ONLINE DATA SUPPLEMENT

Role of B7-H3/IL-33 signaling in pulmonary fibrosis-induced profibrogenic alterations in bone marrow

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SUPPLEMENTAL METHODS

Animals and BLM or FITC induced pulmonary fibrosis

Female 6-8 week old C57BL/6 were purchased from the Jackson Laboratory (Bar Harbor, ME) or Japan Charles River Laboratories (Kanagawa, Japan), and maintained in a specific pathogen-free environment. B7H3 knockout (KO) mice and ST2 KO mice on C57BL/6 background were kindly provided by Professor Tak W. Mak (University of Toronto, Toronto, Canada), and Dr. Stefan Wirtz (Friedrich Alexander Universität Erlangen, Erlangen, Germany), respectively. Pulmonary fibrosis was induced by the endotracheal injection of 2 U/kg body weight BLM (Blenoxane; Mead Johnson, Plainsboro, NJ). Control mice received sterile saline (SAL) alone. In experiments where BLM pretreatment was indicated, this was undertaken as above but with 60% of the normal dose (1.2 U/kg body weight). A subsequent full dose BLM treatment was given after the indicated time interval (9 or 24 weeks) from the pretreatment. At each BLM treatment time, SAL was injected to the control animals. This experiment was repeated using the B7H3 KO mice using the 9-week time interval. As indicated in some experiments 6 weeks after BLM pretreatment, the mice underwent BMT using BM from naïve mice followed by the full dose BLM treatment 6 weeks later (total of 12 weeks after BLM pretreatment). Where indicated 15 mg/kg body weight of FITC (Sigma-Aldrich, St. Louis, MO) in PBS was endotracheal injected to induce lung fibrosis. For *in vitro* experiments using BM cells, whole BM cells were isolated from SAL (control), BLM or

FITC treated WT, B7H3 KO or ST2 KO mice. Where indicated lineage negative BM cells were isolated using a mouse lineage cell cocktail kit (CD3e, CD11b, B220, Ter-119, Ly6C and Ly6G), and purified with MACS separation system (Miltenyi Biotec Inc., San Diego, CA). Obtained BM cells were further treated with or without 5 μg/ml of recombinant mouse B7H3 (R & D Systems, Minneapolis, MN, USA) for 24 hours. Where indicated, whole BM cells obtained from SAL, BLM or FITC treated mice, were stimulated with 10 ng/ml of GM-CSF (R&D Systems) for 5 days, and then further treated with or without sB7H3 for another 24 hours.

BMT and cell transfer

BLM-induced pulmonary fibrosis was established by endotracheal BLM injection in recipient mice 6 weeks after BMT. In some experiments, BLM- or SAL-pretreated mice were used as BM donors. Whole BM cells (2×10^6 cells) or where indicated presorted cells (2×10^3 cells for hematopoietic stem cells [HSCs] or 1×10^6 cells for monocytes) from BLM- or SAL-treated donor BM were transferred by intravenous injection into recipient mice in combination with BM from naïve donors. HSCs ($cKit^+/Sca1^+/Lin^-$) were obtained by fluorescence-activated cell sorting. Monocytes were obtained by magnetic-activated cell sorting system using Monocyte Isolation Kit (Miltenyi Biotec Inc., Auburn, CA), which enriched the monocytic population by depleting differentiated blood cells positive for CD3ε, CD45R/B220, NK1.1, Ly6G, and CD49b, and majority of sorted monocytic cells are Ly6C positive based on a flow staining result provided on the kit data sheet (https://www.miltenyibiotec.com/US-en/products/macs-cell-separation/cell-separation-reagents/microbeads-and-isolation-kits/monocytes-and-macrophages/monocyte-isolation-kit-bm-mouse.html).

Flow-cytometry and cell sorting

Single-cell suspensions of lung cells were obtained by mincing following chemical digestion with collagenase III and DNase I (Worthington Biochemical Corporation, Lakewood, NJ) at

37°C for 90 minutes. Red blood cells in obtained whole lung cell suspensions or collected peripheral blood cells were lysed with ACK buffer (Thermo Fisher Scientific, Waltham, MA). Then, these cell suspensions were blocked with anti-mouse CD16/32 Abs (FcγR, clone 2.4G2; BD biosciences, San Diego, CA), before incubation with the appropriate dilutions of antibodies or their isotype-matched control. We used rat mAbs (Biolegend, San Diego, CA) for anti-mouse CD3 (clone: 17A2), CD4 (RM4-5), CD8 (53-6.7), CD11b/Mac1 (M1/70), CD45R/B220 (RA3-6B2), CD117/cKit (2B8), B7H1/CD274/PD-L1 (10F.9G2), B7H2/CD275/B7RP1/ICOSL (HK5.3), B7H3/CD276/B7RP2 (RTAA15), F4/80 (CI: A3-1), Gr1 (RB6-8C5), Ly6C (HK1.4), Sca1 (Ly6A/E, D7), TER119 (TER-119), and VEGFR1 (Y103, Abcam, Cambridge, MA, USA). We also used hamster mAbs for anti-mouse CD11c (N418, Biolegend). Macrophages were defined as F4-80$^+$. T cells or B cells were defined as CD3$^+$ or B220$^+$ on lymphocytes gated cells, respectively. HSCs were defined as cKit$^+$/Sca1$^+$/Lin$^-$ (lineage markers included CD4, CD8, CD11b/Mac1, CD45R/B220, Gr1, and TER119). A BD LSR II machine (BD biosciences, San Diego, CA) was used for cell analyses, while sorting was accomplished using a BD FACS Aria II machine (BD biosciences). Data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR).

Purification of RNA, reverse transcription and real-time quantitative PCR

Total RNA was extracted from lung tissue using RNeasy Mini Kit (Qiagen, Hilden, Germany). After reverse-transcribing into cDNA with High Capacity cDNA Reverse Transcription Kit (Life Technologies), real-time PCR was performed using an ABI Prism 7500 sequence detector system, and SDS analysis software (Applied Biosystems, Foster City, CA). We used sequence-specific TaqMan inventoried primers and probes (Applied Biosystems) for *Areg* (Mm01354339_m1), *CD276* (Mm00506020_m1), *Il4* (Mm00445259_m1), *Il13* (Mm00434204_m1), *Il33* (Mm00505403_m1), *Ifng* (Mm01168134_m1), *Tgfb1* (Mm03024053_m1), *Col1a1* (Mm00801666_g1), *Acta2*

(Mm01546133_m1), and *18S* (Mm03928990_g1). For each assay, 100 ng total RNA was used as template. The signal of *18S* was used as internal control.

ELISA

sB7H3 levels in human plasma and BALF samples were measured using commercially available ELISA kit (R&D Systems). sB7H3 levels in mouse BALF samples were measured using commercially available ELISA kit (LifeSpan Biosciences Inc., Seattle, WA) according to the manufacturer's protocol.

SUPPLEMENTAL FIGURE LEGENDS

Figure E1

Effect of magnetic-activated cell sorting to enrich monocytic cells

Representative image of bone marrow $Ly6C^+$ monocytic cells before and after magnetic-activated cell sorting. The numbers indicated the % of $Ly6C^+$ cells in bone marrow cells before and after cell sorting.

Figure E2

Effect of low dose bleomycin treatment on pulmonary inflammation and fibrosis

(A) Lung hydroxyproline content in each group at 3 or 6 weeks after SAL- or initial low dose (60% of normal dose) BLM-treatment (n=6/group). (B) BALF total cells obtained 3 week after SAL- or low dose BLM-treatment (n=5-6/group). * $P<0.05$ vs. SAL/BLM group.

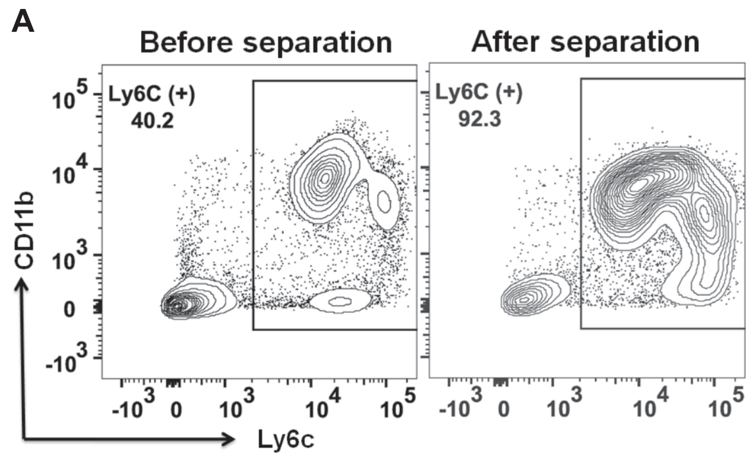

Figure E1

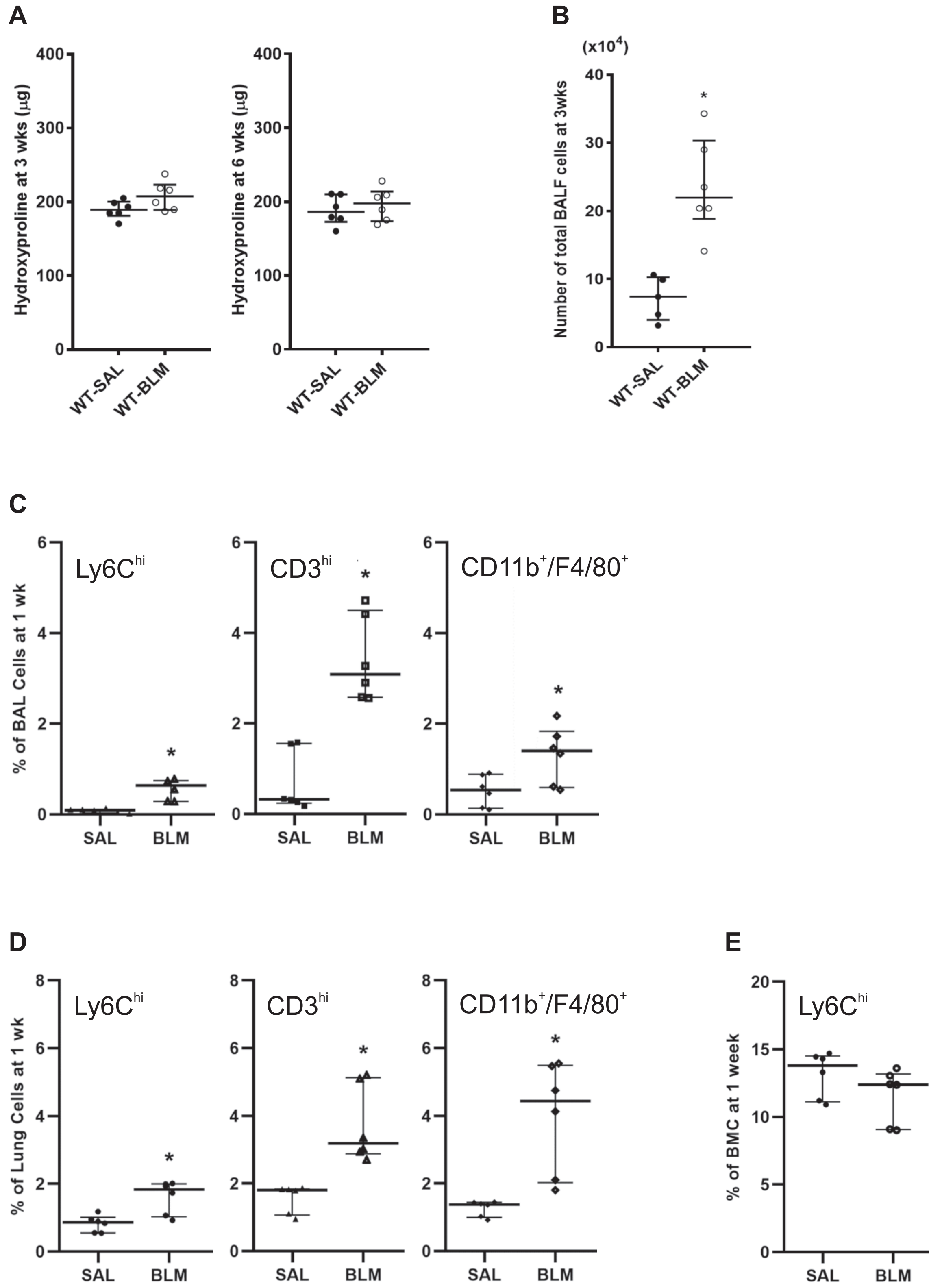

Figure E2