Supplementary Materials

# Supplementary Figures

**Chart, diagram

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**Figure S1** *Magnetic-activated CD4+CD25+CD127- cell sorting purity of human Tregs.* (**A** to **C**) Isolation purity of magnetically sorted CD4+ (A) CD25hiCD127lo FoxP3+ (B) human Tregs with granzyme B and annexin V expression levels (C) immediately post isolation. GrB, granzyme B.

Diagram, engineering drawing

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**Figure S2** *Human Treg phenotyping strategy and activation-induced upregulation of GrB and apoptosis.* Human Tregs were isolated and expanded for three days with α-CD3, α-CD28, and IL-2 (n = 3 technical replicates/condition; 3 representative experiments). (**A** to **B**) Gating strategy identifying magnetically sorted, activated human CD4+ CD25hiCD127lo T cells (A), and therein the FoxP3+ subset (B). (**C**) Flow cytometric analysis of GrB+ cells among the CD4+ CD25loCD127lo T cell and CD4+ CD25hiCD127lo FoxP3+ Treg subsets and accompanying box plots. (**D**) Flow cytometric analysis and box plots of Annexin V+ cells among the CD4+ CD25loCD127lo T cell and CD4+ CD25hiCD127lo FoxP3+ Treg subsets. (**E**) Flow cytometric analysis of Annexin V+ cells among GrB- and GrB+ FoxP3+ Treg subsets, including box plots. (**F**) Box plots of Annexin V MFI among total GrB (GrB+/-), GrB-, and GrB+ FoxP3+ Treg subsets. Data represent boxplots with median, interquartile range, minimum, maximum, and all individual data points of the denoted experimental groups. *P* values were calculated with independent samples two-tailed Student’s *t*-tests, and non-parametric Mann-Whitney *U*-tests were performed when the assumption of homoscedasticity could not be met. For analyses with more than two groups, one-way analyses of variance followed by Holm-Šídák multiple comparison tests were performed. GrB, granzyme B; MFI, mean fluorescence intensity.

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**Figure S3** *Rapamycin experiments phenotyping strategy and supplementary box plots related to figure 1.* (**A**) Gating strategy identifying magnetically sorted, activated human CD4+ T cells. (**B** to **D**) Box plots of GrB (B) and Annexin V (C) mean fluorescence intensities (MFIs), and percentages of live Annexin V- CD25hiCD127lo FoxP3+ Tregs (D) among CD4+ T cells (n = 3 technical replicates/condition; 2 representative experiments of 5). Data represent boxplots with median, interquartile range, minimum, maximum, and all individual data points of the denoted experimental groups. *P* values were calculated with independent samples two-tailed Student’s *t*-tests, and non-parametric Mann-Whitney *U*-tests were performed when the assumption of homoscedasticity could not be met. CT, control; GrB, granzyme B; MFI, mean fluorescence intensity; Rapa, rapamycin.

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**Figure S4** *Rapamycin dose de-escalation increases GrB expression and apoptosis in human Tregs and affects Treg viability discordantly from calcineurin inhibition.* Human Tregs were isolated and expanded for three days with α-CD3, α-CD28, and IL-2, without additional inclusions (CT), with CT stimulants plus 0.1–10 nM rapamycin (Rapa), or with CT stimulants and cyclosporin A (CsA). (**A** to **D**) Flow cytometric analyses of CD25hiCD127lo subset among CD4+ T cells (A), FoxP3+ subset among CD25hiCD127lo cells (B), and GrB+ (C) and Annexin V+ subsets (D) among FoxP3+ Tregs, including the respective box plots (n = 2 technical replicates/condition; 2 experiments). Data represent boxplots with median, interquartile range, minimum, maximum, and all individual data points of the denoted experimental groups. *P* values were calculated with one-way analyses of variance followed by Holm-Šídák multiple comparison tests. CsA, cyclosporin A; CT, control; GrB, granzyme B; MFI, mean fluorescence intensity; Rapa, rapamycin.

Diagram, engineering drawing

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**Figure S5** *GranToxiLux experiments phenotyping strategy and Treg stimulation leads to intracytoplasmically active GrB.* (**A**) Gating strategy identifying an active FSChiSSChi subset of magnetically sorted CD4+ CD25hiCD127lo human Tregs that were expanded for three days with α-CD3, α-CD28, and IL-2. (**B** to **D**) Flow cytometric analysis and box plots of GranToxiLux+ Treg percentages (B and C), and GranToxiLux mean fluorescence intensities (E; n = 3 technical replicates/condition; 2 representative experiments of 4). Data represent boxplots with median, interquartile range, minimum, maximum, and all individual data points of the denoted experimental groups. *P* values were calculated with independent samples two-tailed Student’s *t*-tests, and non-parametric Mann-Whitney *U*-tests were performed when the assumption of homoscedasticity could not be met. GrB, granzyme B; MFI, mean fluorescence intensity.

Diagram

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**Figure S6** *Phosphorylation experiments* *phenotyping strategy.* Gating strategy identifying magnetically sorted CD4+ CD25hiCD127lo human Tregs that were expanded in a 24-hour cell culture window with α-CD3, α-CD28, and IL-2.

Diagram, engineering drawing

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**Figure S7** *In vivo mTORC1 inhibition reduces human Treg GrB expression apoptosis of peripheral Tregs in an in vivo allotransplantation model.* (**A** and **B**) NOD-*scid* IL-2 receptor-γnull (NSG) mice were transplanted with healthy donor skin seven days prior to the adoptive transfer of 5.0×106 human PBMCs and 1.0×106 human CD4+CD25hiCD127lo Tregs (A). Afterwards, the NSG mice received daily intraperitoneal injections of 1× DPBS (PBS) or 1 mg/kg rapamycin (Rapa) for nine days. Fourteen days from the start of the treatment, the mice were euthanized and the splenocytes were extracted (B; n = 3 mice/condition; 1 of 2 experiments). (**C**) Gating strategy identifying splenic CD4+ human T cells. (**D**) Flow cytometric analysis and box plots of CD25+FoxP3+ and CD25++FoxP3+ Tregs among CD4+ T cells. (**E** and **F**) Flow cytometric analysis and box plots of GrB+ cells among FoxP3+ Tregs (E), and box plots of GrB mean fluorescence intensities (MFIs) among FoxP3+ Tregs (F). (**G** and **H**) Flow cytometric analysis and box plots of Annexin V+ cells among FoxP3+ Tregs (G), and box plots of Annexin V MFIs among FoxP3+ Tregs (H). (**I**) Box plots of live Annexin V- CD25hiCD127lo FoxP3+ Tregs among CD4+ T cells. (**J**) Flow cytometric analysis and box plots of p-4E-BP+ cells among FoxP3+ Tregs. Data represent boxplots with median, interquartile range, minimum, maximum, and all individual data points of the denoted experimental groups. *P* values were calculated with independent samples two-tailed Student’s *t*-tests, and non-parametric Mann-Whitney *U*-tests were performed when the assumption of homoscedasticity could not be met. GrB, granzyme B; MFI, mean fluorescence intensity; Rapa, rapamycin.