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Short Communication

Isolation and expansion of thymus-derived regulatory T cells for use in pediatric heart transplant patients

Marco Romano[1], *Monica Sen*[1], *Cristiano Scottà*[1], *Rowa Y. Alhabbab*[2], *Andres Rico-Armada*[3], *Robert I. Lechler*[1], *Michael Burch*[3] *and Giovanna Lombardi*[1]

[1] MRC Centre for Transplantation, School of Immunology and Microbial Sciences, King's College London, Guy's Hospital, London, UK
[2] Applied Medical Sciences Department, Division of Immunology, King Abdulaziz University, Jeddah, Saudi Arabia
[3] Department of Paediatric Cardiology and Transplant Cardiology, Great Ormond Street Hospital, London, UK

Regulatory T-cells (Tregs) are a subset of T cells generated in the thymus with intrinsic immunosuppressive properties. Phase I clinical trials have shown safety and feasibility of Treg infusion to promote immune tolerance and new studies are ongoing to evaluate their efficacy. During heart transplantation, thymic tissue is routinely discarded providing an attractive source of Tregs. In this study, we developed a GMP-compatible protocol for expanding sorted thymus-derived $CD3^+CD4^+CD25^+CD127^-$ (Tregs) as well as $CD3^+CD4^+CD25^+CD127^-CD45RA^+$ (RA^+Tregs) cells. We aimed to understand whether thymic RA^+Tregs can be isolated and expanded offering an advantage in terms of stability as it has been previously shown for circulating adult $CD45RA^+$ Tregs. We show that both Tregs and RA^+Tregs could be expanded in large numbers and the presence of rapamycin is essential to inhibit the growth of IFN-γ producing cells. High levels of FOXP3, CTLA4, and CD25 expression, demethylation of the FOXP3 promoter, and high suppressive ability were found with no differences between Tregs and RA^+Tregs. After freezing and thawing, all Treg preparations maintained their suppressive ability, stability, as well as CD25 and FOXP3 expression. The number of thymic Tregs that could be isolated with our protocol, their fold expansion, and functional characteristics allow the clinical application of this cell population to promote tolerance in pediatric heart transplant patients.

Keywords: Cell therapy · Heart transplant · Regulatory T cells · Thymus

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Heart transplantation is a lifesaving procedure for pediatric patients with end-stage heart disease. Long-term success is limited by chronic inflammation and the use of immunosuppressive drugs [1]; therefore, new strategies are needed to induce indefinite transplant survival. So far, phase I clinical trials have shown safety and feasibility of regulatory T-cells (Tregs) therapy in the setting of transplantation and autoimmunity [2]. Similarly to conventional $CD4^+$ T cells, Tregs arise from thymus following maturation. This organ is routinely discarded during heart

Correspondence: Giovanna Lombardi
e-mail: giovanna.lombardi@kcl.ac.uk

transplantation and is considered to be an attractive source of autologous Tregs, especially for pediatric patients where it is not possible to collect large amounts of blood. In 2015, Dijke *et al.* showed for the first time the feasibility of isolating and expanding thymic resident Tregs [3]. These cells showed higher suppressive ability both in vitro and in vivo in comparison to circulating Tregs. Furthermore, the same group evaluated different expansion and freezing protocols for translating this population into the clinic [4].

One of the main challenges when isolating Tregs for clinical use is the purity of the resultant cell product. Following immunomagnetic isolation, thymic Tregs showed a greater purity compared to peripheral blood Tregs; however, 15–20% of the cells were still negative for FOXP3 [3,4]. This is due to the protocols enrolled for cell isolation and the presence of thymic cells expressing CD25 while they lack FOXP3 [5], a population that might be converted in effectors cells [6].

Good manufacturing practice (GMP) compliant cell sorters are now available in the clinic allowing the isolation of pure $CD4^+CD25^+CD127^-$ cells as well as Treg subpopulation like $CD4^+CD25^+CD127^-CD45RA^+$. We have previously shown that for some specific diseases, such as Crohn, blood-derived expanded $CD45RA^+$ Tregs were functionally superior and more stable compared to total $CD4^+CD25^+CD127^-$ Tregs [7]. Furthermore, blood-derived $CD45RA^+$ Tregs appear to be the ideal candidate population in the evolving landscape of CAR-Treg technology as they are more stable during expansion [8]. In thymic tissue, $CD45RA^+$ Tregs represent the cells that will move into the bloodstream following upregulation of CD31 [9]. As the thymus has the potential to become an alternative Treg source for autologous and also an HLA-matched cell therapies (in the prevention of graft vs. host disease), we aimed to develop an expansion protocol that can be used in future clinical trials with thymic Tregs and compare $CD3^+CD4^+CD25^+CD127^-$ (Tregs) with $CD3^+CD4^+CD25^+CD127^-CD45RA^+$ (RA^+Tregs) to understand whether the latter offered an advantage.

Results and discussion

Evaluation of thymic Tregs: Phenotype and numbers

Thymocytes were obtained from discarded thymi (age range 0.5–149 months, Table 1) following mechanical and enzymatic digestion and the median yield was 0.65×10^9 cells per gram of tissue (range $0.35–1.08 \times 10^9$ thymocytes per gram). This wide range was related to the quality of the tissue collected during the surgery as no correlation between the age of the patients and the number of cells recovered was found (data not shown). Thymocytes were then phenotypically characterized to determine their cellular composition (Supporting Information Fig. S1A and Table S1). The mean percentage of single positive (SP)-CD4 was $35.36 \pm 6.5\%$ of the whole thymocytes with $27.21 \pm 6.93\%$ of the cells being positive for $CD45RA^+$ (Supporting Information Table S1). We then evaluated the proportion of $CD25^+CD127^-$ cells in the SP-CD4 compartment as the expression of these two markers are routinely used for Treg sorting. $CD3^+CD4^+CD8^-CD25^+CD127^-$ cells were $11.8 \pm 3.63\%$ of the whole SP-CD4 with $84.57 \pm 5.68\%$ of them expressing FOXP3 (Supporting Information Fig. S1A and Table S1). The mean expression of $CD3^+CD4^+CD25^+CD127^-FOXP3^+$ in all the samples was $9.86 \pm 2.52\%$ of the total SP-CD4. The difference between the percentage of $CD3^+CD4^+CD8^-CD25^+CD127^-$ and $CD3^+CD4^+CD8^-CD25^+CD127^-FOXP3^+$ was in line with reports showing the presence of thymic $CD25^+$ cells negative for FOXP3 in both mouse [10] and humans [5]. RA^+Tregs represented the $38.7 \pm 5.13\%$ of the total Tregs, and no correlation was found between the age of the patients and the expression of CD45RA (data not shown). Next, the expression of functional and activation markers together with chemokine receptors was evaluated. No statistically significant differences were found in the expression of CD39, CTLA-4, TIGIT, HLA-DR, CCR6, CCR7, CD62L, CXCR3, CXCR4, CD38, CD69, and HELIOS between Tregs and RA^+Tregs (Supporting Information Fig. S1B and C). Additionally, all $CD3^+CD4^+CD8^-CD25^+CD127^-$ cells did not

Table 1. Characteristics of collected samples and yield from Treg isolation from 11 different donors

Donor	Weight (g)	Age (months)	Total thymocytes $\times (10^9)$	Thymocytes/g $\times (10^9)$	Processed thymocytes $\times (10^6)$	Post CD8 depletion $\times (10^6)$	Post CD25 enrichment $\times (10^6)$	Tregs/g $\times (10^6)$
D1	17.00	11	10.58	0.62	600	54.00	1.10	1.14
D2	9.11	20	3.30	0.36	1000	80.00	2.60	0.94
D3	5.00	0.5	2.18	0.44	1000	100.20	5.50	2.40
D4	14.80	18	6.25	0.42	1000	45.00	1.40	0.59
D5	10.33	21	7.40	0.72	1000	45.00	1.80	1.29
D6	6.70	3	6.50	0.97	1300	132.00	3.80	2.84
D7	25.00	18	27.00	1.08	1300	75.00	8.00	6.65
D8	9.60	29	3.35	0.35	1400	160.00	5.50	1.37
D9	8.00	149	3.34	0.42	1400	130.00	4.50	1.34
D10	3.80	85	2.80	0.74	1400	87.00	1.80	0.95
D11	9.00	9	9.70	1.08	1600	88.70	4.00	2.69
Mean	10.76	33.04	7.49	0.65	1181.82	90.63	3.64	2.02
Range	3.8–25	0.5–149	2.18–27	0.35–1.08	600–1600	45–160	1.1–8	0.6–6.65

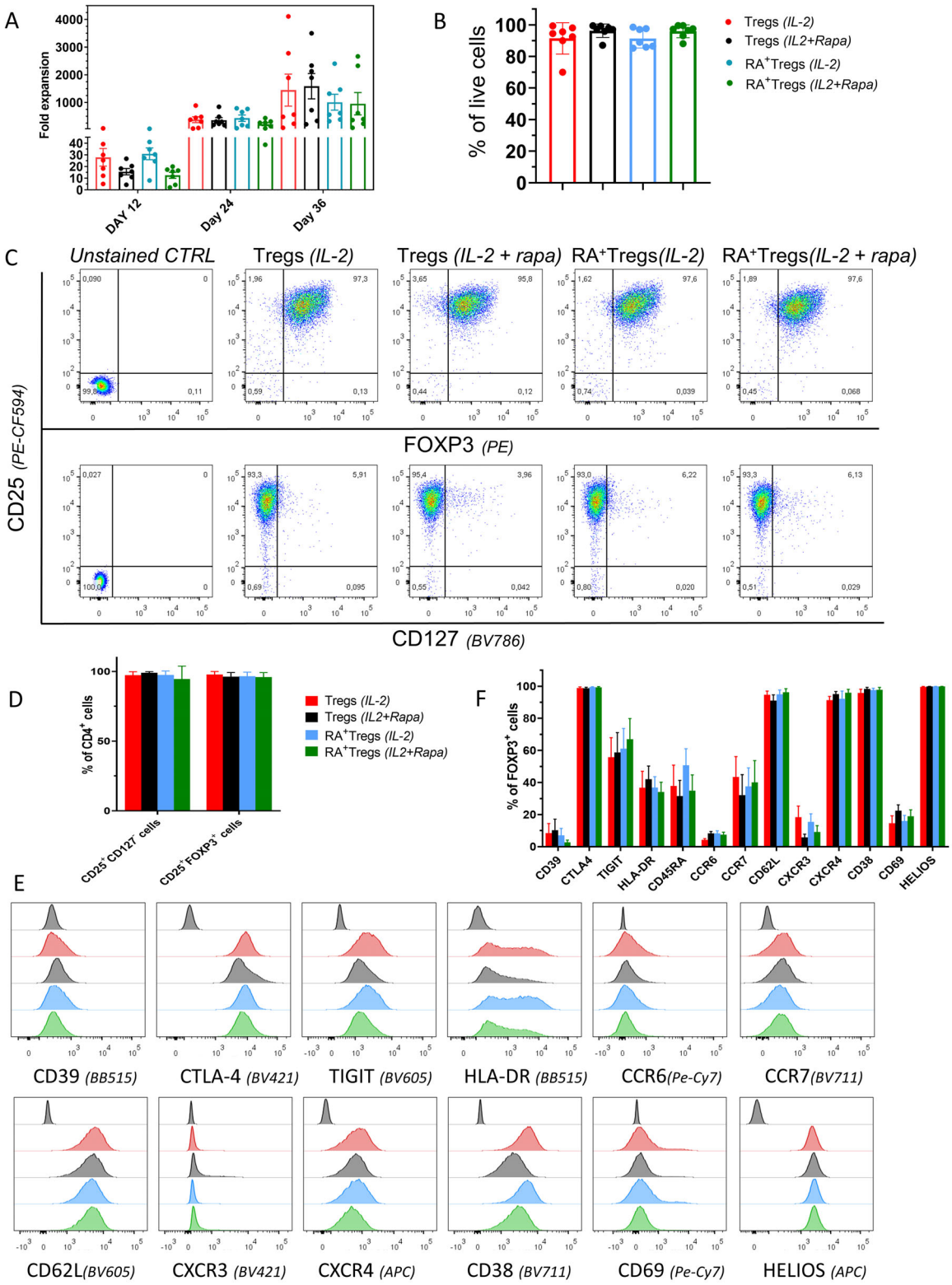


Figure 1. Treg expansion. (A) Fold expansion of the four Treg preparations following 36 days culture. Data are calculated as number of harvested cells/number of cells put in culture. (B) Percentage of live cells evaluated by flow cytometry at the end of the expansion. (C) Representative plots showing the expression of CD25, CD127, and FOXP3 in the cell preparations at the end of the expansion. (D) Cumulative data showing percentages of $CD25^+FOXP3^+$ and $CD25^+CD127^-$ cells in the $CD4^+$ population at the end of the expansion. Representative histograms (E) and cumulative data (F) showing the expression of the indicated markers in the four preparations (seven independent experiments with one donor per experiment). All the data presented in the figure are expressed as mean ± SD and come from seven independent experiments with one donor per experiment.

produce IL-2 following stimulation with PMA/Ionomycin (data not shown).

Highly suppressive and stable Tregs are expanded following cell sorting

$CD3^+CD4^+CD8^-CD25^+$ lymphocytes were isolated by $CD8^+$ depletion followed by CD25 enrichment (Table 1). The yield was 2.02×10^6 Tregs/g (range 0.59–6.65) and more than 80% of the cells were $CD3^+CD4^+CD8^-CD25^+CD127^-$ (range 72–90.8%) (Supporting Information Fig. S2A and B). To compare the expansion of Tregs and RA^+Tregs, the two populations were sorted according to the gating strategy shown in Supporting Information Fig. S2A; while Tregs included 14.3 ± 2.6 $CD4^+CD25^+FOXP3^-$ cells, this population was almost absent in RA^+Tregs (Supporting Information Fig. S2C). Sorted cells were expanded polyclonally (anti-CD3/CD28 beads and IL-2) in the presence or absence of rapamycin. After 36 days, the mean fold expansion rate was greater than 900 in all the Treg preparations (Fig. 1A). The viability of the Tregs was $\geq$90% in all the preparations (Fig. 1B) and no statistically significant differences were found between the four culture conditions. Of note, a higher fold expansion was obtained after 36 days of culture, compared to previously published protocols where nonsorted cells have been expanded for 15 days [3,4]. Furthermore, all the cells were $CD4^+CD25^+CD127^-$ and $CD4^+CD25^+Foxp3^+$ (Fig. 1C and D), while the expansion of nonsorted cells lead to a lower FOXP3 expression [4]. The phenotype at the end of the culture (day 36) was comparable between Tregs and RA^+Tregs and the presence of rapamycin in culture did not modify the expression of the main Treg markers (Fig. 1E and F). In detail, expanded Tregs were all positive for CTLA-4, HELIOS, CD38, and homing receptors such as CD62L and CXCR4. Conversely, activation and functional markers such as CD69 and CD39 and homing receptors such as CCR6 and CXCR3 were poorly or not expressed (Fig. 1E and F). Of note, when expanded for 15 days by using a GMP-compatible method, nonsorted Tregs partially lose the expression of CTLA-4 [4].

Following expansion, Tregs upregulated the expression of TIGIT and CXCR4, whereas the expression of CCR7 and CD69 was lost (Supporting Information Fig. S3B and C). The majority of RA^+Tregs downregulated the expression of CD45RA after expansion indicating an acquired memory phenotype. However, they lacked the expression of markers routinely expressed by expanded Tregs from blood such as CXCR3 and CD39 [11,12].

In line with both MacDonald et al. and Dijke et al. [3,4], expanded Tregs showed a high suppressive ability in vitro in a dose-dependent manner (Fig. 2A).

To evaluate the stability of the expanded cells, Tregs and RA^+Tregs were cultured in the presence of two pro-inflammatory cocktails of cytokines known to convert them into Thelper-17 [13]. As control, low dose of IL-2 only was used. No IL-17-producing Tregs were detected in all the conditions, while IFN-γ-producing cells were detected only in Tregs expanded in absence of rapamycin (Fig. 2B and C). These data confirmed the importance of rapamycin in culture to obtain a stable Treg product suitable for cell therapy. To further define cell stability at the end of the culture, we tested the methylation status of 11 CpG sites within the TSDR region. The mean percentage of methylation of the whole region was less than 10% in all the Treg preparations (Fig. 2D).

In our experience, cell freezing at the end of the Treg expansion has been a very helpful step for the application of Treg therapy. This step will be applied in the proposed clinical trial with thymic Tregs. Compatible with this idea, we have evaluated the phenotype and function of our Treg products soon after thawing. All the cells showed a viability greater than 70% over a period of 2 years (Supporting Information Fig. S3C), the Tregs retained their phenotype (Fig. 2E and Supporting Information Fig. S3D) and suppressive ability (Fig. 2F). Furthermore, almost no IL-2-producing cells were detected (% of $IL\text{-}2^+$ cells $<1\%$ in all the preparations), while the proportion of TNF-α-producing cells was lower than 10% (Fig. 2G and Supporting Information Fig. S3E).

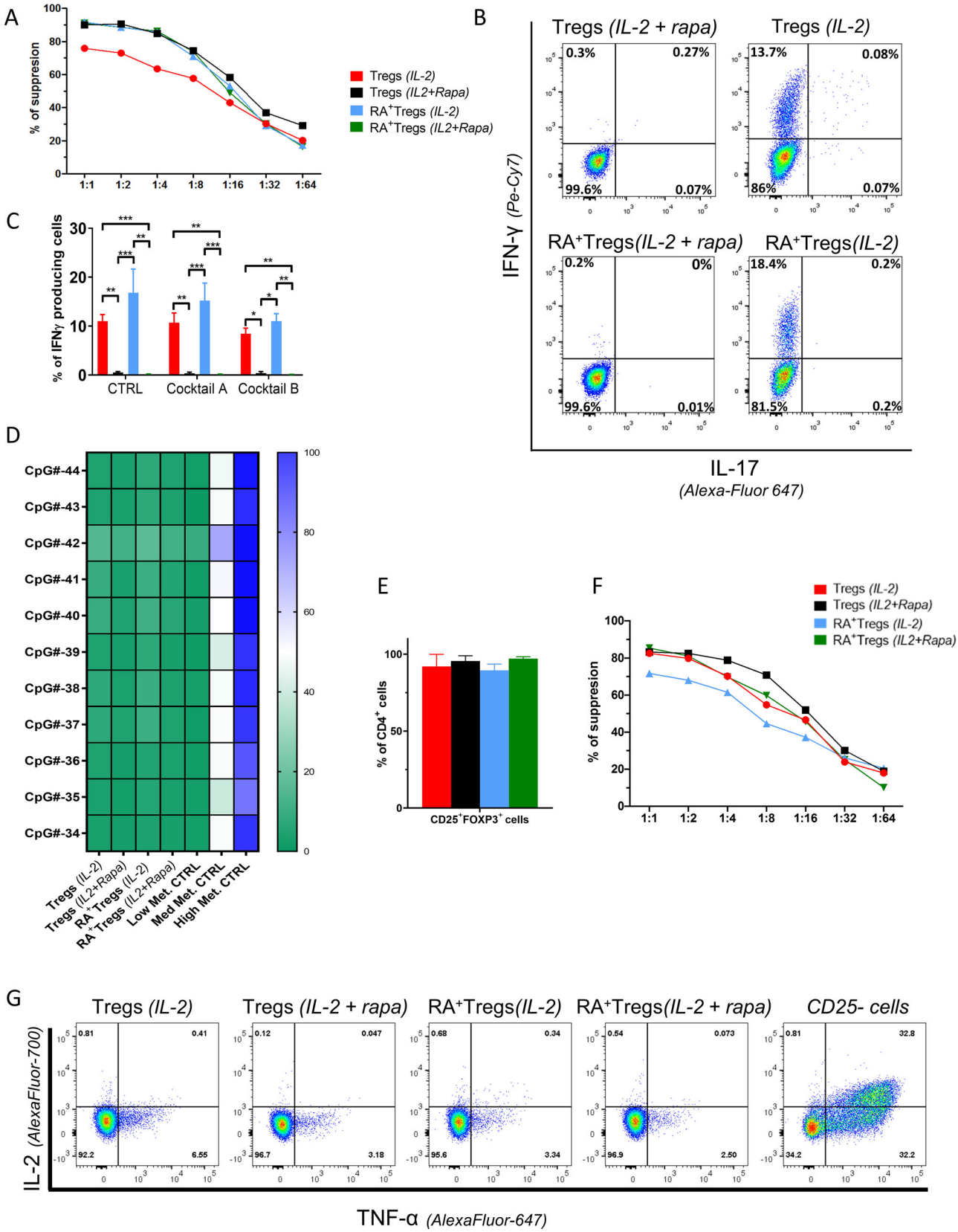
Conclusion

In conclusion, $CD3^+CD4^+CD25^+CD127^-$ Tregs showed the same phenotype, stability, and methylation level as the $CD3^+CD4^+CD25^+CD127^-CD45RA^+$ Tregs, suggesting that the additional step of isolating the $CD45RA^+$ subpopulation is not essential. Most importantly, thymic Tregs can be isolated and expanded to provide sufficient numbers ($>3\times10^7$ cells/kg) for clinical application in a tolerance protocol. In addition, we have shown that the expanded thymic Tregs can go through a process of freezing and thawing without compromising their efficacy or stability. Overall, our data provide additional information for the translation of thymus-derived Tregs to the clinic.

Materials and methods

Tissue processing

Discarded thymi from pediatric heart surgery were received from "Great Ormond Street Hospital" at University College London (UCL). Informed consent was obtained from all the donors prior to enrolment into the study (ethical approval: 06-MI-13(B)). Thymocytes were obtained after mechanical and enzymatic digestion by using dissociation medium consisting of X-Vivo 15 (Lonza) supplemented with 5% of human serum AB male (BioWest), amphotericin B (0.5 μg/mL; Thermo Fisher Scientific), collagenase (0.2 mg/mL; Merk), and deoxyribonuclease I (5 mg/mL; Merk). Thymic fragments were transferred into GentleMacs C tubes (Miltenyi Biotech) for mechanical digestion.



Flow cytometry analysis

Freshly isolated thymocytes and expanded Tregs were stained with two different panels. Panel 1: LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher Scientific), CD4 (clone: OKT4, BD), CD3 (clone: OKT3, BD), CD8 (clone: SK1, BD), CD25 (clone: M-A251, BD), CD127 (clone: HIL-7R-M21, BD), CD45RA (clone: H100, BD), HLA-DR (clone: G46-6, BD), CCR6 (clone: 11A9, BD), CXCR3 (clone: CXCR3-173, BD), CD62L (clone: HRL1, BD), CXCR4 (clone: 2B11, BD), and CCR7 (clone: G043H7, Biolegend). Panel 2: LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit, CD4, CD3, CD8, CD25, CD127, CD45RA, FOXP3 (clone: 236A/E7, BD), CTLA-4 (clone: BNI3, BD), HELIOS (clone: 22F6, BD), CD38 (clone: HIT2), TIGIT (clone: A15153G, Biolegend), CD69 (clone: FN50, BD), and CD39 (clone: TU66, BD).

Intracellular staining was performed using Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instruction. Stained cells were acquired on 5-lasers LSRFortessa X20 (BD) and analyzed using Flowjo. Guidelines for the use of flow cytometry in immunological studies were adhered to Cossarizza et al. [14].

Tregs isolation, expansion, and cryopreservation

Tregs were isolated in two steps: $CD8^+$ T cell depletion followed by enrichment of $CD25^+$ cells using immunomagnetic beads (Miltenyi Biotech). After isolation, cells were labeled with the following dyes for cell sorting (FACSAria, BD): LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit, CD4, CD3, CD8, CD25, CD127, CD45RA (all from Biolegend). After sorting, total and $CD45A^+$ Tregs were counted and cultured in X-Vivo 15 (Lonza, UK) supplemented with 5% of human AB serum (BioWest), IL-2 (1000 IU/mL; Proleukin, Novartis), and in the presence/absence of rapamycin (100 nM; LC-Laboratories, USA). All the cells were activated with MACS GMP ExpAct Treg Kit (1:1 bead to cell ratio; Miltenyi). IL-2 and rapamycin were replenished every 2 days following activation. Cells were re-stimulated twice every 10–12 days. At the end of the expansion, cells have been aliquoted into cryotubes following resuspension in serum free freezing media (Biological Industries) placed at −80°C overnight and then stored in liquid nitrogen for up to 2 years.

Suppression assay

$CD4^+CD25$- T cells (Teffs) were labeled with 2.5 μM CFSE and activated with anti-CD3/CD28 beads (Invitrogen) at 40:1 (cell/bead) ratio. Teffs were then cultured alone (1×10^5) or co-cultured with HLA-A2-mismatched Treg at different ratios. After 5 days, cells were stained with anti-HLA-A2 and data were acquired. The percentage of suppression was calculated based on the proliferation of Teffs alone compared to the percentage of proliferation observed in the presence of iTreg cells.

Stability assay

At the end of the expansion, total and $CD45A^+$ Tregs (± rapamycin) were stimulated with anti-CD3/CD28 beads (Invitrogen) at a bead:cell ratio of 1:1 and cultured for 5 days in complete X-Vivo 15 supplemented with the following cytokine cocktails: Cocktail A IL-2 (10 IU/mL), IL-1β (10 ng/mL), IL-6 (4 ng/mL), and TGF-β (5 ng/mL; all R&D-Systems, USA); Cocktail B IL-2 (10 IU/ml), IL-21 (25 ng/mL; Cell-Sciences, USA), IL-23 (25 ng/mL; R&D), and TGF- β (5 ng/mL; R&D). Cells cultured in complete medium supplemented with IL-2 (10 IU/mL) were used as control. At the end of the stimulation, IFN-γ- and IL-17-producing cells were identified by intracellular staining following stimulation with Leukocyte Activation Cocktail with BD GolgiPlug (BD). Following freezing and thawing, IL-2 (clone: MQ1-17H12 Biolegend) and TNF-α (clone: MAb11 Biolegend) producing cells were identified by intracellular staining following stimulation with Leukocyte Activation Cocktail with BD GolgiPlug (BD) for 5 h.

Methylation assay

CpG methylation analysis has been executed on 250 000 cells from each preparation. CpG methylation analysis was determined by pyrosequencing of bisulfite-modified genomic DNA and was conducted by EpigenDx, as previously described by us [15].

Statistics

Statistical tests were prepared using GraphPad Prism software v8.3. A RM two-way ANOVA was used for the analysis of all the

Figure 2. Treg characterization following expansion. (A) Suppressive ability at different ratios of the four preparations versus HLA-A2-mismatched T-effectors stimulated with anti-CD3/CD28 beads. Mean from seven independent experiments with one donor per experiment are expressed as percentage of inhibition of the T-effectors proliferation. (B) Representative plots showing the percentages of IFN-γ- and IL-17-producing cells in the presence of low dose of IL-2 and (C) cumulative data showing the percentages of IFN-γ producing cells after stimulation in the presence of IL-2 only, Cocktail A (IL-2 (10 IU/mL), IL-1β (10 ng/mL), IL-6 (4 ng/mL), and TGF-β (5 ng/mL)), and Cocktail B (IL-2 (10 IU/mL), IL-21 (25 ng/mL), IL-23 (25 ng/mL), and TGF- β (5 ng/mL)). Data (five independent experiments with one donor per experiment) are expressed as mean ± SD; $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ by RM two-way ANOVA followed by Tukey's multiple comparison test. (D) Mean percentage (five independent experiments with one donor per experiment) of the methylation level in 11 conserved CpGs at the FOXP3 TSDR. (E) Cumulative data showing percentages of $CD25^+FOXP3^+$ cells in the $CD4^+$ population following freezing and thawing (five independent experiments with one donor per experiment). (F) Suppressive ability at different ratios of the four preparations versus HLA-A2-mismatched T-effectors stimulated with anti-CD3/CD28 beads. Mean from three independent experiments with one donor per experiment are expressed as percentage of inhibition of the T-effectors proliferation. (G) Representative plots showing the percentages of IL-2 and TNF-α producing cells following freezing and thawing. All cell preparations as well as thawed $CD25^-$ cells (positive control) have been stimulated with Leukocyte Activation Cocktail for 5 h for inducing cytokine production.

data. Post hoc tests were used as indicated in the figure legends. p values are reported as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

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Conflict of Interest: The authors declare no conflict of interest

Ethics approval: Discarded thymi from pediatric heart surgery were received from "Great Ormond Street Hospital" at University College London (UCL). Informed consent was obtained from all the donors prior to enrolment into the study (ethical approval: 06-MI-13(B)).

Author contributions: M.R. conceptualized the study, designed, and performed experiments; analyzed data; and wrote the first draft of the manuscript. M.S., C.S., and R.Y.A. performed experiments and analyzed data. A.R.-A. and M.B. provided patient samples and scientific input. R.I.L. provided scientific input and critical revision of the manuscript. G.L conceptualized the study, supervised the project, and wrote the manuscript.

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

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Abbreviation: **SP**: single positive

Full correspondence: : Prof. Giovanna Lombardi, MRC Centre for Transplantation, School of Immunology and Microbial Sciences, King's College London, Guy's Hospital, SE1 9RT London, UK
e-mail: giovanna.lombardi@kcl.ac.uk

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