SUPPLEMENTARY APPENDIX

The TLR7 ligand R848 prevents mouse graft-*versus*-host disease and cooperates with anti-interleukin-27 antibody for maximal protection and regulatory T-cell upregulation

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TLR7 ligand R848 prevents mouse graft-versus-host disease and cooperates with anti-IL-27 antibody for maximal protection and Treg upregulation.

Supplementary Data

Supplementary Methods

GVHD experiments

For non-conditioned or sublethally irradiated parent to F1 GVHD, on day -1, B6xDBA/2 F1 (B6D2F1) recipient mice were left untreated or irradiated with 5 Gy from a ^{137}Cs source before aHCT. On day 0, they received an i.p injection of 50–70 x10^6 C57Bl/6 (B6) spleen cells and, for the irradiated mice, 10 million B6 CD3ε$^+$ T cell-depleted BM cells (TCD-BM). For lethally irradiated model, BALB/c recipient mice were irradiated with 2x4Gy at 6 hour interval and transplanted with 2.5x10^6 CD5$^+$ splenocytes + TCD-BM cells from B6 mice. All experiments were performed on adult mice with a weight of ± 20 g. Mice were monitored for survival and weight loss every other day. Mice were bled on the indicated days to monitor plasma cytokines and, at the experimental endpoint, euthanized for spleen and liver analyses.

Liver Histology

For histo-pathology analyses, paraffin tissue blocks of 4% paraformaldehyde fixed liver were prepared using routine methods and six micron consecutive sections were made. The sections were stained with hematoxylin and eosin (H&E) and scanned with Pannoramic P250 digital slide scanner (3D HISTECH).

Mixed lymphocyte culture

MLC was carried out by incubating $1.25x10^6$ responder spleen cells/ml with an equal concentration of irradiated (30 Gy from a ^{137}Cs source) adherent spleen cells. In some cases, responders were CD4 T cells purified by MACS from spleens and seeded at a density of $1x10^6$ cells/ml. Adherent cells were obtained by coating $1x10^6$ splenocytes in a 96-well flat bottomed microtiterplate for 1.5h and removing non-adherent cells by washing the microplate twice with PBS (37°C). DC subpopulations were cultured at 10^4 cells per well. Proliferation was measured after two days by incubation with [^{3}H]-Thymidine at 1 μCi (0.037 MBq)/well for a further 18h. [^{3}H]-Thymidine incorporation was measured using a scintillation counter (Packard Microplate Scintillation Counter).

Flow cytometry and cell sorting

To determine chimerism, splenocytes were stained with anti-H-2D^d-FITC (clone: 34–2–12) and anti-H-2D^b-PE (clone: KH95) (all from Biolegend, San Diego, CA). Spleen cells were also labelled with anti-CD4 (clone: RM4-5 and GK1.5), anti-CD8α (clone: 53-67), anti-TCR-β (clone: H57-597), anti-CD44 (clone: IM7), anti-CD62L (clone: MEL-14), anti-CD69 (clone: H1.2F3), anti-CD25 (clone: PC61) and anti-LAP (clone: TW7-16B4) Abs, all from Biolegend, and a viability marker (LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit, Life Technologies, Eugene, OR). Treg cells were determined in spleens according to manufacturer instructions using a kit from eBioscience (San Diego, CA) with the FJK-16s anti-mouse Foxp3 clone. For MACS cell purification or depletion, we used anti-CD5 (cat. 130-049-301) and anti-CD3ε (cat. 130-094-973) microbeads (Miltenyi Biotec Bergisch Glabach, Germany). For *in vitro* functional assays, DC subpopulations were purified from

spleens as previously described (1). All cells were acquired using a FACS-LSRFortessa according to BD bioscience protocols and analysed by FlowJo software version 9.8.1.

Cytokine measurements

Cytokine production was measured in cell culture supernatants and plasma. ELISA specific for murine IFNγ (R&D Systems, MN, USA) and TNF-α (LifeTechnologies, MD, USA) were performed according to manufacturer instructions. IL-27p28, active TGF-β1 and TGF-β3 were measured using mAbs generated in our laboratory as previously described (2). TGF-β1 and 3 were measured in plasma without previous acid treatment to limit detection of their active forms. In all ELISAs, biotinylated detection Abs were used followed by avidin-HRP (Biolegend). Absorbance readings were made at 450 nm, using a 96-well plate spectrophotometer (VERSAmax, Molecular Devide). Detection limits are less than 10 pg/ml.

RT-qPCR

Total RNA was isolated from spleen and liver with TriPure isolation reagent (Roche, IN, USA). One μg of total RNA was reverse transcribed using oligo(dT) primer (Eurogentec, Vilnius, LT) and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative PCR (qPCR) amplifications were performed on cDNA obtained from 25 ng of total RNA, using primer sets and TaqMan probes (Eurogentec) or MasterMix for SYBR Green (Eurogentec). Primers and probes used for *β-actin* were 5'-CTCTGGCTCCTAGCACCATGAAG-3', 5'-GCTGGAAGGTGGACAGTGAG-3' and 5'-TCGGTGGCTCCATCCTGGC-3'. Primers and probe used for *IFNγ* were 5'-TCAAGTGGCATAGATGTGGAAGAA-3', 5'-TGGCTCTGCAGGATTTTCATG-3' and 5'-TCACCATCCTTTTGCCAGTTCCTCCAG-3'. Primers for *SAA1/2* were

5'- TCTGCTCCCTGCTCCTGGGA-3' and 5'- TGGCTTCCTGGTCAGCCATG-3'. Primers for *SAA3* were 5'-GCTGGTCAAGGGTCTAGAGAC-3' and 5'-GATGACTTTAGCAGCCCAGGC-3'. Samples were first heated for 10 min at 95°C before amplification as follows: 40 cycles of two-step PCR program at 95°C for 10 s and 60°C for 1 min. For SYBR Green qPCR, melting point analysis was carried out by heating the amplicon from 60 to 95°C. A standard curve with known concentrations of a cloned cDNA fragment was used for each gene.

CFSE staining

$CD5^+$ T cells were suspended at $20x10^6$ cells/ml in serum-free media and CFSE (Invitrogen, Oregon, USA) was added at 0.5uM final concentration. Cells were incubated at 37°C for 10 minutes and reaction was stopped with the same volume of cold FCS and washed in media containing 3% FCS. Cells were injected i.p. in recipient mice and after 6 days, they were analysed on a FACS-LSRFortessa.

Statistical analysis

Statistical analysis was performed with Prism 5 (Graphpad Software, La Jolla, CA) using non-parametric tests (Kruskal–Wallis or Mann–Whitney), parametric test (Bonferroni multicomparison), and Log- rank Test for survival curves.

References

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2. Marillier RG, Uyttenhove C, Goriely S, Marbaix E, Van Snick J. IL-27p28 is essential for parent-to-F1 acute graft-versus-host disease. Eur J Immunol. 2014;44(7):2064-2073.

Supplementary Figure Legends

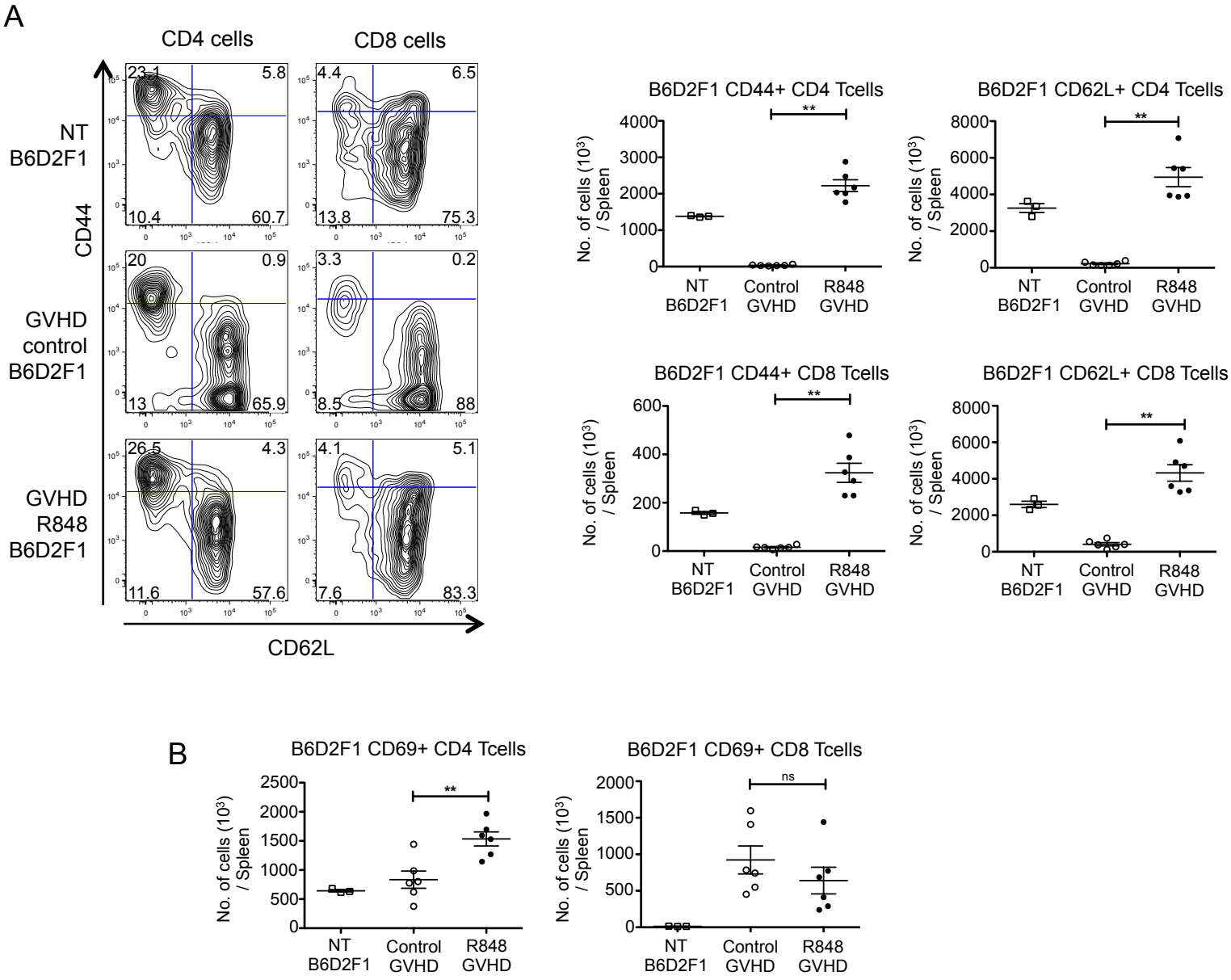
Supplementary Figure 1. Modulation of B6D2F1 T cell populations in control and R848 non-conditioned GVHD. B6D2F1 mice, treated with R848 or not (25 μg at 48 and 24 h before transplantation), received $60x10^6$ spleen cells from B6 mice non-treated or treated with R848, 48 hours before cell transfer. 14 days after B6 spleen cell transplantation, spleen cell subsets were enumerated by FACS, using anti-H-$2D^d$, H-$2D^b$, -CD4, -CD8, (A) -CD44, -CD62L and (B) -CD69 Abs. The levels of T cell activation are represented with density plots for percentage and scatter plots for absolute numbers. Data are representative of two independent experiments (**$p < 0.01$ by Mann–Whitney unpaired t-test).

Supplementary Figure 2. PC61 treatment strongly reduces Treg number in R848-GVHD mice. Tregs were depleted with PC61 Abs in donor and host mice 4 days before R848 treatment and 1 day after B6 cell transfer in ncGVHD model. H-$2D^d$, H-$2D^b$, LIVE/DEAD®, CD4, TCRβ and Foxp3 staining was used to evaluated by flow cytometry Treg population 14, 20 and 50 days after B6 cell transfer. Data are representative of 3 independent experiments (*$p < 0.05$, **$p < 0.01$ Mann–Whitney unpaired t-test).

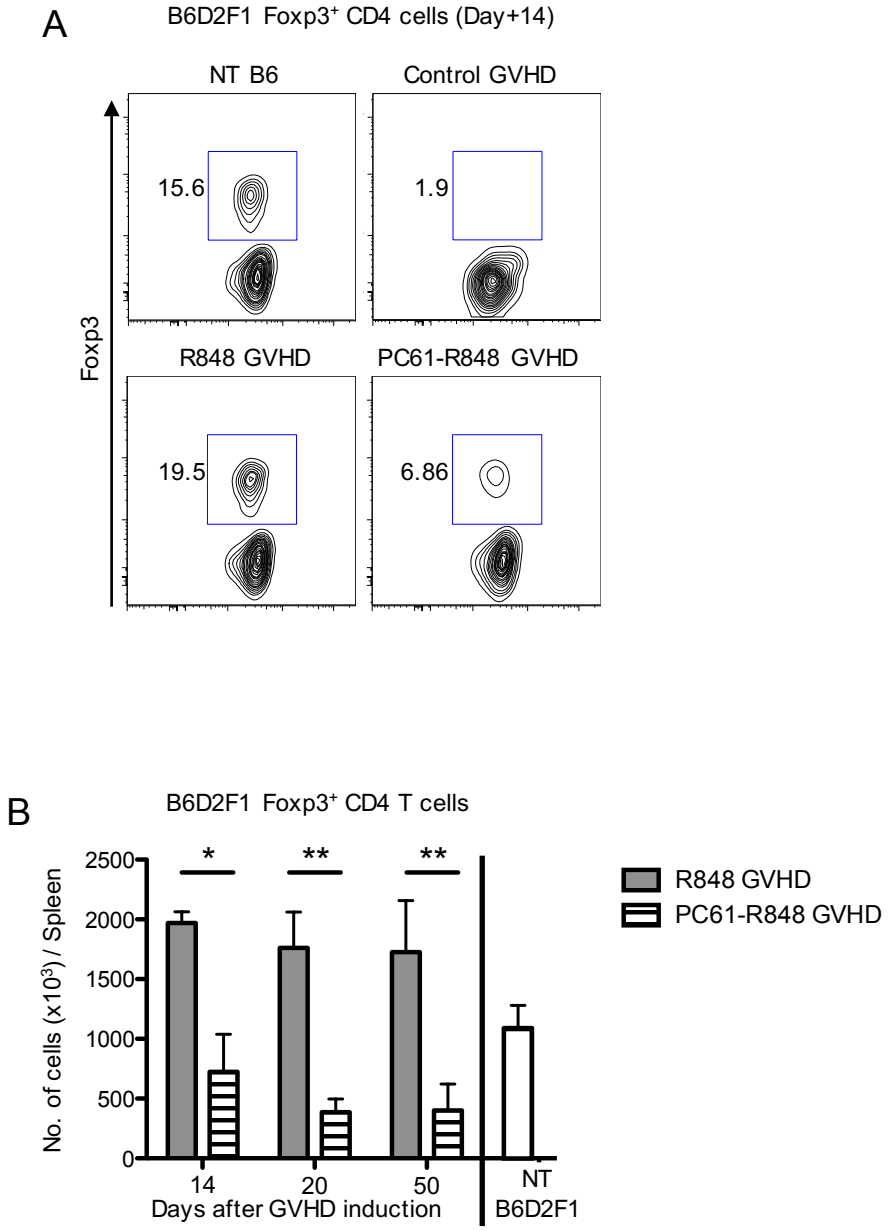
Supplementary Figure 3. T cell subset analysis after acute GVHD prevention by anti-IL-27p28 monoclonal antibody and R848 combination in two irradiated models. (A) BALB/c recipient mice were irradiated with 8Gy and treated or not with R848 (25 μg/mouse) 48 and 0h before transplantation of $2x10^6$ splenocytes from B6 mice. In addition, recipients were treated or not with aIL-27 (0.5) at days 0 and 6. After 6 days of GVHD induction, spleen cells from control GVHD and R848-MM27 GVHD mice were recovered and stained with anti-H-$2D^d$, -H-$2D^b$, -CD4, -CD8, -CD69, -CD44 and -CD62L Abs for FACS spleen cell subset analyses. (B) B6D2F1 recipient mice were irradiated with 5Gy and

treated or not with R848 (25 μg/mouse) 48 and 0 h before transplantation of $40x10^6$ B6 splenocytes. In addition, both recipient groups were treated or not with aIL-27 (0.5 mg) at days 0 and 6. After 7 days of GVHD induction, spleen cells were recovered and stained with anti-H-$2D^d$, -H-$2D^b$, LIVE/DEAD®, anti-CD4, -CD8, -LAP and -Foxp3 Abs to evaluate Treg populations. Data are representative of 2 experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Kruskal-Wallis test with Dunn Multiple Comparison Test).

Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3