Differential effects of rapamycin and retinoic acid on expansion, stability and suppressive qualities of human CD4+CD25+FOXP3+ T regulatory cell subpopulations

Cristiano Scottà,[1,2] Marianna Esposito,[1,2] Henrieta Fazekasova,[1,2] Giorgia Fanelli,[3] Francis C. Edozie,[1] Niwa Ali,[4] Fang Xiao,[1] Mark Peakman,[2,5] Behdad Afzali,[1] Pervinder Sagoo,[1] Robert I. Lechler,[1,*] and Giovanna Lombardi[1,2,*]

[1]Department of Nephrology and Transplantation, King's College London, Medical Research Council (MRC) Centre for Transplantation, Guy's Hospital, London, UK; [2]National Institute for Health Research Biomedical Research Centre at Guy's and St Thomas' NHS Foundation Trust and King's College London; [3]Department of Biology and Biotechnology C. Darwin, University Sapienza of Rome, Italy; [4]St John's Institute of Dermatology, King's College London, Guy's Hospital, London, UK; [5]Department of Immunobiology, King's College London, UK

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ONLINE SUPPLEMENTARY APPENDIX

Antibodies for flow cytometry

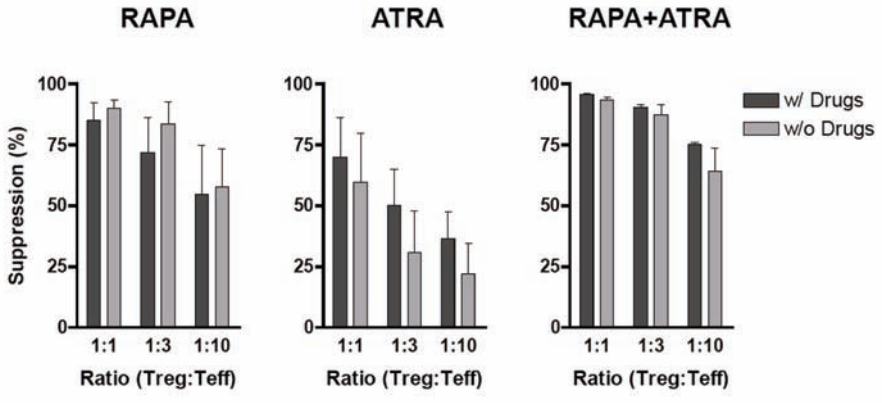
For flow cytometry, CD3-FITC, CD4-FITC (both Sigma-Aldrich); CD25-PE (4E3; Miltenyi-Biotec,UK); CD62L-FITC (Greg-56; Invitrogen,UK); CD152-PE (14D3), CD127-PE-Cy7 (eBioRDR5), GITR-APC (eBioAITR), ICOS-PE-Cy7 (ISA-3), CD39-FITC (eBioA1), Integrin α4/CD49d-PE (9F10), CD161-APC (HP-3G10), IFN-γ-FITC (4S.B3), IL-17-PE (eBio64DEC17) and FOXP3-FITC (PCH101 and 236A/E7) (all eBioscience, USA); CD27-PE (M-T271) and Integrin β7-PE-Cy5 (FIB504) (both BD-Bioscience, UK); HLA-DR-APC (L243), CLA-FITC (HECA-452) and CCR4-PerCP-Cy5.5 (TG6/CCR4) (all Biolegend, USA) and appropriate isotype controls from mouse or rat were used. Prior to use, all mAbs were titrated using normal resting or activated PBMC to establish optimal staining dilutions.

Surface and intracellular staining

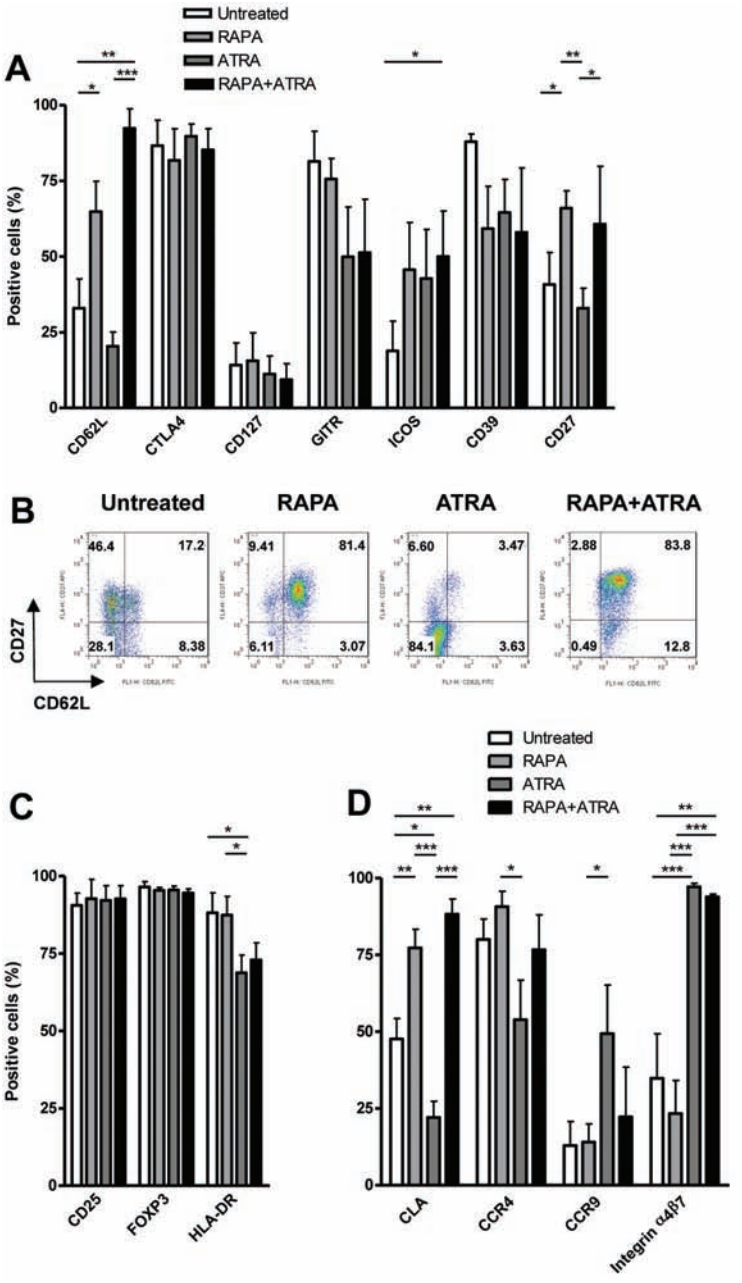
After harvest, cells were washed and surface stained with the above listed mAbs for 15 minutes at 4°C. Appropriate isotype control antibodies were used for each sample. Following staining, cells were examined by flow cytometry. Intracellular staining for FOXP3 was performed according to the manufacturer's protocol. Expressions of IFN-γ and IL-17 were assessed after activation of cells with phorbol-myristate-acetate (PMA, 50 ng/ml, Sigma-Aldrich), Ionomycin (1 μg/mL, Sigma-Aldrich) and Monensin (2 μM, eBioscience) for 5 hours. Subsequent intracellular staining for IFN-γ and IL-17 was performed according to the manufacturer's protocol.

Suppression assay

Cryopreserved T responders (Tres) were thawed and labeled with 2.5 μM CFSE (Molecular Probes, USA). Next, $1x10^5$ Tres were cultured alone or co-cultured at different ratios with Tregs (Treg:Tres ratio = 1:1, 1:3 and 1:10) in X-Vivo 15 medium supplemented with 5% human serum and anti-CD3/CD28 beads (Invitrogen,UK) in U-bottom 96-well plates and incubated at 37°C, 5% CO_2 for 5 days. After harvest, data were acquired on a FACSCalibur (BD-Bioscience, UK) and analyzed with FlowJo software (TreeStarInc, USA). Suppression of CFSE-labeled Tres proliferation by Tregs was analyzed using standard methods.[1] The percentages of suppression were calculated based on the proliferation of responder cells alone compared with the proliferation of cultures containing responder and suppressor cells.



Online Supplementary Figure S1. Suppressive ability of Treg lines after drug withdrawal. (A) suppression of autologous Teffs proliferation by 28-day Treg lines that were either kept in the presence of drugs (w/) or "starved" of RAPA, ATRA and RAPA+ATRA (w/o) for a further 2 weeks. Data, mean±s.e.m. of 3 independent experiments, are expressed as percentage of inhibition of Teff proliferation. Statistical analysis shows no significantly different data.



Online Supplementary Figure S2. Phenotypic analysis of untreated, RAPA-, ATRA- or RAPA+ATRA-treated Tregs. (A) Expression of common Treg markers on 28-day Treg lines. Data, mean ± s.d. of 7 independent experiments. (B) CD27 and CD62L co-expression on differently treated Treg lines. Representative dot plots from 7 experiments. (C) Expression of HLA-DR with CD25 and FOXP3. Data, mean ± s.d. of 3 independent experiments. (D) Expression of homing receptors on Treg lines. Data, mean ± s.d. of 7 independent experiments. Statistical analysis shown only on significantly different data. **P*<0.05, ***P*<0.01, ****P*<0.001.

ELISA

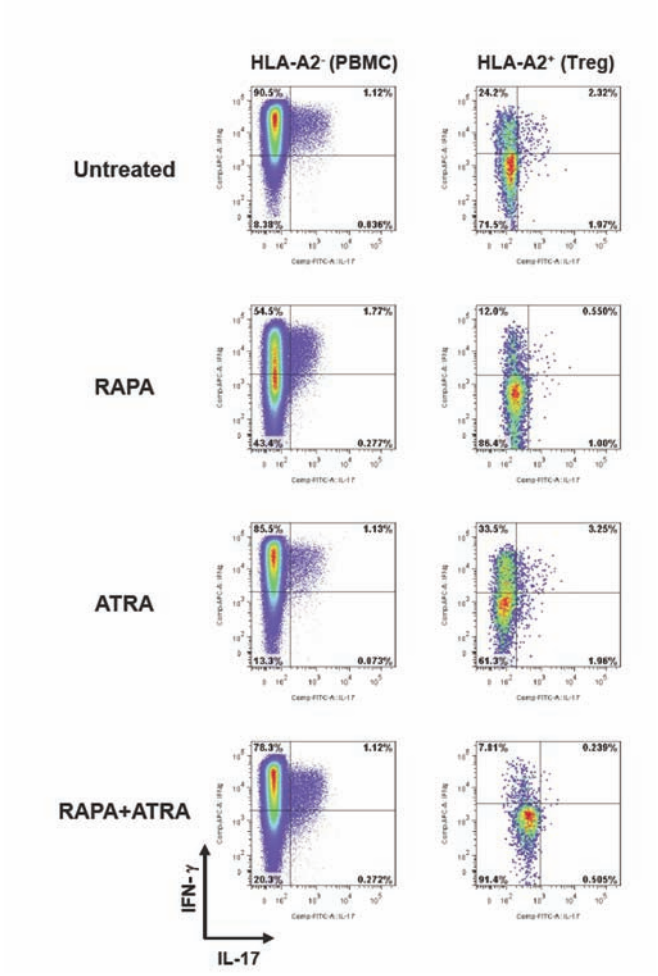
IL-17 and IFN-γ in supernatants were analyzed by an indirect sandwich ELISA, using purified and biotinylated anti-IL-17 and anti-IFN-γ (BD Pharmingen, USA), and visualized with TMB (Zymed, USA) measuring the optical density at 450 nm on an automatic plate reader (Titertek-Multiscan-PLUS, Finland). The amount of each cytokine was calculated from a standard curve.

In vitro *Treg stability assay*

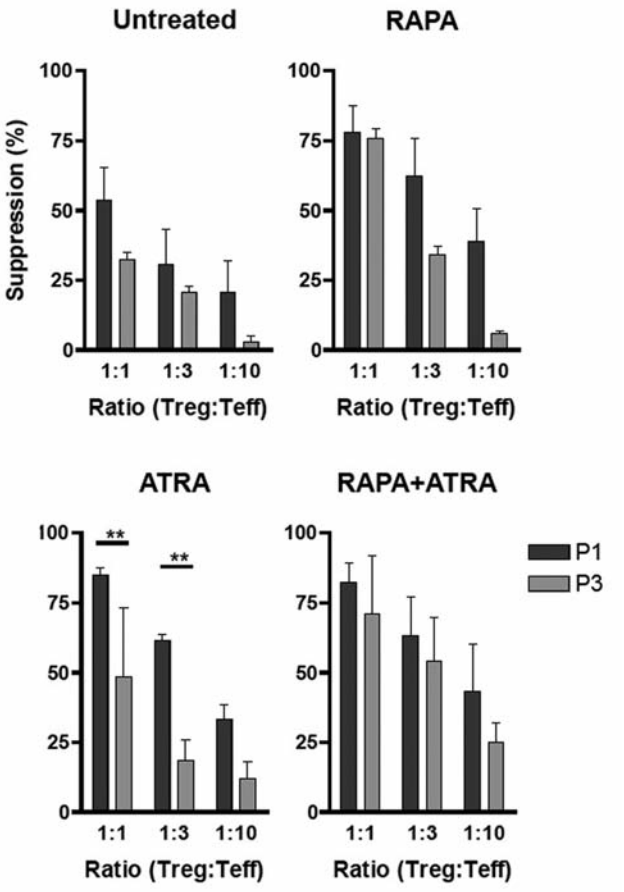
Fresh ($5x10^5$), untreated, RAPA and/or ATRA treated Tregs were activated with anti-CD3/CD28 beads at a bead:cell ratio of 1:1 and cultured for 5 days in X-Vivo 15 medium supplemented with 5% human serum supplemented with the following cytokine cocktails: Cocktail-A IL-2 (10 IU/mL), IL-1β (10 ng/mL), 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 (25ng/ml, Cell-Sciences, USA), IL-23 (25ng/ml, R&D) and TGF-β (5ng/ml, R&D). Cells cultured in complete medium supplemented with IL-2 (10 IU/mL) were used as control.

Xenogeneic GVHD and in vivo *stability assay*

NOD/Scid/IL-2Rγc$^{-/-}$ mice (NOD.cg-PrkdcscidIl2rgtm1Wjl/SzJ mice, abbreviated to NSG, provided by Prof Adrian Hayday, King's College London, UK) were used between 8-14 weeks of age. Mice were maintained under specific pathogen-free conditions and handled in accordance with the Institutional Committees on Animal Welfare of the United Kingdom Home Office (the Home Office Animals Scientific Procedures Act, 1986, reference PPL 70/7302). In order to establish whether injected Tregs could maintain a stable phenotype



Online Supplementary Figure S3. Interleukin17 and IFN-γ production from *in vitro* expanded and *in vivo* infused Tregs. Representative dot plots from 2 independent experiments showing IL-17⁺ and IFN-γ⁺ cells in HLA-A2-(PBMC) and differently treated HLA-A2⁺ (Treg) subsets infused in a humanized mouse model of xeno-GvHD.



Online Supplementary Figure S4. Treg subsets suppressive abilities in the presence of different treatments. Comparison of P1 and P3 suppressive abilities at different ratios of Treg:Teff after 4 rounds of stimulation and cultured in the presence of different treatment. Data, mean±s.e.m. of 5 independent experiments, are expressed as percentage of inhibition of Teff proliferation. Statistical analysis shown only on significantly different data.**P<0.01.

in the presence of inflammation, a Xeno-GvHD model was induced by intravenous transfer of 20x10⁶ PBMCs depleted of CD25⁺ cells into NSG mice 6 hours after total body irradiation (2.4Gy). Animals were monitored three-times weekly for body weight, other GvHD symptoms (hunched back, fur loss, skin inflammation) and weekly assessment of human CD45⁺ cell engraftment. Mice were euthanized on weight loss of below 15% of baseline, or when severe symptoms of GvHD became apparent. Tregs (5x10⁶) were introduced into this inflammatory environment after 3 weeks of GvHD induction when animals were reconstituted (less than 10% human CD45⁺ cells) and experiencing active disease based on weight-loss. Spleen and lymph nodes were harvested 2 days after Treg transfer and analyzed by flow cytometry as described above.

References

1. Venken K, Thewissen M, Hellings N, Somers V, Hensen K, Rummens JL, et al. A CFSE based assay for measuring CD4+CD25+ regulatory T cell mediated suppression of auto-antigen specific and polyclonal T cell responses. J Immunol Methods. 2007;322(1-2):1-11.