patients with CD 18 and that there is indeed a greater number of CD4+FOXP3+ cells in the lamina propria of CD patients compared to healthy controls (HC) 19. However, considerable evidence exists to support the hypothesis that the Tregs present in the lamina propria are locally induced and can develop IL17 secreting capabilities under pro-inflammatory conditions, which may reduce their suppressive capacity 20, 21. We thus sought to assess whether an imbalance in regulatory and effector T cells committed to traffic to the gut existed in CD. Treatment with highly suppressive, lineage committed...
autologous ex vivo expanded Tregs, that are not skewed to produce IL-17 under pro-inflammatory conditions and are licensed to traffic to the gut would aim to correct such an imbalance.

We have previously shown that Tregs purified from peripheral blood (PB) of CD patients play a critical role in controlling both phenotype and expansion of auto-reactive T cells. Retinoic acid (RA) regulates the expression of the primary gut homing integrin α4β7 and we have previously defined the mechanisms by which RA controls the stability of T cell lineage commitment. We have also shown that RA can induce the expression of α4β7 on normal (HC) Tregs following in vitro culture.

RA is effective at inducing the expression of integrin α4β7 and has been suggested to have an effect on improving Treg suppressive ability. However, the stabilizing effect of all-trans retinoic acid (ATRA) on Tregs has been found to be transient and serum dependent, and there are ongoing concerns about the ability of retinoic acid to also skew Tregs towards a pro-inflammatory phenotype. Additionally, ATRA binds to the retinoic acid receptors (RARA, β, and γ) with similar affinity and their activation in the presence of this ligand is relatively non-selective. Therefore, all RARs and RXRs will be activated within the cell, some of which may be associated with adverse off-target effects. Thus we focused our attentions on using a specific agonist of RARA (RAR568) to induce the expression of integrin α4β7. This RARA agonist has a selectivity profile against human RARs with an EC50 of 0.59nM and 290-fold greater selectivity for RARA over RARβ and >13,000 fold selectivity over RARγ. RAR568 was also chosen due to negative cytotoxicity and genotoxicity screens when compared to other lead compounds and commercially available RARA agonists.

We show that ex vivo expanded Tregs derived from patients with CD retain optimal suppressive ability and phenotypic stability when treated with RAR568 compared to standard culture conditions (IL2 and Rapamycin). Additionally, we show that RAR568 has superior efficacy compared to ATRA by virtue of selective ligation of RARA. We also demonstrate superior gut homing of RAR568 treated Tregs using a dynamic in vitro system as well as in a humanized xenograft mouse model.
Materials and Methods

Patient Samples

CD PBMCs and tissue samples were obtained from patients attending endoscopy and outpatients at Guy’s and St Thomas’ NHS Trust. Ethics approval for human blood and tissue collection was obtained from NRES Committee – London Riverside (REC reference: 15/LO/0151) and Guy’s and St Thomas’ NHS Trust R&D (R&D REF: RJ115/N122)

Cell Culture Media and Buffers

“Complete X-VIVO-15” (Lonza, Walkersville, MD) was used for ex vivo Treg expansion, Treg cytokine challenge experiments and Treg suppression assays. This was supplemented with 100nM or 10nM Rapamycin and all-trans retinoic acid (ATRA) 2μM or 1nM, or Rapamycin and RAR568 1nM.

Other experiments were performed in RPMI 1640 medium (PAA Laboratories, Pasching, Austria) 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% foetal calf serum (all PAA).

CD4 Isolation and Cell Sorting

Peripheral blood mononuclear cells (PBMCs) were isolated via Ficoll-Paque. CD4⁺ cells were enriched by MACS enrichment as per manufacturer’s instructions. CD4⁺ cells were FACS sorted (BD FACSaria) into CD4⁺CD25highCD127lowCD45RA⁺ and effector T cell (CD4⁺CD25⁻) populations.

LPMC isolation
Colonic biopsies collected from CD patients and HC were washed in HBSS containing 1mM EDTA. Samples were then digested using Collagenase Ia (Sigma) 1mg/ml and DNAse I (Roche) 1ul/ml. Following digestion, cells were passed through a 100um cell filter and counted.

**In vitro Treg expansion**

FACS-sorted Treg populations were plated at 1 x 10^6 or 0.5 x 10^6 in X-VIVO-15 medium and activated with anti-CD3/anti-CD28 coated beads (Dynabeads®, Invitrogen, Paisley, UK) at 1:1 bead: cell ratio. Rapamycin was added at day 0 of culture at a final concentration of 100nM +/- ATRA 1nM or RAR568 1nM. rhIL-2 (Proleukin®, Novartis, Camberley, UK) 1,000 IU/ml was added at day 5 of culture. Cells were re-stimulated every 10-12 days and expanded for a total of 24-30 days. The phenotype and suppressive ability was assessed at the end of the culture period.

**Assessment of Treg suppressive ability**

Effector T cells (Teff) were labeled with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) according to standard protocols. Cells were washed with phosphate buffered saline (PBS) to remove excess protein. Cells were then incubated with a 1μM CFSE solution in the dark at room temperature for 4 minutes. The reaction was then quenched with 9ml of complete medium.

Teff were activated with anti-CD3/anti-CD28 micro beads at a bead: Teff ratio of 0.02:1. 1 x 10^5 Teff were then cultured either alone or with Tregs in serial dilutions. The ratios of Teff: Treg were 1:1, 2:1, 4:1, and 8:1. This was done in X-VIVO-15 and proliferation rates were assessed by flow cytometry after 5 days incubation.

Percentage suppression (S) of proliferation was calculated using the following formula:

\[ S = 100 - ((c/d) \times 100) \]

\[ c = \text{percentage of proliferating precursors in the presence of Tregs} \]
Flow Cytometry Analysis

Flow cytometry panels have been designed to assess the subtypes of regulatory T cells in patients with CD as well as their expression of gut homing molecules. Gating was performed based on natural populations when assessing for CD4^+CD25^{high}CD127^{low} populations, as well as for CD45RA^+ Treg populations. Additionally, to minimize bias in the assessment of expression of gut homing markers and transcription factors, a fluorochrome minus one (FMO) panel was added for each marker of interest in each experiment that was performed.

Ibidi Flow Chamber Experiments

Ibidi μ-Slides VI^0.4 were coated with recombinant human MAAdCAM-1 (R&D Systems), at a concentration of 10μg/ml and incubated overnight.

Cells from two CD patients that had previously undergone ex vivo expansion under two parallel conditions (Rapa and Rapa+RAR568) and had been frozen in liquid nitrogen were defrosted and rested overnight. Rested cells were then activated with rhCCL25 (R&D systems) and passed through the coated Ibidi flow chamber at a rate of 1 dyne/cm^2. The total number of cells, as well as those rolling, adherent and crawling was quantified from six randomly selected fields of view per treatment.

Estimation of Cytokine Concentrations

Cytokine concentrations were measured using the Ready-SET-Go sandwich ELISA kits from eBioscience.

Assessment of IL-17 and IFNγ production under pro-inflammatory conditions

Ex vivo expanded Tregs were activated with CD3/CD28 beads at a 1:20 ratio and cultured at 10^6 cells/ml in X-VIVO for 5 days at 37°C/5% CO2, supplemented with the following cytokine cocktail: 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), IL-21 (25 ng/mL), IL-23 (25 ng/mL) (all R&D Systems). Supernatant IL-17 and IFNγ concentrations were measured by ELISA.

**C.B-17 SCID mouse human intestinal xenograft model**

The C.B-17 SCID mouse human intestinal xenograft model has been previously described28, 29. 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). Tregs were labeled with CFSE (Invitrogen) prior to transfer, as per manufacturer’s instructions. Xenografts were processed as per LPMC digestion protocol. CFSE positive cells were detected by flow cytometry. Additionally, CFSE positive cells were detected by immunofluorescence on frozen sections from treated xenografts.

**Immunofluorescent Staining**

Fresh xenograft sections were fixed and stored in OCT. Fixed cryostat sections were blocked with 20% FCS and stained with rat anti human CD45 (Invitrogen) and mouse antihuman FOXP3 (Biolegend), followed by donkey anti-rat AF594 (Invitrogen) and donkey anti-mouse NL637 (RnD Systems). Negative controls were obtained from sections from xenografts that did not receive Treg transfer.

**Statistical Analysis**

Flow cytometric data were analysed with FlowJo 10.4.2 for MacOsX. Statistical analysis was performed with GraphPad Prism 6.0h for MacOsX. Continuous data are presented as mean ± standard deviation for continuous (approximately) symmetrically distributed variables; as medians and interquartile ranges for skewed variables. Comparison of means and / or medians were performed using paired parametric and nonparametric tests as appropriate (paired t test or Wilcoxon signed rank test, respectively). For comparison of matched values (such as Treg and Teff in the same patient) the Wilcoxon matched pairs signed rank test was used. Mann Whitney Test with a two tailed p value was used to determine significance level in all unmatched values (such as comparisons between CD and HC). The CD and
HC groups were matched by age and gender. A p value of less than 0.05 was considered statistically significant throughout.

Gene array analyses were carried out using Partek® software with a 1-way ANOVA to assess for differential gene expression.
Results

Patients with CD have a lower proportion of Tregs licensed to traffic to the gut than Teffs

Peripheral blood samples were taken from 64 CD patients attending outpatient clinics, the IBD infusion unit or endoscopy at Guy’s and St Thomas’ NHS Trust and 41 HC (patients attending outpatients for the management of irritable bowel syndrome, or undergoing colonoscopy for polyp surveillance/positive fecal occult blood test). Table 1 outlines patient demographics. HC were matched for age and sex (Table 1). Colonic biopsies were also obtained from 19 CD patients and 22 HC. PBMCs and LPMCs were isolated using standard Ficoll density gradient and DNase/collagenase digestion protocols respectively. Tregs were identified as CD4+CD25+CD127+FOXp3+. Teff were identified as the CD4+CD25-CD127-FOXp3+ population (gating strategy is shown in Figure 1a). Significantly more Teff in the peripheral blood expressed integrin β7 compared to Tregs (27.91 ± 18.19 vs 10.81 ± 7.919, p<0.0001). In addition to a reduced percentage of cells expressing β7, there was also a difference in expression per cell, as assessed by the mean fluorescence intensity (MFI) of β7 (932 ± 800.7 vs 575.4 ± 509.4, p<0.0001) (Representative Flow plots Figure 1a, Summary data Figure 1b). Similarly, there was a significantly higher proportion of β7 positive Teff than Treg in the lamina propria of CD patients (30.94 ± 26.4 vs. 23.75 ± 25.56, p=0.0004). This difference was again associated with a reduced expression per cell of β7 on Tregs as assessed by MFI (p<0.05) (Fig 1b). When compared to HC, there was a lower proportion of α4β7 positive Tregs in the circulation of patients with CD compared to HC, representative flow plots of α4β7 gating in Supplementary Figure 1, summary data Figure 2a (5.26, 3.61-8.73 vs 6.75, 5.25-9.65, p<0.05). This difference was even more profound when we compared CD patients with active disease only to HC (4.51, 3.8-7.05 vs. 6.71, 5.1-9.65, p=0.0063) (Fig 2a), (inactive CD data Supplementary Figure 2). The proportion of α4β7+ circulating Tregs was not affected by thiopurine or biologic treatment (Supplemental Figure 2). Given the efficacy of the anti-α4β7 monoclonal antibody Vedolizumab in CD, we sought to examine the balance between regulatory and effector T cells in the lamina propria in CD patients and to compare this to HC. There was a significantly higher proportion of α4β7+ Teff compared to Treg in the lamina propria of patients with CD (30.94 ± 26.40 vs. 23.75 ± 25.56, p=0.0016) (Fig 2b). No such difference existed in HC (Fig 2b). When compared to HC, the lamina propria of CD patients had a significantly increased proportion of α4β7+ Tregs (14.2, 6.29-
30 vs 6.38, 3.62-10.32, p=0.049). However, there was an even greater increase in the proportion of α4β7+ Teff in CD compared to HC (21.30, 14.7-34.1 vs 5.05, 2.76-10.8, p=0.0002) (Fig 2b), suggesting an impaired balance of Teffs to Tregs in diseased tissue.

To ascertain whether the reduction in α4β7+ circulating Tregs was an isolated impairment or the result of a global Treg deficiency, we analyzed the proportion of circulating Tregs in patients with CD and compared it to HC. We found that there was no difference in the percentage of circulating Tregs between CD patients and HC (7.24, 6.00-9.07 vs 6.52, 5.65-7.43, p=ns). Patients with CD however did have a significantly lower proportion of circulating Teff than HC (90.95, 88.20-92.58 vs 92.2, 91.00-93.5, p<0.05). When CD patients were separated based on disease activity, those with active disease had a significantly higher proportion of circulating Tregs compared to HC (7.43, 6.26-9.25 vs 6.52, 5.65-7.44, p<0.05) (Fig 2c). Thus we conclude that the decrease in α4β7+ circulating Tregs is not due to a global Treg deficiency in CD. These findings are contrary to previous reports that there is an overall increase in the proportion of circulating Tregs in CD, which contracts during periods of disease activity but still remains higher than the proportion of circulating Tregs in HC 19, 30, 31.

In order to assess whether the defect was specific for α4β7 expression or extended to other major gut trafficking molecules, we assessed the expression of the intestinal homing chemokine receptors GPR15 and CCR9 on Treg and Teff of patients with CD and compared these to HC. There was no difference between the proportion of GPR15+ Treg in circulation between patients with CD and HC. However, there was a significantly higher proportion of GPR15+ circulating Teff in patients with CD (2.11, 0.86-5.93 vs 1.06, 0.43-3.45 p<0.05) (Fig 2d). On assessment of CCR9 expression, we found that significantly more Treg (1.82, 0.73-4.5 vs 1.23, 0.67-2.09 p<0.05) and Teff (1.55, 0.43-17.3 vs 0.49, 0.28-1.24 p=0.0004) expressed CCR9 in patients with CD compared to HC (Supplementary Figure 2).

We then assessed the proportions of GPR15+ cells in the lamina propria. CD patients had a higher proportion of GPR15+ Treg than Teff (20.37±17.03 vs 12.83±10.77 p=0.0039). CD patients had similar proportions of GPR15+ Treg in the lamina propria as HC, however there was a significant difference in the proportion of GPR15+ Teff (9.61, 4.56-18.8 vs 4.21, 3.02-8.27, p<0.05) (Figure 2e).

To complete our understanding of the dynamics of gut homing Treg and Teff in CD we assessed whether the proportions of α4β7+ Treg and Teff were affected by thiopurine or anti-TNF therapy. Neither
thiopurine nor anti-TNF therapy appeared to affect the proportions of Treg or Teff licensed to traffic to the gut (Supplementary Figure 2), implying that trafficking and pro-inflammatory pathways are mechanistically separable.

**RAR568 induces α4β7 more efficiently and robustly than ATRA**

To address the balance between regulatory and effector T cells in the lamina propria of patients with CD, we sought to develop a highly suppressive, phenotypically stable population of autologous ex vivo expanded Tregs that were licensed to traffic to the gut by high level expression of α4β7. These cells could then be utilized as an autologous cell based therapy for CD. We compared the efficacy of ATRA (all trans retinoic acid) with RAR568 at inducing α4β7, to determine which agent would be more suitable for downstream application in a clinical trial of Treg therapy for CD. As previously defined, our standard culture conditions utilized the CD4+CD25hiCD127loCD45RA+ naïve Treg subset, cultured in the presence of rapamycin (RAPA) and high dose IL2. When compared to cells cultured under standard conditions (Rapa), a higher proportion of cells cultured under standard culture conditions but with the addition of RAR568 (Rapa+RAR568) expressed α4β7 (95.9 ± 1.93 vs 5.947 ± 3.18, p<0.0001; gating strategy is shown in Figure 3a). Additionally, a higher proportion of cells cultured in the presence of RAR568 expressed α4β7 than those cultured in the presence of ATRA (95.89 ±1.93 vs 74.21 ± 25.89, p=0.024; Figure 3b). The efficacy of RAR568 to induce the expression of integrin β7 was apparent at much lower concentrations, when compared to ATRA (Figure 3c), with an EC50 of 0.01nM for RAR568, versus an EC50 for ATRA of 1.5nM. Representative flow plots for the RAR568 and ATRA dose titrations are shown in Supplementary Figure 3. Importantly, the standard deviation of α4β7 expression for cells cultured in the presence of RAR568 was much lower than those cultured in the presence of ATRA (1.93 vs. 25.89), which has important implications for downstream quality control when these agents are employed for cell based therapy. The expression of CD62L, required for homing to the lymph nodes and the effective interaction between integrin α4β7 and its ligand MAdCAM-1 was maintained following ex vivo expansion, irrespective of retinoid treatment (Supplementary Figure 4).

**Treatment with RAR568 does not affect Treg stability or suppressive ability**
Cells expanded in the presence of RAR568 express high levels of FOXP3 (96.99% ± 3.51). This value is not significantly different to cells expanded under standard conditions (96.03 ± 6.18) and those expanded in the presence of ATRA (86.15 ± 19.88) (Figure 3d). However, Tregs expanded in the presence of ATRA showed a less consistent level of FOXP3 expression (range 40.2-99.7, SD 19.88), when compared to those grown in the presence of RAR568 (range 91.5-99.8, SD 3.51).

Cells expanded in the presence of RAR568 were highly suppressive even at the lowest (8:1) titration. Conversely, cells grown in the presence of ATRA became less suppressive at the lowest titration (p<0.005) (Figure 3e). Tregs expanded ex vivo in the presence of either ATRA or RAR568 did not produce IL-17 or IFNγ following pro-inflammatory cytokine challenge (Figure 3f).

Treatment with RAR568 avoids off target RARγ effects and skewing to a pro-inflammatory phenotype

Gene expression analyses were performed on Tregs from CD patients expanded in the presence of Rapa + ATRA, Rapa + RAR568 or rapamycin only (n=3 in each group). A key difference between the ATRA-treated cells and RAR568-treated Tregs was a significant increase in transcripts for CD161 in the ATRA treated group compared to rapamycin only (p<0.05). CD161 has previously been described as a marker of Th17-like Tregs 32. This was not observed in the RAR568-treated group. Additionally, Tregs treated with ATRA had a > 2 fold increase in the expression of STAT4, IL18R1, CD38 and GPR174 (p<0.05) (Figure 4a). IL18R1 and STAT4 are responsible for Th1 lineage commitment and IFNγ production, both have been independently identified as IBD disease related polymorphisms on GWAS 33-36. CD38 has been identified as a marker associated with mature T cells, signaling reduced proliferation, but an increased ability to produce pro-inflammatory cytokines such as IFNγ 37. Ligation of GPR174 negatively affects Treg accumulation and function 38. No clear difference in transcripts for canonical pathways were identified when ATRA treated cells were compared to RAR568 treated cells (Figure 4a).
To assess for off target RARγ effects, we compared the gene expression profiles of RAR568 and ATRA treated cells to a published dataset of RARγ target genes \(^3\). Eleven out of 94 RARγ target genes were upregulated in the ATRA-treated samples, compared to only one in the RAR568-treated samples (Figure 4b). Given the efficacy at inducing α4β7 and lack of off target effects, RAR568 fulfilled the target product profile for an agent that could be used for ex vivo Treg expansion for cell based therapy purposes. Therefore, we probed this effect of RAR568 on Tregs from CD patients in functional in vitro and in vivo trafficking assays.

**The Induction of α4β7 is functionally relevant in vitro and in vivo**

In order to assess the physiological relevance of the induction of α4β7 expression by RAR568 and ATRA, treated and untreated Tregs from CD patients were passed through a MAdCAM-1 coated flow chamber (experimental set up Supplementary Figure 5). The total number of cells adherent to the chamber, as well as their stages of rolling, adhesion and crawling, were compared to cells that were expanded under standard conditions. There were significantly more total cells as well as cells at each condition of migration when RAR568-treated cells were passed through the chamber compared to their counterparts expanded under standard conditions. RAR568 was significantly more effective in inducing these migratory steps than ATRA. All stages of cell migration were blocked when the cells were treated with a monoclonal antibody to integrin α4β7 (Vedolizumab; Figure 5). Videos of cell trafficking are available in supplementary material. This demonstrates that not only is the induction of α4β7 relevant in vitro, but that it is dependent on the interaction of α4β7 and MAdCAM-1 under conditions of physiological shear flow, with maximum interaction induced by the selective ligation of RARγ.

To assess if the induction of α4β7 was functionally relevant in vivo, cells treated with RAR568 or cells expanded under standard conditions were fluorescently labelled and transferred into a SCID mouse xenografted with human fetal intestinal small bowel by intra-venous injection. Inflammation was induced in the xenografts with Mycobactrium Avium Paratuberculosis (MAP). It has previously been demonstrated that MAP can infiltrate into the xenografts and induce inflammation detectable histologically and by the production of pro-inflammatory cytokines \(^2\). Experimental design is illustrated
in Figure 6a. Representative sections from inflamed and un-inflamed xenografts are shown in Supplemental Figure 6b, highlighting lymphocyte infiltration in inflamed xenografts.

RAR568-treated cells were significantly more likely to traffic to xenografts 72 hours following Treg transfer compared to Tregs expanded under standard conditions (p=0.0056; representative FACS plots Figure 6b, cumulative data Figure 6c). The difference in Treg trafficking to the xenografts was further increased by the presence of inflammation; significantly more RAR568-treated cells trafficked to the inflamed xenografts than those grown under standard conditions (p=0.0095; Figure 6d). The presence of CFSE labelled FOXP3+ Tregs was also evident in immunofluorescent labelled cryosections from the inflamed xenografts of mice which had received the RAR568-treated cells (Figure 6g), but not in the xenografts of controls or those who received Rapa-treated cells (Figure 6e-f). Given the concerns that adoptively transferred human cells may be located outside the gastrointestinal system, we assessed Treg trafficking to the spleen. There were no human CD45 positive cells found in the spleens of mice treated with cells either grown under standard conditions or those treated with RAR568 (Supplemental Figure 6a).

Discussion

Contrary to previous reports, we found integrin β7 to be more highly expressed on effector T cells in the peripheral blood of CD patients rather than on regulatory T cells. Furthermore, patients with active CD have a significantly lower proportion of circulating α4β7+ Tregs than their HC counterparts, and a significantly higher proportion of Teff licensed to traffic to the gut. This deficiency does not affect all gut homing receptors, with CD patients having a comparable proportion of GPR15+ Treg in the circulation and a higher proportion of CCR9+ Tregs than their HC counterparts. The reduction in the proportion of α4β7+ Tregs is also not a function of a global reduction in the proportion of circulating Tregs, as there is a higher proportion of circulating Tregs in patients with active CD compared to HC. Thus, while the absolute difference in α4β7+ Treg proportions between CD patients and HC controls is modest, the fact that this difference does not exist with any other marker in addition to the fact that the α4β7 pathway is already being therapeutically exploited with monoclonal antibodies for the treatment of CD, would suggest that this difference is biologically significant. The Treg/Teff imbalance is also apparent in the lamina propria of CD patients. There is a higher proportion of α4β7+ Teff in CD, whereas in HC there is an equal balance between α4β7+ Treg and Teff. A limitation of this finding was that the HC LPMC donors
were slightly older than their CD counterparts; this is an unavoidable function of the patients who present for a colonoscopy in the absence of CD. Studies of Tregs in older subjects have suggested an increase in nTreg and a decline in iTreg\textsuperscript{40}. Whilst this may explain a better balance between Treg and Teff in HC, it does not explain the lower Treg and significantly lower Teff numbers seen in HC compared to CD patients. The imbalance between gut homing Treg and Teff could be a potential pathogenic mechanism underlying the disease. Thus it would follow that by therapeutic expansion of the circulating population of Tregs that is licensed to home to the inflamed bowel, we could re-set the balance between regulatory and effector T cells in this organ which might contribute to disease resolution.

The profound and consistent induction of α4β7 by RAR568 confers Tregs with the ability to traffic to the diseased organ for which they are therapeutically destined. A far more robust induction of α4β7 by RAR568, a highly specific agonist of RARA, is consistent with the fact that it is the downstream function of this receptor, rather than RARβ or RARγ\textsuperscript{41}. Although standard retinoic acid (ATRA) is somewhat effective at inducing the expression of integrin α4β7, there are ongoing concerns about the ability of ATRA to also skew Tregs towards a pro-inflammatory phenotype\textsuperscript{13,25}. ATRA can interact with RARA, RARβ and RARγ, however it has a much higher affinity for RARγ. The higher standard deviation observed in FOXP3 expression, IL17 and IFNγ production when cells are treated with ATRA compared to RAR568 suggests that ATRA’s previously noted ability to skew cells towards a pro-inflammatory phenotype may be due to activation of RARγ and could therefore be avoided when using a RARA specific agonist. It could be argued that the observed heterogeneity in FOXP3 expression is simply due to sample purity. However, given that the expansion in the presence of ATRA or RAR568 took place side by side from a sample that was derived from the same donor and therefore underwent an identical flow sorting protocol, this possibility is less likely.

The increased expression of CD161 transcripts in ATRA-treated cells demonstrates that they may be skewed towards a Th17-producing phenotype. As an immune imbalance skewed towards a Th1 response has been implicated in the pathogenesis of CD\textsuperscript{42}, it would be imprudent to introduce an expanded cell population that has the ability to secrete IL17 into the inflamed gut of CD patients. Similarly, the induction of STAT4 and IL18R1 and CD38 on ATRA treated cells, may confer them with an increased ability to skew to a Th1 like phenotype under pro-inflammatory conditions and secrete
IFNγ. Given the aim of treating cells with ATRA in vitro is to induce migration to the gut, the induction of GPR174 by ATRA, which impedes Treg migration, would hinder that aim. The near complete lack of induction of RARγ target genes in the RAR568-treated cells further confirms the alpha selectivity of the agonist thus allowing us to feel confident that we will not see any off target effects when it is used for large scale Treg manufacture in clinical trials.

Patient-derived Tregs grown under standard conditions do express low levels of α4β7. However, they displayed negligible levels of rolling, adherence and activation when presented with MAdCAM-1 in the Ibidi flow chamber experiments. By contrast, RAR568-treated cells interacted very efficiently with this ligand and to a much greater extent than cells treated with the non-selective RAR agonist ATRA. This suggests that high levels of α4β7 expression are required in order for a cell to progress through the stages of endothelial migration and also that integrin activation in addition to cell surface expression is critical. The complete blockade of interaction between MAdCAM-1 and RAR568-treated Tregs in the flow chamber by treatment with Vedolizumab proves that this process is dependent on α4β7. Taken together, we propose that when RAR568-treated cells are transferred into a pro-inflammatory environment, they will home to tissues where MAdCAM-1 is upregulated, such as the inflamed gut in CD.

To confirm further that ex vivo expanded Tregs remain viable in vivo and have the ability to migrate to the inflamed bowel, we transferred cells grown either under standard conditions or in the presence of RAR568 into a SCID mouse xenografted with human foetal small bowel. The grafts in this model are known to express MAdCAM-1 and develop into tissue that is functionally and morphologically identical to normal adult human gut. MAP was chosen to induce inflammation in the xenografts as it causes granulomatous inflammation, which provides a suitable model for the inflammation occurring in CD. Furthermore, the ability of MAP to invade goblet cells and induce inflammation in this model has been previously described.

Significantly more RAR568-treated cells found their way into the xenografts, particularly when inflammation was induced. We can therefore surmise that by inducing the expression of MAdCAM-1, the inflammatory process in this model draws more of the RAR568-treated cells, which are uniformly
α4β7+, to the inflamed xenografted human gut. This parallels our in vitro findings and would suggest that after treatment with RAR568, Tregs will home to the inflamed gut when they are administered in upcoming trials of cell-based therapy for CD.

It has previously been suggested that the analysis of human Treg trafficking following intravenous administration of the cells in a mouse model is not feasible due to sequestration of cells in non target organs\textsuperscript{18}. However, the absence of human lymphocytes in the spleens of mice who received Treg transfer, suggests that after a 72-hour period, cells have had the opportunity to recirculate from secondary lymphoid organs and off-target tissues and direct themselves to the tissue which their pre-conditioning has programmed them for. Ultimately, a mouse model cannot fully recapitulate the complexity of the human system. Therefore, the final test of gut selectivity and indeed efficacy of these ex-vivo expanded Tregs will be when CD patients receive RAR568 treated, Deuterium labelled cells in the forthcoming trial of Treg therapy for CD (TRIBUTE - NCT03185000).

Conclusions
In conclusion, we have identified a primary defect in the number of Tregs expressing the gut homing molecule α4β7 in CD. Additionally, we have defined the optimal conditions for licensing highly suppressive and phenotypically stable autologous Tregs to traffic to the inflamed gut. These cells maintain their function both in vitro and in vivo and therefore form the optimal therapeutic option for adoptive cell based therapy of CD.

Table 1
Demographics of CD patients and HC included in the study. No significant difference between the mean age and gender proportions between CD and HC groups.

<table>
<thead>
<tr>
<th>Crohn's Disease Patients</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean)</td>
<td>40.35 (±11.55)</td>
</tr>
<tr>
<td>Female sex (%)</td>
<td>28 (44)</td>
</tr>
</tbody>
</table>
### Disease Distribution

| L1 (%)       | 12 (19) |
| L2 (%)       | 10 (16) |
| L3 (%)       | 40 (65) |

### Medical Therapy

| Biologic (%) | 31 (50) |
| Thiopurine (%) | 35 (56) |
| Vedolizumab (%) | 3 (5) |

### Disease Activity

| Active Disease (%) | 26 (42) |
| Evidence of Mucosal Inflammation (%) | 23 (37) |

### Healthy Controls

| Age (mean)       | 45 (±12.23) |
| Female sex (%)   | 27 (57) |
Figure Legends

Figure 1. Expression of gut homing molecules in CD. (a) Gating strategy to define Treg and Teff population and their expression of integrin β7 (b) Differential expression of integrin β7 in peripheral blood and colon of CD patients. Wilcoxon matched pairs signed rank test was used to determine statistical significance in all matched values. ***p<0.001, **p<0.005, *p<0.05, ns=p>0.05 were used throughout. (N=63 CD Peripheral Blood, N=20 CD colon)

Figure 2. a) Patients with Crohn’s Disease have significantly less α4β7 positive Treg in circulation compared to healthy controls (p=0.006). Mann Whitney Test with a two tailed p value was used to determine significance in all unmatched values. (N=56 CD Peripheral blood, N=41 HC Peripheral blood, N=24 active CD) (b) There is a higher proportion of α4β7+ Teff than Treg in the lamina propria of Crohn’s disease patients (p=0.001). There is no difference in proportions of α4β7+ Treg to Teff in healthy controls. (N=15 CD colon, N=16 HC colon) (c) Patients with active Crohn’s Disease have significantly more circulating Tregs than healthy controls (p=0.04). There is a reduced proportion of circulating Teff in CD vs HC (p=0.01 HC vs Active CD, p=0.03 HC vs Inactive CD). (N=64 CD, N=41 HC) (d) The colonic homing marker GPR15 is expressed on a greater proportion of Teff in CD compared to HC (p=0.04). (N=64 CD, N=41 HC) (e) Higher proportion of Treg and Teff express the small bowel homing molecule CCR9 (p=0.03 Treg, p=0.0004 Teff). (N=43 CD, N=37 HC). (f) Higher proportion Treg than Teff in CD colon express GPR 15 (p=0.0039, N=19). When compared to HC CD Teff express more GPR15 (p=0.02, N=19 CD, N=22 HC).

Figure 3. RARa is more efficient at inducing α4β7 during in vitro culture (a) Gating strategy to define α4β7 expression on CD25highCD127lowCD45RA+Tregs freshly isolated and following expansion (b) Cumulative data demonstrating significant and consistent induction of α4β7 in cultures treated with RAR568 (c) Dose response curve demonstrating greater efficacy of RAR568 at inducing expression of integrin β7 (d) FOXP3 expression is unchanged in Tregs expanded in the presence of retinoids compared to standard conditions. Figure 2cont. In vitro treatment with retinoids maintains suppressive ability and phenotypic stability (e) Suppression assay comparing cells expanded
in the presence of ATRA or RAR568 (f) Stability assay demonstrating that cells treated with retinoids maintain their phenotype under pro-inflammatory conditions

Figure 4. Treatment with RAR568 reduces off target retinoid effects. Genes upregulated with \( \geq 2 \) fold increase, with \( p \leq 0.05 \). Cells treated with RAR568 or ATRA compared to those treated with Rapamycin only. Gene expression compared against a published list of RAR\( \gamma \) target genes. (a) Volcano plot demonstrating increased expression of genes associated with pro-inflammatory T cell lineage in cells treated with ATRA (top panel) and more specific upregulation of \( \alpha 4 \) in cells treated with RAR568. (b) Increased expression of RAR\( \gamma \) target genes in cells treated with ATRA.

Figure 5. Induction of \( \alpha 4\beta 7 \) is functionally relevant \textit{in vitro}. (a) \textit{In vitro} trafficking assay demonstrating a significant improvement in RAR568 treated Treg crawling, rolling and adhesion when exposed to the \( \alpha 4\beta 7 \) ligand MadCAM when compared to either ATRA or Rapa only treated cells. (b) Cumulative data from N=3 trafficking assays.

Figure 6. Induction of \( \alpha 4\beta 7 \) is functionally relevant \textit{in vivo} (a) Experimental design: C.B17 SCID mice transplanted with human foetal small bowel that has matured over 12-16 weeks, have inflammation induced with Mycobacterium Avium Paratuberculosis (MAP) in the xenografts at day -3 prior to Treg transfer. Mice are injected with anti-asialo GM1 antibody at day -2 prior to transfer in order to deplete natural killer (NK) cells. On the day of Treg transfer, mice were treated with Tregs that were either expanded with Rapamycin alone or with the addition of RAR568. Mice also received 1000IU of rhIL-2 IP on the day of Treg transfer, to support the Treg in circulation. After three days in circulation, the presence of CFSE labelled Tregs was assessed by FACS in digested xenograft samples and immunofluoresence on frozen sections. (b) Representative FACS plots from Treg transfer into SCID mouse xenografted with human foetal small bowel, demonstrating the presence of CFSE labeled human Tregs in xenografts after transfer of either Rapa or Rapa+RAR568 treated cells. (c) Cumulative data from two independent experiments, N=5 Rapa, N=6 Rapa+RAR568 (d) Cumulative data demonstrating increased trafficking of RAR568 treated cells to inflamed xenografts.

Figure 5 cont. Induction of \( \alpha 4\beta 7 \) is functionally relevant \textit{in vivo}
(e) Control XG, no Tregs, (f) XG from mouse treated with Rapa Tregs (g) XG from mouse treated with Rapa+RAR568 Tregs

References


Figure 3

(a) Pre-Expansion vs Post Expansion

(b) ATRA vs RAR568 dose

(c) % Treg Expressing FOXP3

(d) % Cells Expressing β7

(e) Pro-Inflammatory Cytokine Cocktail: IL2, IL1, IL6, IL21, IL23, TGFβ

(f) IFNγ

(g) IL17
Figure 4.

(a) Greater expression in RAPA + ATRA Greater expression in RAPA + RAR568

(b) RARy Target Genes 83

RAR568 Treated Cells

ATRA Treated Cells

F2R
PLAGL1
ELOVL4
NUDT4
NUDT4P1
NRIP1
RAMP3
GAS1
Mras
COMT

RAR!
Target Genes 83

RARA Treated Cells

RAPA
RAR568
Figure 6.

(a) Human CD45+ CFSE

(b) Rapa Rapa+RAR568

Day 0 – CFSE labeled Rapa or RAR568 treated Treg IV and rhIL-2 IP

Day +3 Animals sacrificed and grafts processed

Human CD45+

CFSE

(c) %CFSE+ cells All

**p=0.0056

(d) %CFSE+ Inflamed vs Un-Inflamed

**p=0.0095  
p=ns

Day 0 – CFSE labeled RAPA or RAR568 treated Treg IV and rhIL-2 IP

(D) Left panel: Mature xenografts (circled) are visible subcutaneously on the dorsum of the mouse. Right panel: Dorsal skin has been removed in an anesthetized mouse to reveal the mucus filled xenograft in situ (right panel). Scale bars=1cm.
**Need to Know**

**Background:**
The CD45RA+ subset of T-regulatory cells derived from thymic tissues of patients with Crohn’s disease can be expanded in vitro to produce cells that can be used therapeutically. Expanded T-regulatory cells were previously shown to be safe and effective in prevention of graft vs host disease and in patients with type 1 diabetes. Intestinal tissues from patients with Crohn’s disease have an imbalance of inflammatory and anti-inflammatory immune cells, and agents that block immune cell trafficking are effective therapies.

**Findings:**
We found that in patients with Crohn’s disease, T-regulatory cells have defects in their ability to localize to the intestine. This defect can be corrected by activation of the retinoic acid receptor alpha on T-regulatory cells in ex vivo culture. We expended gut-specific T-regulatory cells ex vivo and found them to be immune suppressive and not produce inflammatory cytokines.

**Limitations:**
These studies were performed in cultured cells and mice, but are being tested in a phase 1 and 2 trials. Clinicaltrials.gov no: NCT03185000

**Implications for patient care:**
Cell-based therapy with gut specific ex vivo expanded T-regulatory cells might be used to correct the trafficking defect in patients with Crohn’s disease and restore the intestinal balance between regulatory and effector T cells.

**Lay Summary**
Patients with Crohn’s disease develop intestinal inflammation partly because of an imbalance between inflammatory and anti-inflammatory immune cells. We developed a method to expand anti-inflammatory T cells that travel to the gut and correct this imbalance.
Supplementary Figure 1. Gating strategy for $\alpha_4 \beta_7$ expression and $\beta_7$ MFI for CD and HC samples

HC
Treg $\alpha_4 \beta_7$

Treg $\beta_7$ MFI

CD
Treg $\alpha_4 \beta_7$

Treg $\beta_7$ MFI

$\alpha_4$ FMO

Teff $\alpha_4 \beta_7$

Teff $\beta_7$ MFI

Teff $\alpha_4 \beta_7$

Teff $\beta_7$ MFI

$\beta_7$ FMO
Supplementary Figure 2. Effect of CD disease activity, thiopurines and biologics on expression of gut homing molecules, CCR9 expression in peripheral blood.
Supplementary Figure 3: Rapa+RAR568 vs Rapa+ATRA dose titration

β7 FMO

Rapa (0uM)  1uM  0.1uM  0.01uM  0.001uM  0.0001uM  0.00001uM  0.000001uM

Rapa+RAR568

8.1%  99.5%  99.8%  99.5%  98.6%  93.6%  49.4%  31.8%

Rapa+ATRA

7.4%  93.8%  87.3%  82.8%  80.8%  37.5%  4.2%  8%

FSC-A

Integrin β7
Supplementary Fig 4. High expression of CD62L is maintained following expansion and is not affected by RAR568 treatment.
Supplementary Fig 5. Experimental set up for in-vitro trafficking experiments using MAdCAM-1 coated ibidi flow chamber.

MAdCAM-1 Coated ibidi Chamber

Defrosted post ex-vivo expansion

Rapa Tregs
Rapa+RAR568 Tregs
RAPA+RAR568 +Vedo
RAPA+ATRA

Flow Chamber System

Recording
Supplemental Figure 6. (a) Representative plots from spleens of mice treated with either Rapa or Rapa+RAR568 Tregs (b) Representative sections from inflamed and un-inflamed xenografts, demonstrating CD45 infiltration in inflamed xenograft. Panel A-F is the control xenograft, panel G-L is the MAP infected xenograft.
Supplementary Methods

RNA Extraction and Gene Arrays

1) RNA extraction was performed using Qiagen RNEasy mini/micro kits as per manufacturer's instructions. The samples were checked for RNA quality using the Agilent 2100 Bioanalyzer and quantified using the Nanodrop (ND-1000 Spectrophotometer). Samples which passed QC (RIN>8) were chosen such that input amount of each sample was 3 ng.

2) SPIA cDNA was generated using the "Ovation Pico WTA System V2" kit from Nugen, following the manufacturer's instructions.

3) The SPIA cDNA was subjected to a QC check to assess quality (Agilent 2100 Bioanalyzer) and quantity (Nanodrop ND-1000 Spectrophotometer) for the next stage.

4) The SPIA cDNA was fragmented and Biotin-labelled using the "Encore Biotin Module" from Nugen according to the manufacturer's instructions and passed through QC checks to assess fragmentation size (Agilent 2100 Bioanalyzer).

6) Hybridization cocktails were prepared of the fragmented labelled-cDNA according to Nugen's recommendations and hybridized at 45°C overnight in an oven.

7) The arrays were washed and stained using wash protocol FS450_0002 (Affymetrix protocol recommended for Human Gene 2.0 Arrays on the GeneChip Fluidics station 450).

8) The arrays were scanned using the Affymetrix GeneChip Scanner.

List of flow cytometry antibodies

<table>
<thead>
<tr>
<th>Marker</th>
<th>Flurophore</th>
<th>Cat No</th>
<th>Clone</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live Dead</td>
<td>350/450</td>
<td>L34962</td>
<td>N/A</td>
<td>Thermofisher</td>
</tr>
<tr>
<td>CD4</td>
<td>BUV737</td>
<td>564305</td>
<td>SK3</td>
<td>BD</td>
</tr>
<tr>
<td>CD25</td>
<td>PE</td>
<td>356104</td>
<td>M-A251</td>
<td>Biolegend</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HIL-7R-</td>
</tr>
<tr>
<td>CD127</td>
<td>BV786</td>
<td>563324</td>
<td>M21</td>
<td>BD</td>
</tr>
<tr>
<td>CD45RA</td>
<td>FITC</td>
<td>304148</td>
<td>HI100</td>
<td>Biolegend</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>------------</td>
<td>-----</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>GPR15</td>
<td>APC</td>
<td>025</td>
<td>367902</td>
<td>R&amp;D</td>
</tr>
<tr>
<td>CD49d</td>
<td>APC-H7</td>
<td>658332</td>
<td>9F10</td>
<td>BD</td>
</tr>
<tr>
<td>β7</td>
<td>BV605</td>
<td>564284</td>
<td>FIB504</td>
<td>BD</td>
</tr>
<tr>
<td>CCR4</td>
<td>BV510</td>
<td>359416</td>
<td>L291H4</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CCR9</td>
<td>Percp-Cy5.5</td>
<td>358906</td>
<td>L053E8</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD62L</td>
<td>BV421</td>
<td>104436</td>
<td>MEL-14</td>
<td>Biolegend</td>
</tr>
<tr>
<td>FOXP3</td>
<td>AF700</td>
<td>56-4776-41</td>
<td>PCH101</td>
<td>Ebioscience</td>
</tr>
<tr>
<td>FOXP3</td>
<td>FITC</td>
<td>11-4776-42</td>
<td>PCH101</td>
<td>Ebioscience</td>
</tr>
</tbody>
</table>
Click here to access/download

**Video (supplemental)**
Rapa.mov
Click here to access/download
Video (supplemental)
Rapa+RAR568.mov
Click here to access/download

**Video (supplemental)**
Rapa+RAR568+Vedo.mp4