Low-dose decitabine modulates T cell homeostasis and restores immune tolerance in immune thrombocytopenia

Supplemental Methods

RNA sequencing

RNA of PBMCs were extracted from patients and the RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) after running electrophoresis using 1% agarose gels. RNA concentration was measured using Qubit® RNA Assay Kit in Qubit®2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total of 3 μg of RNA per sample were used as input material for the RNA sequencing library preparations. Sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's recommendations. To select cDNA fragments of preferentially 250-300 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μl of USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min, followed by 95 °C for 5 min before PCR. PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and 125 bp/150 bp paired-end reads were generated.

Next, using a previously described method,[1] raw data were filtered and mapped to human transcriptome hg19, and read counts for each gene were generated by feature Counts v1.5.0-p3. Next, Fragments Per Kilobase Million (FPKM) of each gene was calculated based on the length of the gene and reads count mapped to this gene. Differential expression analysis of two groups was performed using the EdgeR package (1.16.1). Data were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted $P < 0.05$ found by EdgeR were assigned as differentially expressed. Gene Ontology (GO) and KEGG

enrichment analysis were performed to identify potential upstream transcriptional regulators, associated diseases and functions, and enriched canonical pathways of the differentially expressed genes. We used cluster Profiler R package to test the statistical enrichment.

Western blot analysis

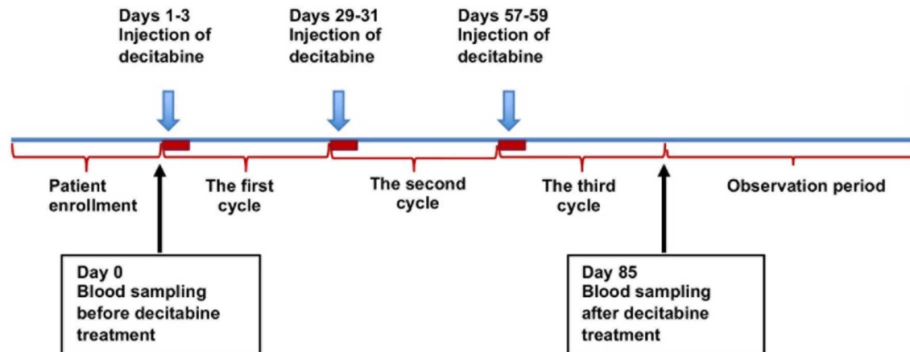
PBMCs from patients were lysed in radio-immunoprecipitation assay (RIPA) buffer (Bestbio). The protein levels were determined using a BCA kit (Beyotime, Shanghai, China). The proteins (30-50 mg per lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were then blocked with 5% bovine serum albumin and incubated overnight with antibodies against p-STAT3, total STAT3, p-Akt, total Akt, and GAPDH (Cell Signaling Technology, NJ, USA) at dilutions specified by the manufacturer's instructions. Anti-rabbit secondary antibodies and an ECL chemiluminescence detection system (Pierce Biotechnology Inc, Rockford, IL) were used to scan and semi-quantitatively analyze the proteins.

Reference:

1. Bao ZS, Chen HM, Yang MY, et al. RNA-seq of 272 gliomas revealed a novel, recurrent PTPRZ1-MET fusion transcript in secondary glioblastomas. *Genome Res*. 2014; 24(11):1765-1773.

Supplemental Figures

Figure S1. The timeline for decitabine treatment and blood sampling of ITP patients.

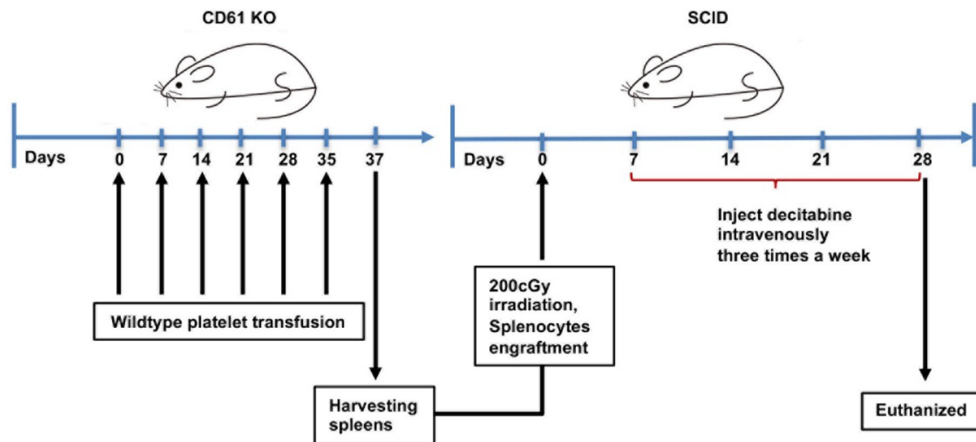


Patients received decitabine at 3.5 mg/m^2 intravenously for three days per cycle for three cycles with a four-week interval.[1] Blood samples were taken before the first injection (day 0) and after 12 weeks of decitabine therapy (day 85), respectively.

Reference:

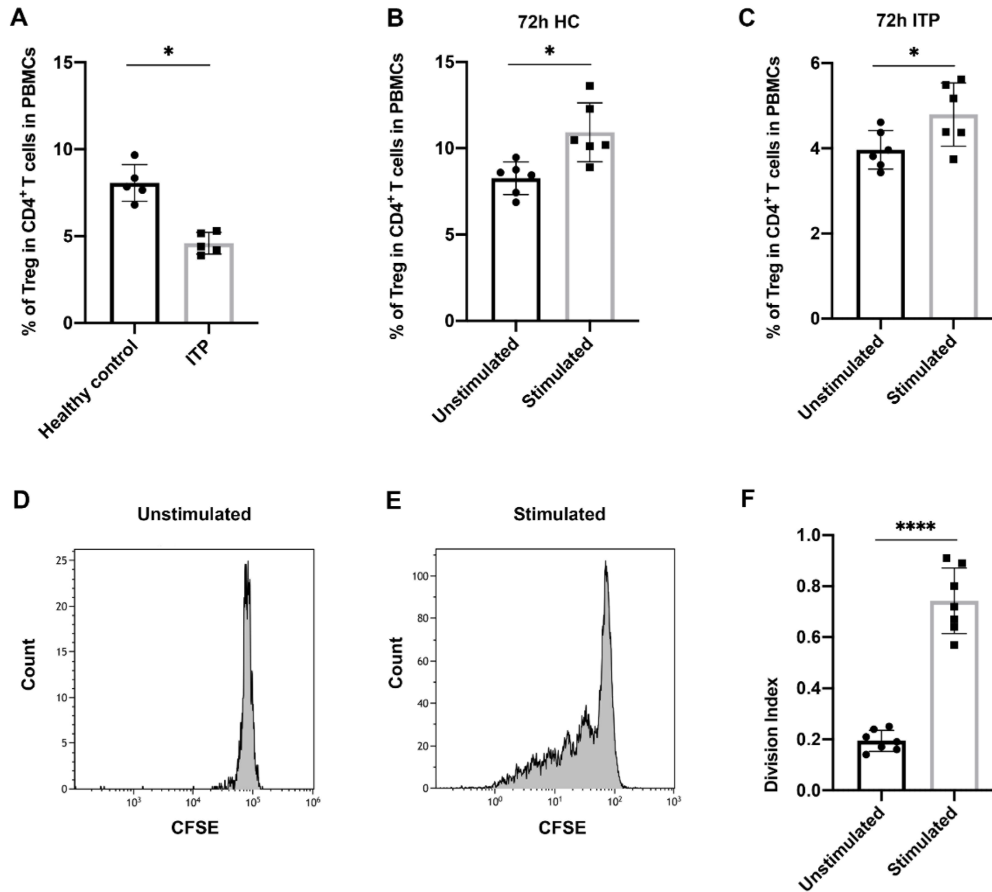
1. Zhou H, Qin P, Liu Q, et al. A prospective, multicenter study of low dose decitabine in adult patients with refractory immune thrombocytopenia. Am J Hematol. 2019; 94(12):1374-1381.

Figure S2. The timeline for ITP murine models.



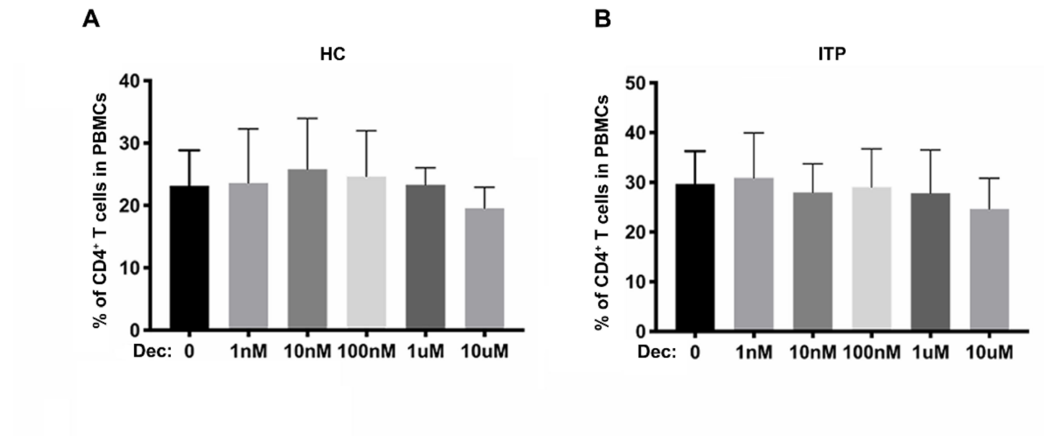
CD61 knockout (KO) mice were transfused weekly with 10^8 platelets from wild-type C57BL/6J mice for 6 consecutive weeks. The spleens of CD61 KO mice were taken and prepared into splenocyte suspensions. On the day of splenocyte transfer, severe combined immunodeficient (SCID) mice were subjected to 200 cGy total body irradiation to inhibit recipient innate immune responses and enhance engraftment. Within 3 hours of irradiation, SCID mice were injected intraperitoneally with 2×10^4 splenocytes of CD61 KO mice. Different doses of decitabine were administrated intravenously three times a week for three weeks. Platelets were monitored weekly. SCID mice were euthanized at day 28 and splenocytes were obtained for analysis.

Figure S3. The baseline Treg cells and CD4$^+$ T cell proliferation with or without stimulation in healthy controls and ITP patients.



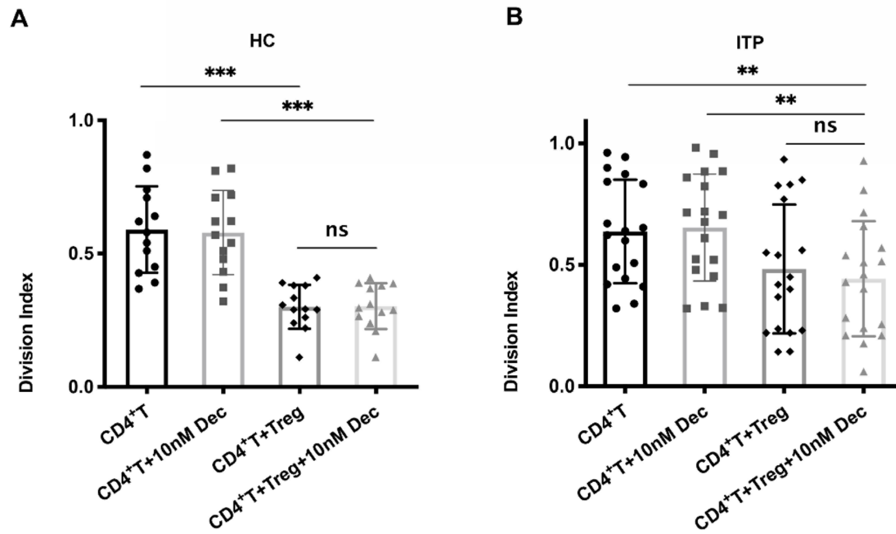
(A) The direct assay of Treg cells in CD4$^+$ T cells in PBMCs from ITP patients and healthy controls. (B) The percentage of Treg cells in CD4$^+$ T cells in PBMCs from healthy controls after 72 hours culture with or without stimulation. (C) The percentage of Treg cells in CD4$^+$ T cells in PBMCs from ITP patients after 72 hours culture with or without stimulation. (D-F) T cell receptor agonists and co-stimulation are essential to maintain CD4$^+$ T cell proliferation. HC, healthy controls. *$P < 0.05$, ****$P < 0.0001$.

Figure S4. Decitabine had no influence on the percentage of $CD4^+$ T cells in PBMCs from healthy controls or ITP patients.



The percentages of $CD4^+$ T cells in PBMCs co-cultured with decitabine were not significantly changed in healthy controls (A) or ITP patients (B). HC, healthy controls.

Figure S5. Decitabine (10 nM) did not enhance the immunosuppressive effect of Treg cells in healthy controls (A) or ITP patients (B).



Teff cells (2×10^5 cells per well) were seeded in 96 well-plate with or without Treg cells (5×10^4 cells per well) in the presence of 10 nM decitabine. Cell division index is calculated based on the dilution of CFSE fluorescence measured by flow cytometry and represent the average number of cell divisions that Teff cells in the original population have undergone [division index = sum (i * N(i) / 2^i) / sum (N(i) / 2^i), "i" is division number (undivided = 0), and "N(i)" is the number of cells in division "i"]. HC, healthy controls; ns, no significance. **P < 0.01, ***P < 0.001.

Figure S6. Splenocyte transfer itself did not affect platelet counts in SCID mice.

The three lines represent the platelet counts in unirradiated SCID mice transferred with 2×10^4 non-immunized splenocytes from CD61 knockout (KO) mice (▼), irradiated SCID mice transferred with either 2×10^4 non-immunized splenocytes from CD61 KO mice (■) or 2×10^4 splenocytes from CD61 KO mice immunized by platelets (▲), respectively. n = 6. ***$P < 0.001$.

Figure S7. High-dose decitabine can’t be tolerated in active ITP mice.

Different doses of decitabine (0, 0.01 mg/kg, 0.03 mg/kg, 0.06 mg/kg, 0.1 mg/kg, 0.3 mg/kg and 0.6 mg/kg) were administrated intravenously. Decitabine at high doses (0.1 mg/kg, 0.3 mg/kg and 0.6 mg/kg) resulted in almost all ITP mice death. The intermediate dose (0.06 mg/kg) of decitabine caused more than half of ITP mice death, although the survivals showed a little higher platelet count. As a result, we chose 0.03 mg/kg as the dose of decitabine for ITP murine studies.