Supplementary materials

Low-dose IL-2 therapy compensates for metabolic shifts and rescues anxiety-like behavior in PD-1-deficiency-induced autoimmunity

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Materials and methods

Experimental animals and treatment

Pdcd1$^{-/-}$ mice (C57BL/6N) were provided by Dr Tasuku Honjo [1]. Mice were housed in groups of four and given seven days to acclimate to the housing facility under specific-pathogen-free (SPF) conditions. Environmental conditions were a temperature of 22 °C ± 1 °C, humidity of 50% ± 5%, and a reversed 12/12h light/dark cycle (lights off at 8:00 a.m. and on at 8:00 p.m.). During housing, animals were monitored twice daily for health status. No adverse events were observed. Heterozygous breeding generated matched wild-type (WT) for experiments as needed. 2-month-old male *Pdcd1*$^{-/-}$or WT mice were intraperitoneally administered IL-2 (recombinant human interleukin-2Ser125, Beijing SL Pharma) at a dose of 30000 IU or PBS every other day for 3 months. The mice were randomly assigned. The experimenters were blinded to the pharmacological treatment while processing data and making exclusion decisions. All animal experiments were carried out under an Shandong University of Traditional Chinese Medicine Laboratory Animal Welfare Ethics Review Committee approved license (number SDUTCM201804011) and in accordance with the Animals (Scientific Procedures) Act, 1986 (UK) (amended 2013). The OFT was conducted using 43 animals, n = 10 (WT + Vehicle), n = 12 (WT + IL-2), n = 11 (*Pdcd1*$^{-/-}$ + Vehicle), and n = 10 (*Pdcd1*$^{-/-}$ + IL-2). The EMP test was conducted using 10 animals per group. The metabolic analysis was conducted using 7 animals per group. Flow cytometry analysis was conducted using 5 (*in vivo*) or 8 (*in vitro*) animals per group.

Behavioral tests

Open-field and Elevated-plus maze tests were performed as described previously [2]. All experiments were conducted during the dark phase of the light/dark cycle under dim red light conditions (10:00 a.m. to 5:00 p.m.).

The Open-field test (OFT) was performed in an open-topped Plexiglas arena (50 cm × 50 cm × 50 cm). Each animal was placed in the arena and was allowed to explore it for 6 min, and all movements of the animals within the arena were automatically recording using the

XR-SuperMaze video tracking and analysis system (Shanghai SOFTMAZE Information Technology Co., Ltd, Shanghai, China). The behaviors within the arena were analyzed according to the total distance moved, the distance travelled in the center areas, the time spent in the center and the center entries.

The Elevated-plus maze (EPM) was made of black perspex with two open arms (35 cm × 5 cm) and two closed arms of the same size. The closed arms were enclosed by walls (15 cm), and the open arms had 0.5 cm edges in order to maximize entries into the open arm. The arms and center square (5 cm × 5 cm) elevated to a height of 80 cm above the floor. Individual mice were placed in the center square facing an open arm, and were allowed to explore the maze for 5 min. The time spent in an arm of the maze was recorded starting when two paws had crossed the line into the arm. The number of entries into the arms and the time spent in the arms were used as a measure of the locomotor activity of the mice in the maze.

Measurement of serum amino acid

Sample preparations for serum metabolic analysis were performed as described previously [1]. In brief, 50 μl of serum was mixed with 250 μl of solvent (methanol : water : chloroform = 2.5 : 1 : 1) containing 6 μl of 0.1 mg/ml 2-isopropylmalic acid (Sigma-Aldrich), which was used as an internal standard. The mixture was shaken at 1200 r.p.m. for 30 min at 37 °C. After centrifugation at 16,000g for 5 min at 25 °C, 150 μl of supernatant was collected and mixed with 140 μl of purified water by a vortex mixer for 5 s. After centrifugation at 16000g for 5 min at 25 °C, 50 μl of supernatant were collected for Liquid chromatography-tandem mass spectrometry (LC-MS/MS, Thermo Fisher Scientific), according to the published methods [1].

Measurement of brain neurotransmitters and amino acid

Extraction of metabolites from brain for metabolomics was performed as described previously [1]. In brief, the frozen tissue was homogenized in ice-cold methanol (500 μl) using a manual homogenizer (TIANGEN, OSE-Y30), followed by the addition of an equal volume of chloroform and 0.4 times the volume of ultrapure water. The suspension was then

centrifuged at 15000g for 15 min at 4 °C. After centrifugation, the aqueous phase was filtered using an ultrafiltration tube. Neurotransmitters and amino acids were analyzed by high performance liquid chromatography coupled to an electrochemical detector (HPLC-ECD, SYKAM) or Liquid chromatography-tandem mass spectrometry (LC-MS/MS, Thermo Fisher Scientific), according to the published methods [1].

Flow cytometry analysis

Lymphocyte isolation and analysis were performed as described [3]. In brief, spleens were mashed and disrupted to form a single-cell suspension. Then the cells were carried on cell-surface and intracellular staining. The following fluorochrome-labelled monoclonal antibodies were purchased from BD Pharmingen or Biolegend and used according to the manufacturers' protocols: anti-CD8α (Clone: 53-6.7), anti-CD4 (Clone: RM4-5), anti-CD44 (Clone: IM7), anti-B220 (Clone: RA3-6B2), anti-CD3 (Clone: 17A2), anti-CD62L (Clone: MEL-14), anti-CD25 (Clone: PC61) and anti-Foxp3 (Clone: MF23). 7-AAD (Thermofisher) was used to exclude dead cells. For intracellular staining, cells were permeabilized using Fixation/Permeabilization solution (eBioscience) after surface staining and incubated with anti-Foxp3 for 30min at 4°C. Samples were then washed and stored at 4°C until acquisition. Flow cytometry was performed using a BD FACSAria III (BD Bioscience) and analyzed by FlowJo software.

Treg in vitro suppression assay

Treg *in vitro s*uppression assay was performed as described [3]. In brief, lymph nodes (axillary, brachial and inguinal) and spleens were harvested and disrupted to form a single-cell suspension. Tregs ($CD25^+$ $CD4^+$ $B220^-$ $CD3^+$) or naïve T cells ($CD62L^+$ $CD44^{lo}$ $CD25^-$ $CD4^+$ $B220^-$ $CD3^+$) were sorted using a FACSAria III cell sorter (BD Bioscience). Naïve T cells from WT mice were labled with CFSE (Invitrogen) and stimulated by Mouse T-Activator CD3/CD28 Dynabeads (Invitrogen) in the absence or presence of Treg cells for 3 d. T cell proliferation was measured by flow cytometry. Percentages of divided cells and division indices (the average number of cell divisions that a cell in the original population has

undergone) were calculated as per instructions (FlowJo).

Statistical analysis

Group comparisons by two-tailed unpaired Student's t-test or ANOVA were performed using GraphPad Prism 7. Correlation analysis by multiple linear regression was performed using the SPSS statistical software (SPSS 25.0).

References

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3 He, J. et al. Low-dose interleukin-2 treatment selectively modulates CD4(+) T cell subsets in patients with systemic lupus erythematosus. *Nat Med*. **22**, 991-993 (2016).