Supplemental materials

Prolactin mediates psychological stress-induced dysfunction of regulatory T cells to facilitate intestinal inflammation

Running title: Stress and mucosal inflammation

Wei Wu*, Mingming Sun*, Huan-Ping Zhang[ξ], Tengfei Chen*, Ruijin Wu*, Changqin Liu*, Gui Yang[†], Xiao-Rui Geng[†], Bai-Sui Feng*, Zhigang Liu[¶], Zhanju Liu*, Ping-Chang Yang[¶]

*Department of Gastroenterology, The Shanghai Tenth People's Hospital of Tongji University, Shanghai 200072, China; [¶]Shenzhen Key Laboratory of Allergy & Immunology, Shenzhen University School of Medicine and State Key Laboratory of Respiratory Disease for Allergy at Shenzhen University, Shenzhen, China. [ξ]Department of Respirology, the First Hospital, Shanxi Medical University, Taiyuan, China. [†]Longgang Central Hospital, ENT Hospital, Shenzhen ENT Institute, Shenzhen, China.

Wu W, Sun M and Zhang HP share the first authorship.

Corresponding authors: Dr. Zhanju Liu (301 Yanchang Road, Zhabei District, Shanghai 200072, China. Email: liuzhanju88@126.com; Tel: +86 21 66301164; Fax: +86 21 66303983) and Dr. Ping-Chang Yang (pcy2356@szu.edu.cn; Room 204 of Medical School Building; 3688 Nanhai Ave., Shenzhen, China).

Reagents

Lipopolysaccharide (LPS), TNBS, DSS, Adrenocorticotropic Hormone (ACTH), corticotropin releasing factor (CRF), prolactin (PRL), cabergoline (the inhibitor of PRL), horseradish peroxidase (HRP, type II), recombinant IL-4 and GM-CSF, o-dianisidine dihydrochloric acid, hexadecyl-trimethylammonium bromide and ortho-dianisidinedihydrochloride were purchased from Sigma-Aldrich (Shanghai, China). The antibodies of IL-17, TNF-α, Foxp3, IL-6, IL-23 and BAY 11-7082 were purchased from Santa Cruz Biotech (Shanghai, China). Neutralizing anti-IL-6 and IL-23, the ELISA kits of IL-6, IL-23, IL-17, interferon (IFN)-γ and TNF-α were purchased from R&D Systems (Shanghai, China). The reagent kits for magnetic cell sorting (MACS) were purchased from Myltenyi Biotech (Shanghai, China). The reagents for quantitative real time RT-PCR (qRT-PCR) were purchased from Invitrogen (Shanghai, China). The antibodies for flow cytometry were purchased from BD Biosciences (Shanghai, China). The endotoxin levels in all reagents were detected using the Limulus assay (Limulus amebocyte lysate QCL 1000, BioWhittaker, Walkersville, MD, USA). The reagents used in this study contained < 0.2U endotoxin/10 µg reagents.

LPMC isolation and culture

The small intestine was excised from mice and washed with RPMI1640 medium; the visible Peyer's patches were removed with scissors. The intestine was opened longitudinally, cut into 5 mm pieces and then incubated in 0.5 mM EDTA in calcium and magnesium free Hanks' for 20 min at 37°C with shaking to release intraepithelial lymphocytes and epithelial cells. Then, the tissue was incubated 30 min at 37°C in 20 ml RPMI1640 medium containing 1 mg/ml collagenase, 10% FCS, 25 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. After brief shaking the sample tubes by hands, the cells were filtered through a cell strainer (70 μm in diameter). After washing, cells were layered onto Percoll in a 50 ml conical tube with a 30:70% gradient. Cells were centrifuged at 2200 xg for 20 min. As checked by trypan blue exclusion assay, the viability of the cells was greater than 90%. The cells were cultured in RPMI1640 medium for further experiments.

Isolation of CD4$^+$ CD25$^+$ Treg cells

The mouse spleen single cells and lamina propria mononuclear cells (LPMC) were prepared with our established procedures. The cell suspension was added with the antibody cocktail (containing biotinylated antibodies against CD8, CD11b, CD45R, CD49b and Ter-119) and incubated at 4 °C for 20 min followed by incubating with magnetic beads conjugated to anti-biotin antibody. Elution through a column under a magnetic field enriched the unlabeled CD4$^+$ T cells. The cells were washed with PBS

and incubated with PE-labeled anti-mouse CD25 Ab and anti-PE magnetic microbeads. The cells were positively selected by passing through a column under a magnetic field. Cell purity was greater than 98% as evaluated by flow cytometry.

Generation of bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BMDCs) were prepared from bone marrow isolated from the femurs of male BALB/c mice. The cells were flushed from the femurs with RPMI1640 medium; the red blood cells were removed by adding lyse buffer to the cells; the cells were washed three times and re-suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum. The cells were cultured in 75 cm^2 flasks at a density of 1×10^6 cells/ml supplemented with 40 ng/ml recombinant murine GM-CSF and 10 ng/ml recombinant murine IL-4. The medium was changed every other day. On day 6, the cells were collected and treated with a DC-isolation kit; the CD11c$^+$ CD11b$^+$ B220$^-$ DCs were isolated by MACS following the manufacturer's instruction.

Western blotting

The protein extracts were separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and transferred onto nitrocellulose membranes. Western blotting was performed using the indicated antibodies (detailed in figure legends). The immune blots were photographed with the UVP BioSpectrum Imaging System (Upland, CA, USA).

Quantitative real time RT-PCR (qRT-PCR)

Total RNA was isolated from the cells. cDNA was synthesized using a cDNA synthesis

Kit. The qRT-PCR was performed on a MiniOpticon real-time PCR detection system

(Bio-Rad Laboratories, Shanghai, China) using the SYBR Green Master Mixes. The

sequences of the primers using in this study were listed in Table 1. Samples were run

in duplicate, and a reaction without cDNA was used as a control. The relative

quantification of expression of the genes was determined by the $2^{-\Delta\Delta CT}$ method.

Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-6, IL-23, IL-17, interferon (IFN)-γ and TNF-α were determined by ELISA

with commercial reagent kits following the manufacturers' instructions.
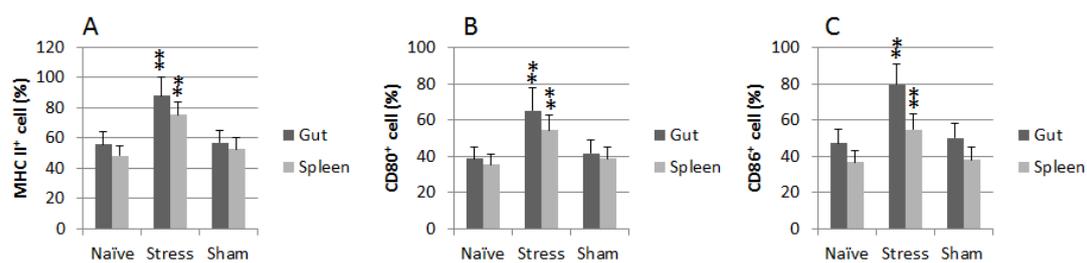
Figure S1 Stress activates DCs in mice. BALB/c mice were treated with stress or sham

stress as described in the text. With the same procedures of Fig 2, intestinal and

spleen DCs were isolated and analyzed by flow cytometry. The bars indicate the

frequencies of MHC II$^+$ (A), CD80$^+$ (B) and CD86$^+$ (C) DCs. The data are presented as

mean ± SD; **, p<0.01, compared with naïve groups. Each group consists of 9 mice.

Specimens from 3 mice were pooled as one sample to process. The data represent 3

separate experiments.
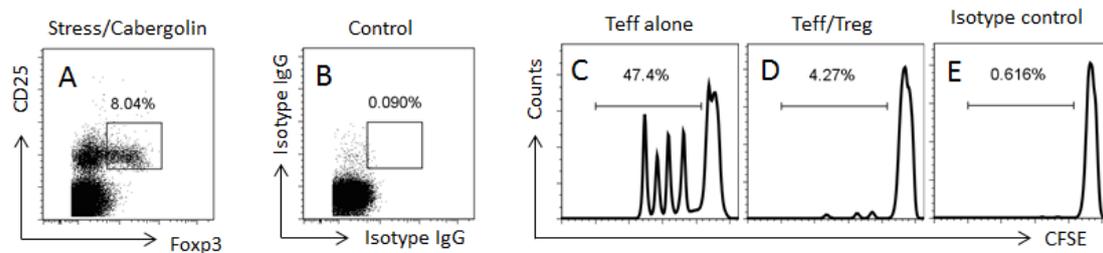
Figure S2 Antagonists of PRL on preventing the inhibitory effect of stress on Treg's

suppressor function. Affiliated to Figure 1, mice were treated with stress as

described in Figure 1. Cabergoline was administered to the mice (i.p.) 30 min prior to

each stress session at a dose of 500 µg/kg body weight. After sacrifice, samples were

taken from mice and processed in the same procedures of Figure 1. A, the gated dot

plots indicate the frequency of $CD25^+$ $Foxp3^+$ Tregs in $CD4^+$ T cells ($CD4^+$ T cells were

gated first) in the intestine. B, an isotype IgG control. C-E, the histograms indicate the

Teff cell proliferation with the same procedures of Figure 1. C, Teff cells were isolated

with commercial reagent kits; the Teff cells were cultured in the presence of

anti-CD3/CD28 antibodies for 3 days. D, Teff cells were cultured with isolated Tregs at

a ratio of 10^6:2 × 10^5 (Teffs:Treg) with the same procedures of panel C. Panel D is a

control. The data were from 9 mice; cells from 3 mice were used as one sample.
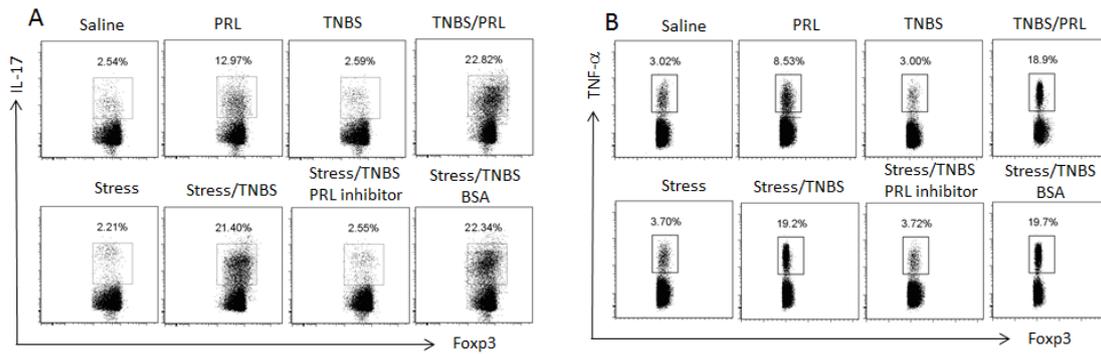
Figure S3 PRL modulates Treg phenotypes in the intestine. Affiliated to Figure 6, the gated dot plots indicate the frequencies of IL-17$^+$ Foxp3$^+$ cells (A) or TNF-α^+ Foxp3$^+$ cells (B) in the gated Tregs in Figure 6C. The summarized data of A and B are presented in Figure 6E.
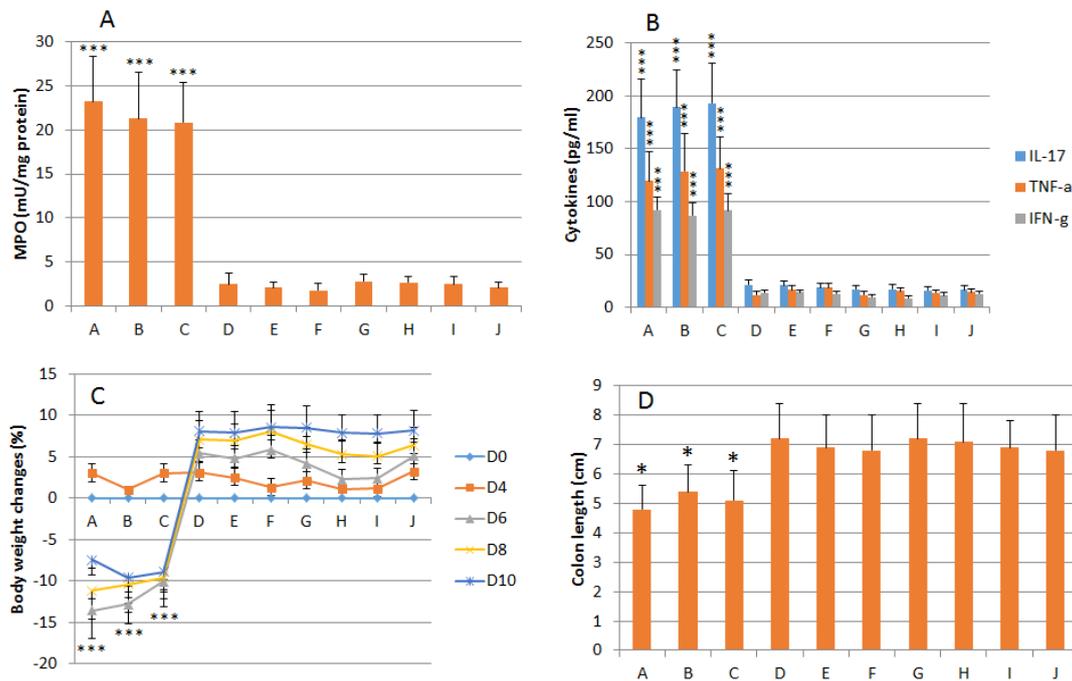
Figure S4 The levels of MPO, cytokines and body weight changes of mice affiliated to Figure 7. The treatment of mice and sample process are described in Figure 7. A, the bars indicate the levels of MPO in the colon extracts. B, the bars indicate the serum levels of IL-17, TNF-α and IFN-γ. C, the curves indicate the mouse body

changes in the course of treatment. D, the bars indicate the colon length. The groups are labeled on the x axis, which are the same as the image labels of Figure 7. The data are presented as mean ± SD. *, p<0.05, ***, p<0.001, compared with group J (the saline control group). Each group consists of 6 mice. Samples from individual mice are processed separately.