MHC disparity hampers thymus-dependent T-cell recovery post-hematopoietic transplantation through dysregulation of TGF-β1 and LRP6 pathways

Ning Wu,[1*] Wei Ding,[1*] Jiashuo Wu,[1,2] Min Ling,[1,2] Keli Yue,[1,2] Cong Cheng,[1] Mingzhu Jia,[1] Jiangying Liu[1] and Xiao-Jun Huang[1,2]

[1]Peking University People's Hospital, Peking University Institute of Hematology, National Clinical Research Center for Hematologic Disease, Beijing Key Laboratory of Cell and Gene Therapy for Hematologic Malignancies, Peking University and [2]Peking-Tsinghua Center for Life Sciences, Academy for Advanced Interdisciplinary Studies, Peking University, Beijing, China

**NW and WD contributed equally as first authors.*

Correspondence: J. Liu
xys_liujiangying@pkuph.edu.cn

X-J. Huang
huangxiaojun@bjmu.edu.cn

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Supplementary Methods

Monitor of engraftment and immune recovery in transplant recipients

Following transplantation, peripheral blood samples were collected from recipient mice at 14, 28, 45, 60, and 120 days, and stained with fluorochrome-conjugated monoclonal antibodies：PE-anti-H2Kd, BV421-anti-H2Kb, APC-Cy7-anti-CD45, FITC-anti-CD3, PE-Cy5-anti-CD4, BV510-anti-CD8a, AF700-anti-CD69, BV605-anti-CXCR4, PE-DAZZLE594-anti-CTLA4 and BV786-anti-PD-1 (Biolegend, USA) at room temperature for 15 mins. After incubation, red blood cells were lysed with a lysis solution (BD Biosciences, USA) and then washed twice with PBS. Polychromatic flow cytometric analyses were performed on a BD LSRFortessaTM Cell Analyser and further analyzed using BD FACSDivaTM software.

Ex-vivo functional assay for the recovered T cells

Spleen tissues were isolated from recipient mice approximately 45 days following transplantation and subsequently homogenized in a 10 mL Petri dish. After filtration and centrifugation, primary splenocytes (1×10^6 cells/100μL) were cultured in 96-well round-bottom plates (Costar, Cambridge, MA) with 0.2 mL RPMI 1640 medium plus 10% FBS and stimulated with 5 μg/mL Concanavalin A (ConA, Sigma, USA) for 48 hours at 37°C. The Protein Transport Inhibitor Cocktail (500x, eBioscience, USA) was administered 6 hours prior to detection. Then cells were harvested and stained with APC-Cy7-anti-CD45 and FITC-anti-CD3, PE-DAZZLE594-anti-CTLA4 and BV786-anti-PD-1 (Biolegend, USA). Following fixation and permeabilization with the FIX&PERM kit (MultiSciences Biotech, China), cells were stained with APC-anti-Ki67, BV711-anti-CD107a, and AF700-anti-TNF-α (Biolegend, USA). Polychromatic flow cytometric analyses were performed on a BD LSRFortessaTM Cell Analyser and further analyzed using BD FACSDivaTM software.

Hematoxylin and eosin (H&E) staining and immunohistochemistry assay

Thymic tissues were isolated from recipient mice approximately 45 days post-transplantation, and were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned into continuous slices with a thickness of 4 μm. For H&E staining, deparaffinized sections underwent

rehydration through a graded ethanol series, followed by staining with hematoxylin for nuclear visualization. Then the sections were counterstained with eosin to highlight cytoplasmic and extracellular matrix components before being mounted with neutral balsam. Immunohistochemistry assays were conducted on the paraffin-embedded thymic sections using the following primary antibodies: Rabbit Anti-FoxN1, Rabbit Anti-EpCAM, and Rabbit Anti-IL22BP antibody (Bioss, China). The PV-9000 two-step immunohistochemical staining kit (Zhongshan Jinqiao Biotechnology Company, China), which includes DAB staining followed by hematoxylin counterstaining, was used in accordance with the manufacturer's instructions. Subsequently, the stained slides were examined under an inverted microscope (XDS-2B, Chongqing Optical & Electrical Instrument Co., China) and captured using the HPIAS-1000H high-definition color pathological image analysis system.

Detection of T cell receptor rearrangement excision circle (TREC)

Peripheral blood samples were collected from recipient mice at 30 and 45 days following transplantation. Genomic DNA was extracted using the TIANamp Genomic DNA Kit (TIANGEN BIOTECH, China) with subsequent RNAase treatment. The concentration and purity of the extracted DNA were assessed using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA). Signal joint TREC (sjTREC) levels were assessed through quantitative PCR performed on the ViiA 7 Real-Time PCR Systems (Applied Biosystems). β-actin was used internal reference gene to determine the DNA quality.

Primer sequence information is as follows:

sjTREC-forward: 5′-CAA GCT GAC AGG GCA GGT TT-3′;

sjTREC-reverse: 5′-TGA GCA TGG CAA GCA GTA CC-3′;

β-actin-forward: 5′-GGC TGT ATT CCC CTC CAT CG-3′;

β-actin-reverse: 5′-CCA GTT GGT AAC AAT GCC ATG T-3′;

Detection of the thymus-specific gene expression

Thymic tissues were isolated from recipient mice around 45 days following transplantation. Total RNA was extracted with RNA-Quick Purification Kit (ES Science, China). First-strand cDNA was reverse transcribed with a Fast All-in-One RT kit (ES Science, China). Quantitative

PCR was performed on a ViiA 7 Real-Time PCR System (Applied Biosystems) with 2× SYBR Green qPCR master mix (TIANGEN BIOTECH, China).

Primer sequence information is as follows:

Foxn1-forward: 5′-TGA CGG AGC ACT TCC CTT AC-3′;

Foxn1-reverse: 5′-GGG AAA GGT GTG GGT AGG TC-3′;

Wnt4-forward: 5′-CTC AAA GGC CTG ATC CAG AG-3′;

Wnt4-reverse: 5′-TCA CAG CCA CAC TTC TCC AG-3′;

DLL4-forward: 5′-TAT AAC CCT TTG GCC CAC TG-3′;

DLL4-reverse: 5′-ATG GGG AGG TCT GTT TTG TG-3′;

BMP4-forward: 5′-AAC CGA ATG CTG ATG GTC GT-3′;

BMP4-reverse: 5′-CTC TGG GAT GCT GCT GAG GT-3′;

β-actin-forward: 5′-GGC TGT ATT CCC CTC CAT CG-3′;

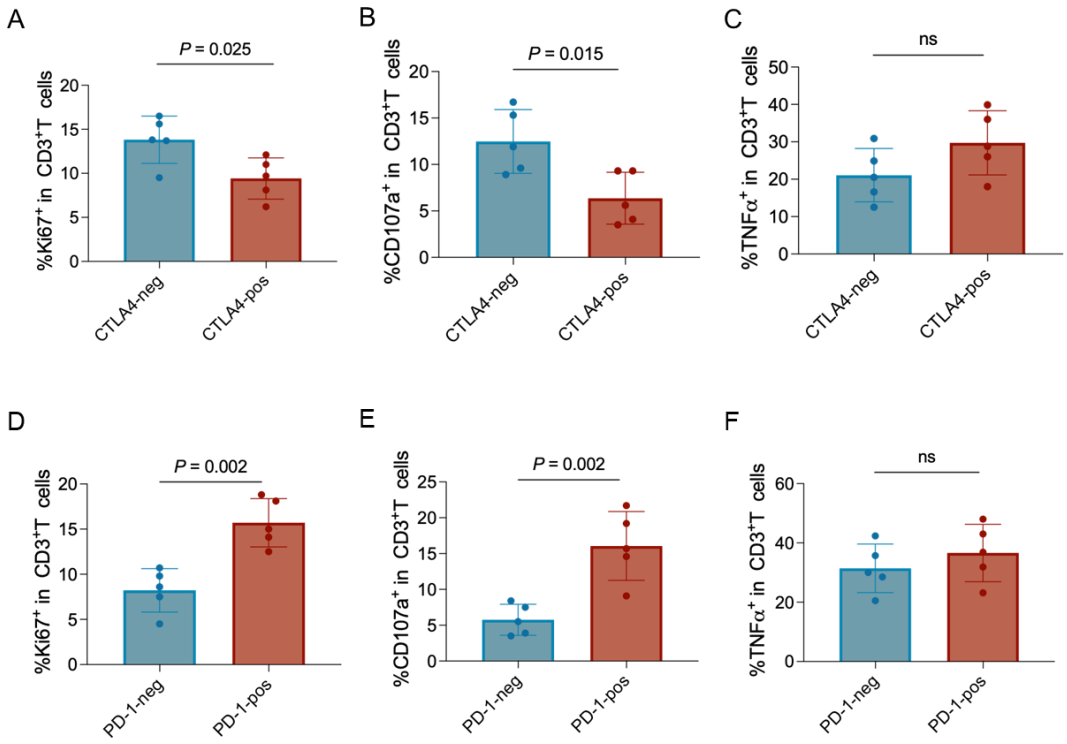
β-actin-reverse: 5′-CCA GTT GGT AAC AAT GCC ATG T-3′;

Ex-vivo treatment of thymocytes

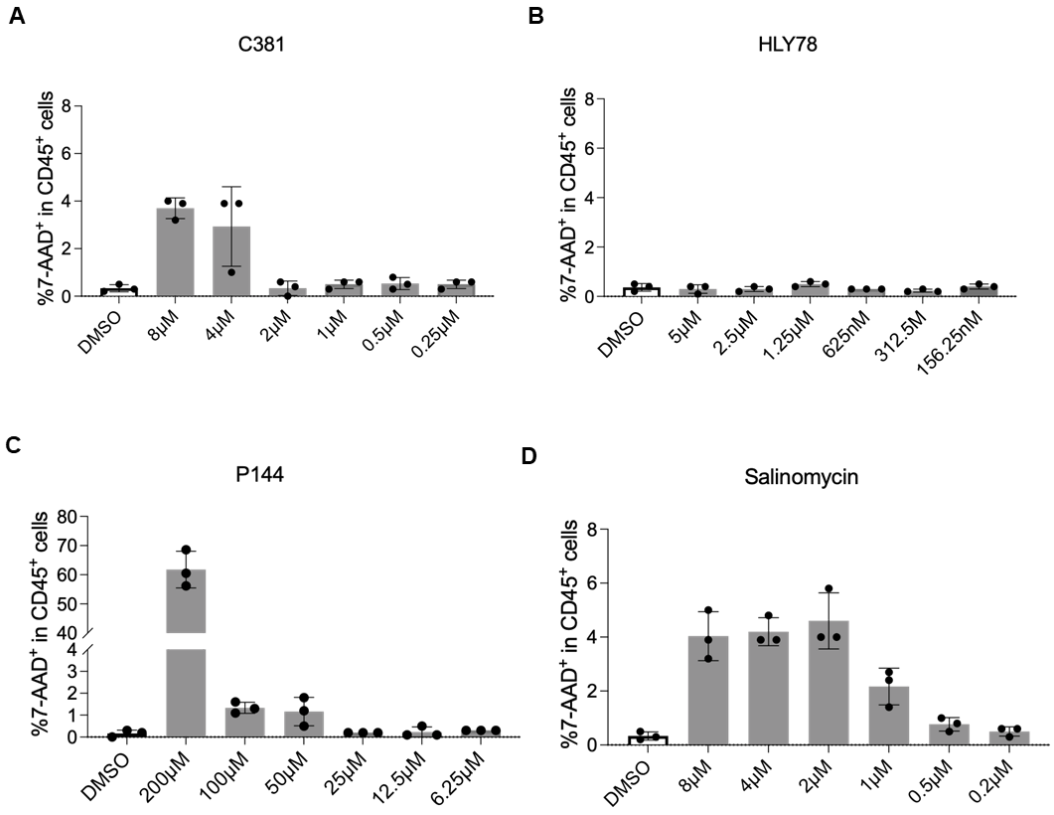
Thymic tissues were harvested from either normal C57BL/6 mice or haploHCT recipient mice. The tissues were digested with 0.2% collagenase IV for 30 minutes, followed by red blood cell lysis. The isolated thymocytes were cultured in 48-well plates containing RPMI 1640 medium supplemented with 10% FBS (Gibco, USA), mouse IL-7 (10 ng/mL, Stemcell Technologies，Canada), mouse FLT3/Flk2 Ligand (5 ng/mL, Stemcell Technologies, Canada) at 37℃ for 48 hours. Normal C57BL/6 thymocytes were treated with TGF-β1 agonist C381 and/or the LRP6 agonist HLY78 (Selleck Chemicals, USA) at varying concentrations. In contrast, thymocytes from haploHCT recipient mice were treated with different concentrations of the TGF-β1 inhibitor P144 and/or the LRP6 inhibitor Salinomycin (Selleck Chemicals, USA).

Cell apoptosis was assessed through staining with 7-AAD (Biolegend, USA). To detect the expressions of molecules related to thymocyte differentiation and maturation, cells were stained with the following fluorochrome-conjugated monoclonal antibodies: APC-Cy7-anti-CD45, FITC-anti-CD3, PerCP-Cy5.5-anti-CD4, BV510-anti-CD8a, BV650-anti-CD127; PE-Cy7-anti-CD62L; and BV605-anti-CXCR4 (Biolegend, USA) at room temperature for 15 minutes. Polychromatic flow cytometric analyses were performed on a BD LSRFortessaTM

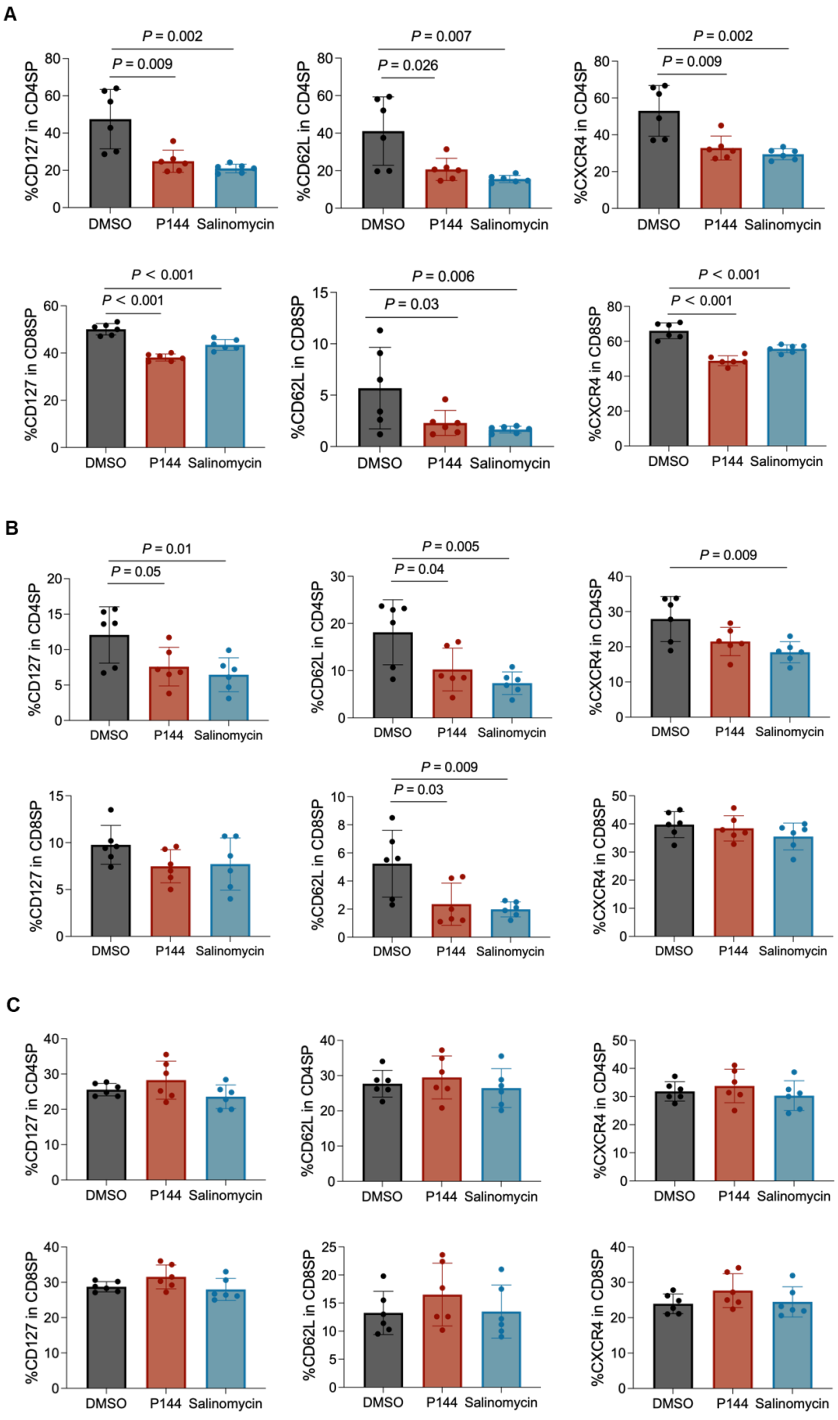
Cell Analyser and analyzed using BD FACSDivaTM software.



Supplementary Figure 1. Functional assay for CTLA4 and PD-1 positive T cells. Splenocytes were harvested from recipient mice following haploHCT and stimulated with 5 μg/mL ConA for 48 hours. The Protein Transport Inhibitor Cocktail was administered 6 hours prior to detection by flow cytometry. (A-C) The expressions of Ki67, CD107a, and TNF-α in $CD3^+$ T cells were compared between the CTLA4- negative (neg) and positive (pos) fractions. (D-F) The expressions of Ki67, CD107a, and TNF-α in $CD3^+$ T cells were compared between the PD-1-neg and PD-1-pos fractions. n = 5; *P* values are indicated on the graphs.



Supplementary Figure 2. The cytotoxic effects of TGF-β1 and LRP6 pathways-related modulators on primary thymocytes. Primary thymocytes were treated with the indicated concentrations of TGF-β1 and LRP6 agonists (C381 and HLY78, A, B) or inhibitors (P144 and Salinomycin, C, D) for 24h. The percentage of 7-AAD$^+$ fraction in CD45$^+$ cells were analyzed by flow cytometry (n = 3).



Supplementary Figure 3. Dose-dependent effects of pathway inhibitors on primary thymocytes from haploHCT recipient mice. The expressions of CD127, CD62L, and CXCR4 on CD4SP and CD8SP thymocytes were assessed by flow cytometry following treatment with varying doses of TGF-β1 inhibitor P144 and LRP6 inhibitor Salinomycin. (A) Treatment with 25 μM of P144 and 200 nM of Salinomycin. (B) Treatment with 2.5 μM of P144 and 100 nM of Salinomycin. (C) Treatment with 20 nM of P144 and 5 nM of Salinomycin.