

Impact of immunosuppressive drugs on the therapeutic efficacy of ex vivo expanded human regulatory T cells

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SUPPLEMENTAL DATA

Section 1: Materials and Methods

Section 2: Supplementary Figures

Section 1

MATERIALS AND METHODS

Cell isolation and separation (extended)

Peripheral blood mononuclear cells (PBMC) were isolated by Lymphocyte (PAA) density gradient centrifugation. CD4⁺CD25⁺ T cells were purified by negative selection of CD4⁺ T cells followed by positive selection of CD25⁺ T cells using miniMACS CD4⁺CD25⁺ T Regulatory Cell Isolation Kit (Miltenyi-Biotec). The purity of CD4⁺CD25⁺ T cells was between 90-98%. CD4⁺CD25⁺ T cells were expanded while aliquots of CD4⁺CD25⁻ T cells (effector T cells, Teff) were cryopreserved and used as autologous responder cells in suppression assays.

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 IL-10, IFN- γ and IL-17 were assessed after activation of cells with phorbol-myristate-acetate (PMA, 50ng/ml, Sigma-Aldrich), Ionomycin (1 μ g/ml, Sigma-Aldrich) and Monensin (2 μ M, eBioscience) for 5 hours. Subsequent intracellular staining for IL-10, IFN- γ and IL-17 was performed according to the manufacturer's protocol.

Analysis of cytokine concentration in culture supernatant

Interleukin-17, IL-10 and IFN- γ in supernatants were analyzed by cytometric bead array assay kit (BD Biosciences) and analyzed on a BD Fortessa flow cytometer (BD Biosciences).

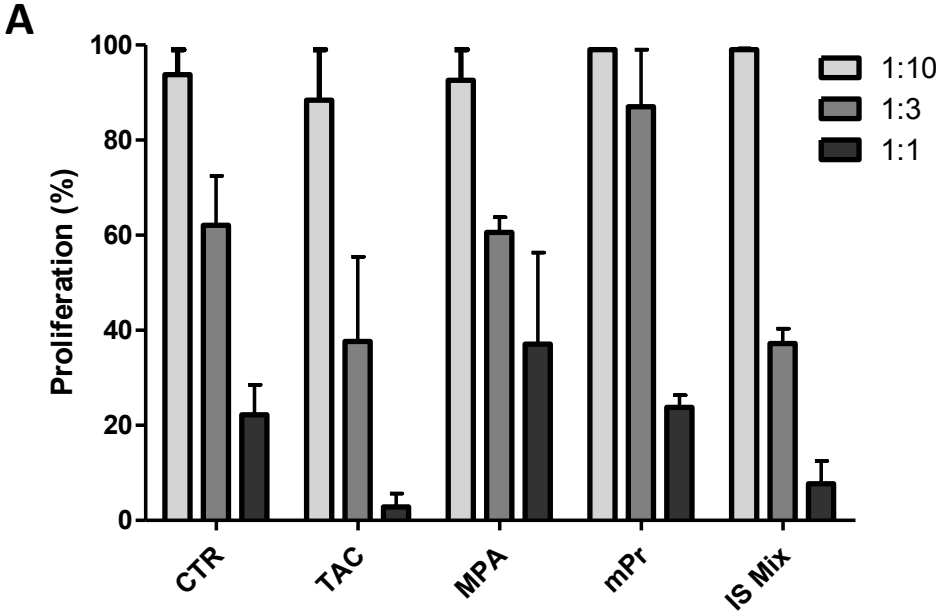
Suppression assay

Cryopreserved T responders (Teff) were thawed and labeled with 2.5 μ M CFSE (Molecular Probes). Next, 1×10^5 Teff were stimulated with anti-CD3/CD28 beads (Invitrogen) and co-cultured at different ratios with Tregs (Treg:Teff ratio = 1:1, 1:3 and 1:10) in U-bottom 96-well plates at 37°C for 5 days. Data were acquired on a BD LSRFortessa™ cell analyzer (BD-Bioscience) and analyzed with FlowJo software (TreeStar Inc). Treg suppressive ability was calculated according to the inhibition of CFSE-dilution in Teff cells and analyzed as previously described (6). The percentage of suppression was calculated based on the proliferation of Teff alone compared with the proliferation of cultures containing responder and suppressor cells.

Statistical analysis

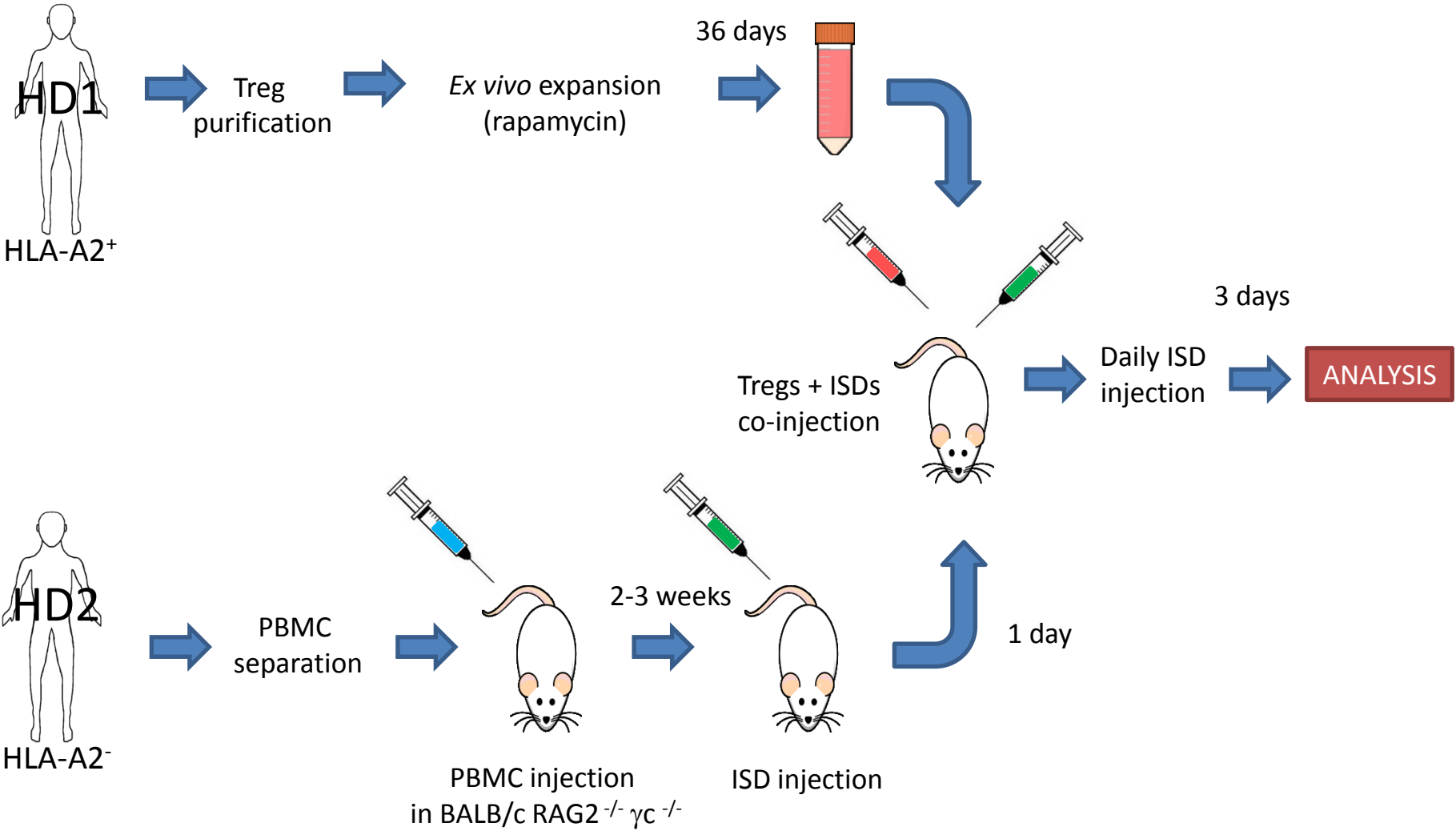
Statistical analysis was carried out using GraphPad Prism software (GraphPad software Inc.). Parametric and non-parametric data were calculated as the mean \pm s.d. and median (interquartile range, IQR) respectively. For comparison of parametric and non-parametric data, t-test, one- or two-way ANOVA with Bonferroni test comparison and Kruskal-Wallis test were used where appropriate.

SUPPLEMENTARY FIGURE 1



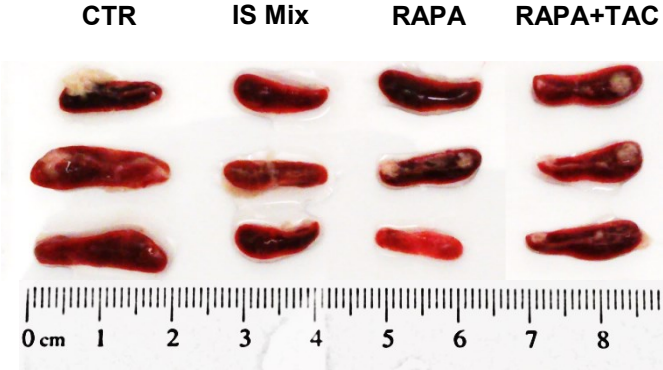
Supplementary Figure 1. Suppressive ability of Tregs cultured for 5 days with only medium (CTR), single immunosuppressive agent (TAC, MPA and mPr) or their combination (IS Mix). Data are combined from 3 independent experiments. Note: No significant difference between Teff proliferation in the presence of untreated (CTR) or treated Tregs

Schematic of the *in vivo* experiment

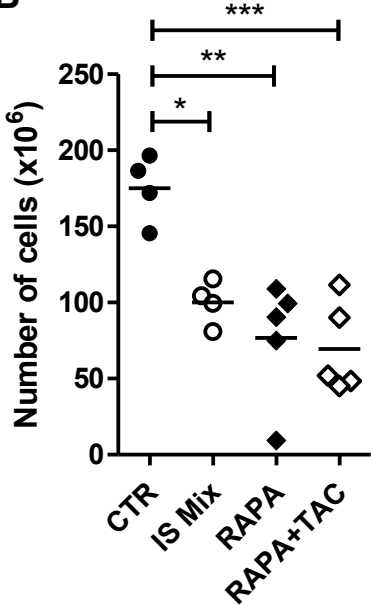


SUPPLEMENTARY FIGURE 3

A



B



Supplementary Figure 3. Panel A, image of representative spleens from mice receiving immunosuppressive treatment (IS Mix, RAPA and RATA+TAC) and controls (CTR). Panel B, total number of splenocytes recovered from the four groups of animals.