SUPPLEMENTAL MATERIALS

NAD+ regulates Treg cell fate and promotes allograft survival via a systemic IL-10 production that is CD4+CD25+Foxp3+ T cells independent

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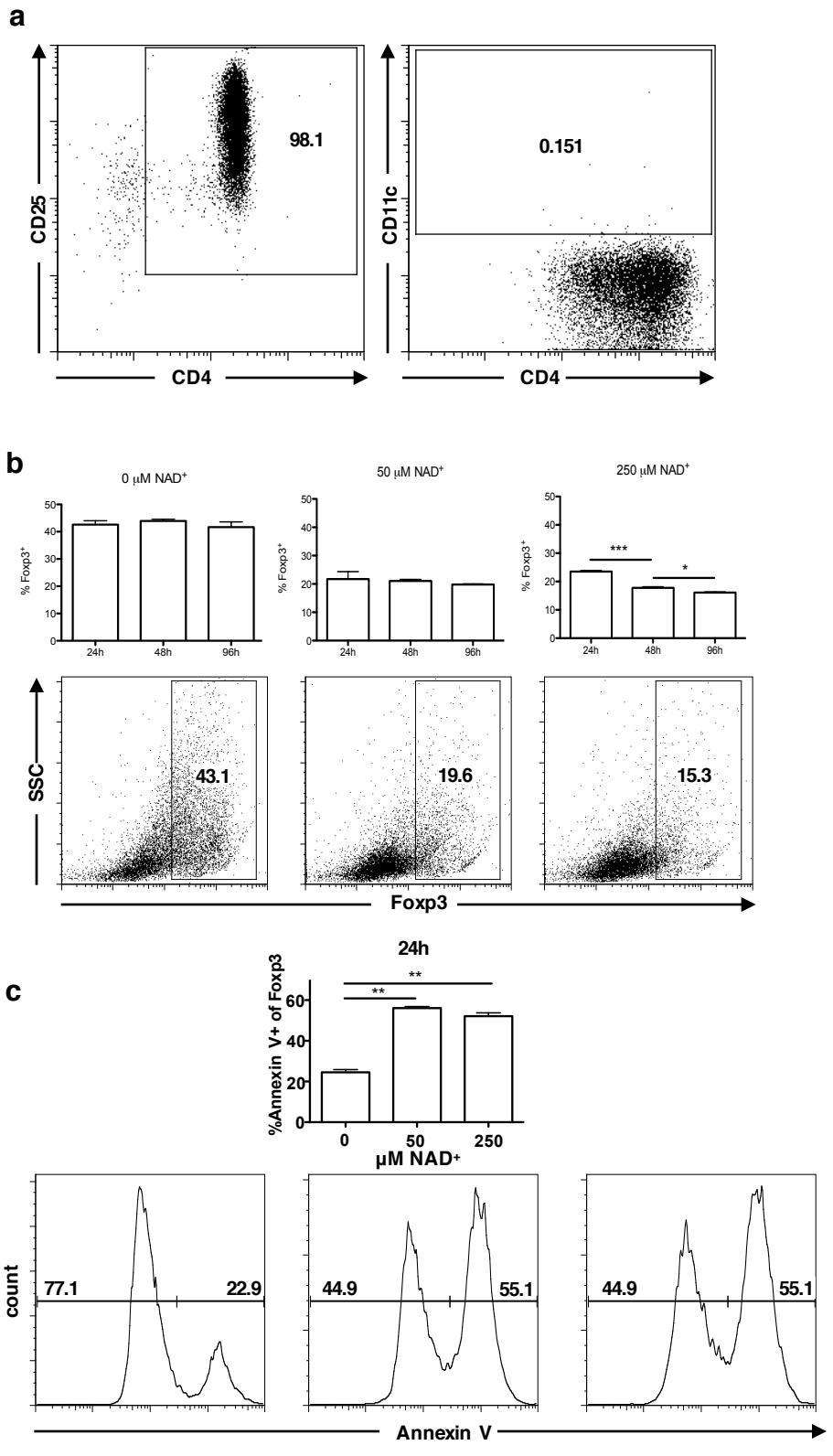
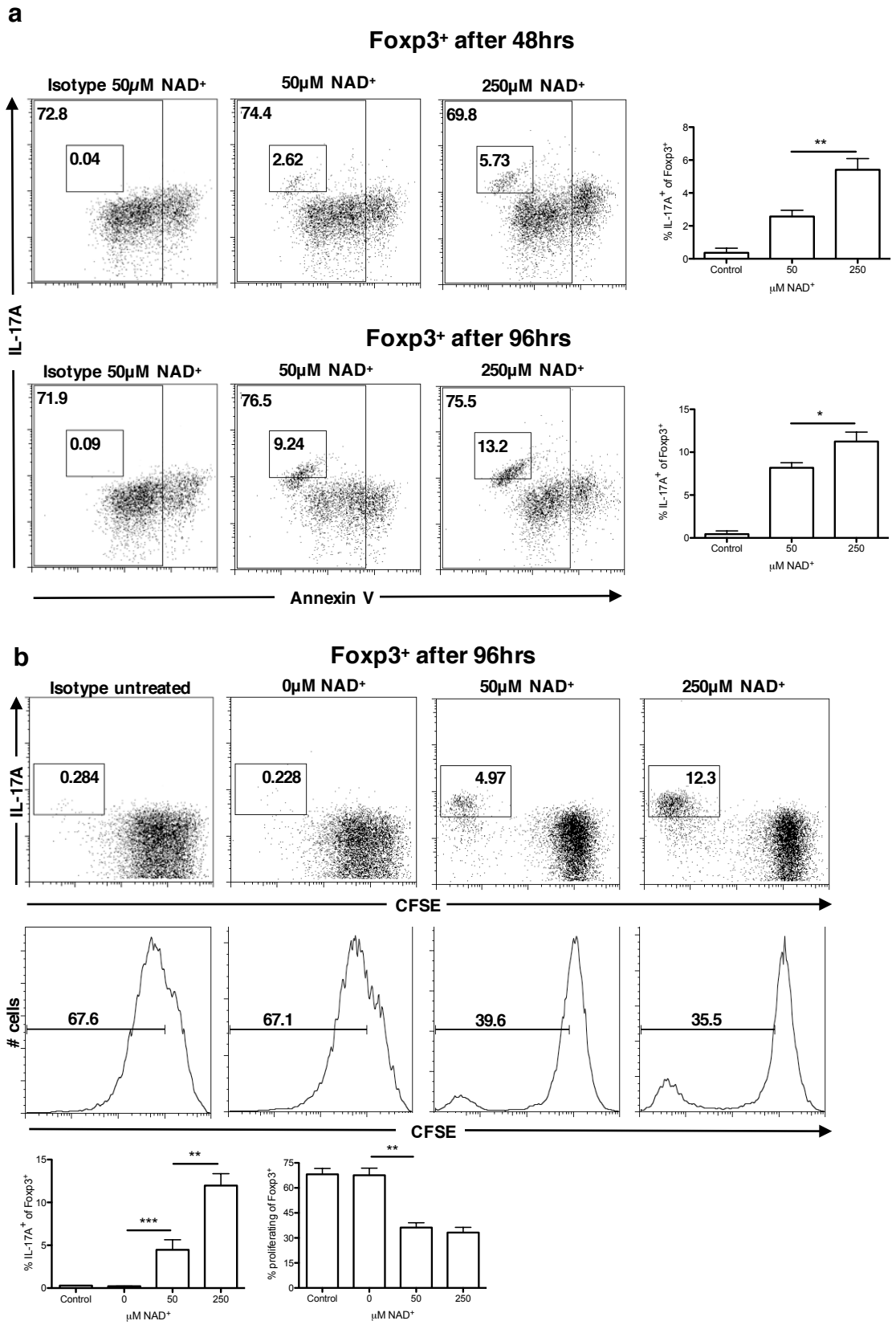
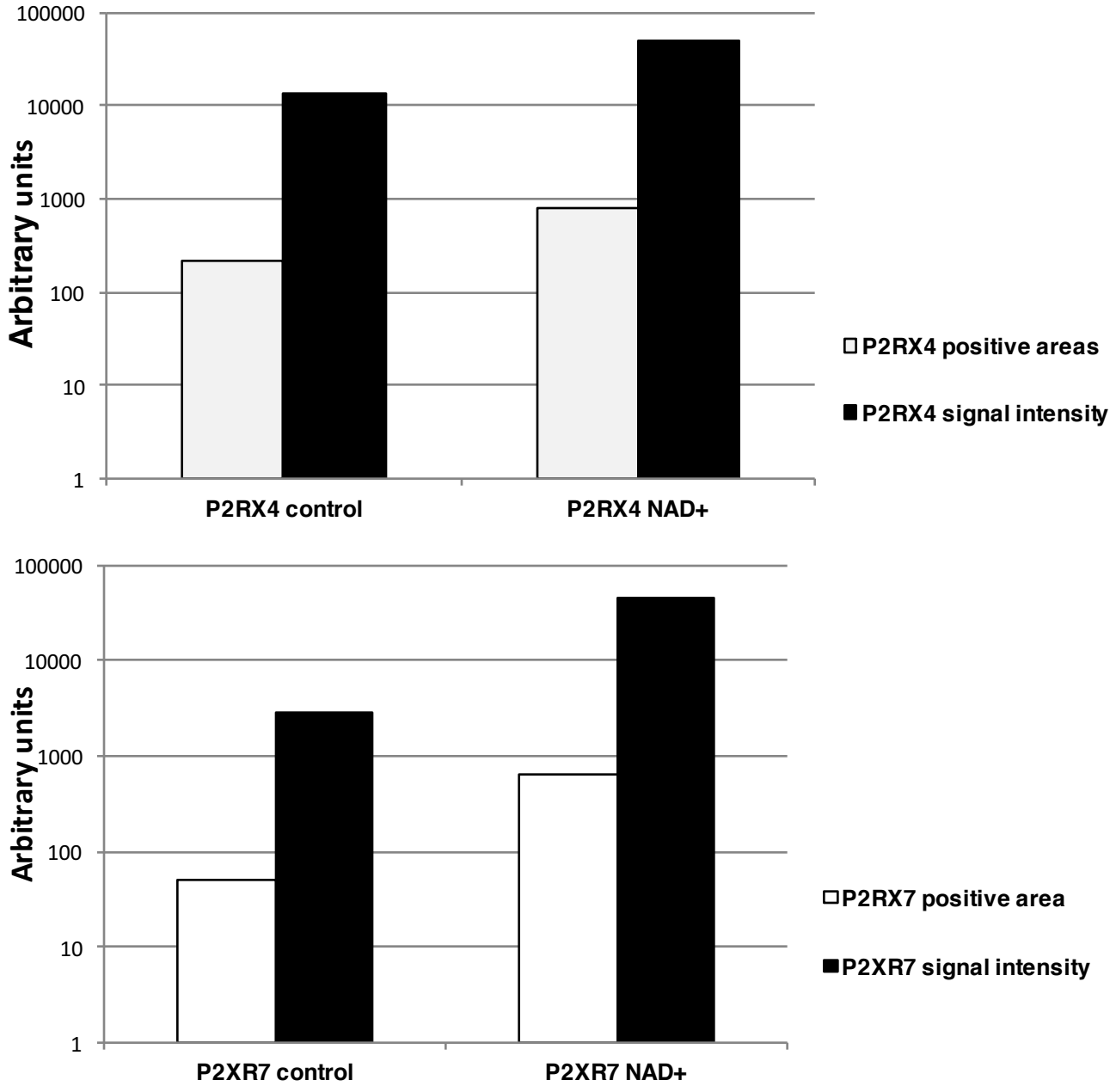


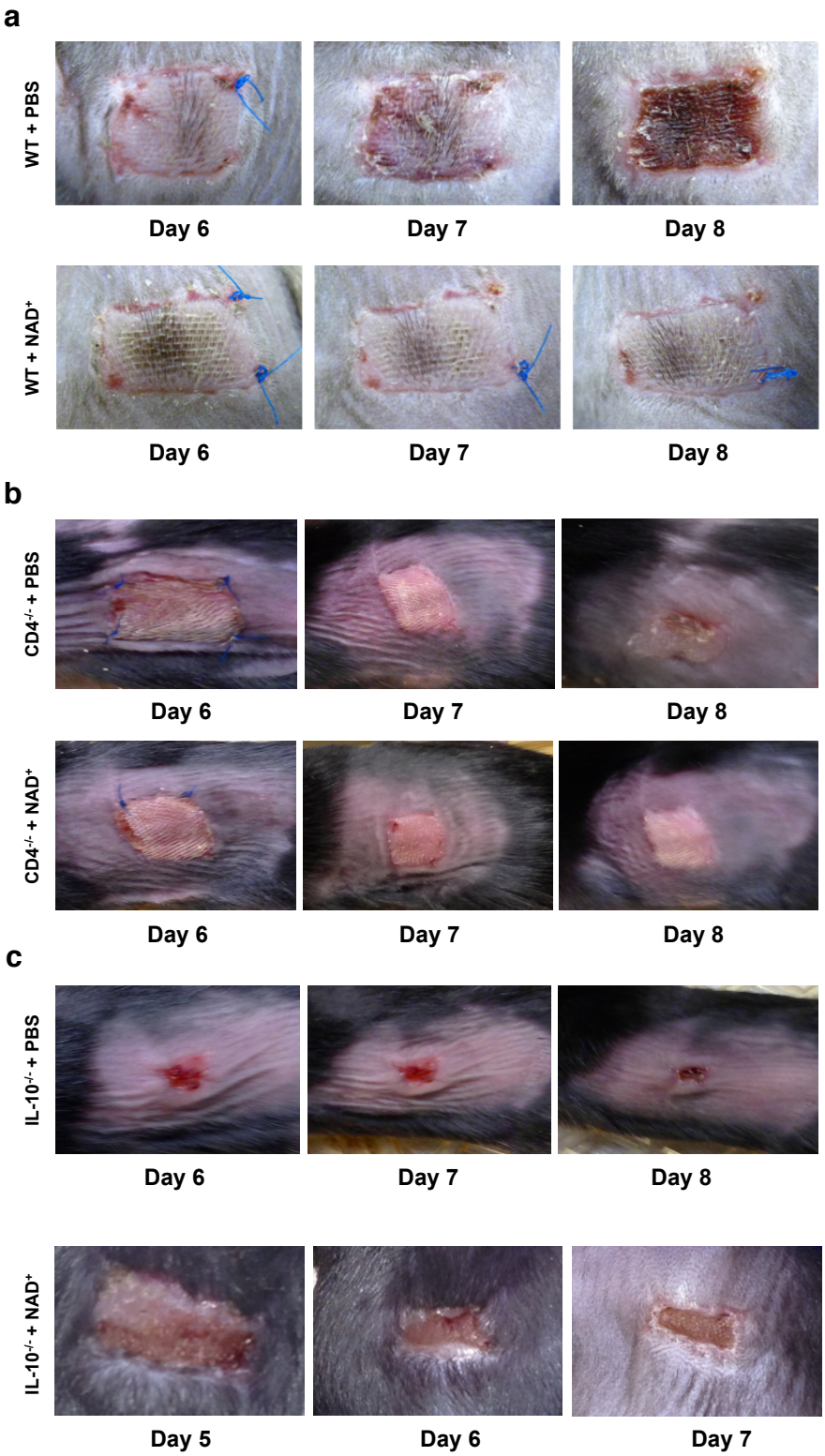
Supplementary Figure S1: NAD⁺ induces Treg apoptosis and loss of Foxp3 expression in vitro. (a) Purities of $CD4^+CD25^+$ Tregs after isolation from spleens of DBA mice were >98%, containing no contaminating $CD11c^+$ cells (plots shown are representative for three independent experiments). (b) Frequencies of Tregs that were cultured in presence of α-CD3, α-CD28, and IL-2 and increasing concentrations of NAD^+ after 24hrs, 48hrs and 96hrs (n=6 per group, representative plots shown). (c) Percentages of Annexin V^+ cells among $CD4^+CD25^+Foxp3^+$ Tregs after 24hrs of culture in presence of α-CD3, α-CD28, and IL-2 and increasing concentrations of NAD^+ (n=6 per group, representative plots shown). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. Student's t-test was used to compare between groups.



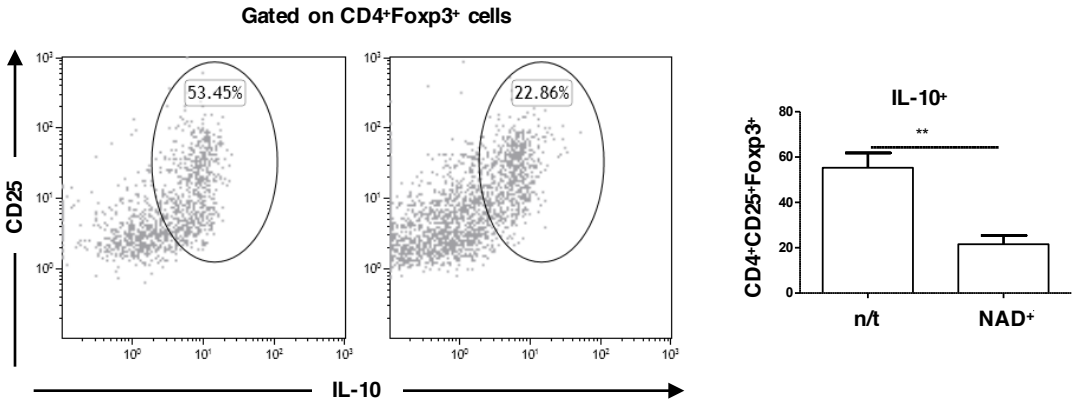
Supplementary Figure S2: Tregs converted into IL-17A producing cells remain resistant to apoptosis and proliferate in presence of NAD+ without exogenous IL-23 cytokine. $CD4^+CD25^+Foxp3^+$ cells were cultured in presence of α-CD3, α-CD28, and IL-2 with increasing concentrations of NAD^+ and (a) apoptosis of $CD4^+CD25^+Foxp3^+IL\text{-}17A^+$ cells was assessed with Annexin V after 48hrs and 96hrs in a dose-dependent manner (*n*=6 per group; data derived from two independent experiments; representative plots shown). (b) Proliferation was assessed with CFSE in a dose-dependent manner (*n*=6 per group; data derived from two independent experiments; representative plots shown).



Supplementary Figure S3: NAD+ increases P2RX4 and P2RX7cell surface expression. Isolated T cells were cultured for 24hrs in presence of vehicle alone (right column) or 50μM NAD+ (left column). Cells were collected and stained at 4°C for either P2RX4 (top row) or P2RX7 (bottom row) without prior fixation or permeabilization. Stacks of 20, 2-channel images were acquired under each condition; resulting stacks were de-convolved and re-constituted for further analysis. Results shown Quantification of positive area as well as signal intensity.



Supplementary Figure S4: NAD+ promotes allograft survival. Photos of skin grafts from day 6 to day 13 after transplantation of mice treated with NAD+ or with a placebo solution. Fully MHC-mismatched tail skin allografts from C57BL/6 mice were transplanted onto DBA/2 recipient mice that received (A) control solution (PBS) or daily doses of NAD+ (40 mg). (B) Fully MHC-mismatched DBA tail skin allografts were transplanted onto *CD4*$^{-/-}$ mice (C57BL/6 background) that were treated daily with NAD+ (40 mg). (C) Fully MHC-mismatched DBA tail skin allografts were transplanted onto *IL-10*$^{-/-}$ mice (C57BL/6 background) that were treated daily with NAD+ (40 mg). Photos of skin grafts were taken daily from day 6 to day 13. For *IL-10*$^{-/-}$ mice photos of skin grafts were taken daily from day 5 to day 7. *n*=5 mice per group; representative images are shown.



Supplementary Figure S5: Increased systemic production of IL-10 is not mediated through Tregs. Fully MHC-mismatched C57BL/B6 tail skin allografts were transplanted onto DBA/2 mice that received daily doses of NAD^+ (40 mg in 100µl PBS) or control solution (PBS). $CD4^+$ T cells were isolated from spleens 8 days after transplantation and gated on $CD4^+Foxp3^+$ cells and frequencies of $IL\text{-}10^+CD25^+$ were analyzed by flow cytometry (*n*=6 per group, representative plots shown).

Supplementary Movie S1: NAD^+ promotes cell surface expression of P2RX4 and P2RX7 receptors on Tregs. P2RX4 expression by Tregs in presence of (a) PBS solution or (b) 50μM of NAD^+ after 24hrs of culture. P2XR7 expression by Tregs in presence of (c) PBS solution or (d) 50μM of NAD^+ after 24hrs of culture.