**Supplementary information for**

**Myeloid cell-derived soluble IL-6 receptor diminishes the differentiation of tumor-specific Th1 cells to exacerbate tumor progression**

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Supplementary Materials and Methods

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**Supplementary Materials and Methods**

***In vivo* cell depletion**

Mice were injected with 200 µg anti-Gr-1, anti-Ly6G Abs (BioXCell), control IgG Ab (Millipore), 180 µl clodronate liposome or control liposome (Hygieia Bioscience) 10 days after MCA-OVA inoculation.

**Cell isolation**

CD8+ T cells, B cells, NK cells, CD11c+ cells, or CD11b+ cells were isolated from tumor-free or tumor bearing mice using anti-CD8, anti-CD19, anti-DX5, anti-CD11c, or anti-CD11b microbeads (Miltenyi Biotec), respectively. For the isolation of Gr-1+ cells and/or Ly6G+ cells, spleen cell suspension was labeled with biotin-conjugated anti-Gr-1 Ab or Ly-6G Ab, and then were separated using anti-biotin microbeads (Miltenyi Biotec) according to the manufacture’s instruction of MDSC isolation kit.

**Measurement of TACE activity**

After the isolation of cell fractions from splenocytes, TACE activity was measured using the SensoLyte 520 TACE activity assay kit (ANASPEC) according to the manufacturer’s instruction.

**Processing of metastatic tumor in the lung**

Lung tumors were minced with razorblade and then digested in RPMI/1% FBS containing collagenase D (Roche Life Science; 2.5 mg/mL) and DNase I (SIGMA; 0.2 mg/mL) for 30 min at 37C. After passage through 100 m cell strainers and washing with PBS twice, cell suspensions were used for flow cytometric analysis.

**Knockdown of ADAM10/17 expression in tumor cells**

Sequence-verified viral vector (pLKO.1-puro) encording a short hairpin RNA library against ADAM10 and ADAM17 (ADAM10-shRNA and ADAM17-shRNA) or control shRNA were purchased from Sigma. Vector plasmids used for virus assembly were generated in DH5α competent cells. Preparative isolation and purification of plasmids for transfection were performed using Plasmid Maxi kits (Qiagen) according to the manufacturer’s instructions. For the preparation of lentiviral vectors, 293T cells were transiently co-transfected with vector plasmids, pCAG-HIVgp and pCMV-VSV-G using Lipofectamine 2000 (Invitorogen). The virus-containing supernatants were collected 72 hours after transfection, and then were concentrated by ultracentrifugation at 25,000 × g for 2 h. The precipitates were re-suspended with a small amount of PRMI medium. MCA-OVA were transfected with ADAM10-shRNA, ADAM17-shRNA or control shRNA for 48 hours, then exposed to puromycin (80 μg/ml) for 72 hours. Several ADAM10/17-knockdown clones were obtained via limiting dilution. After ADAM10/17-knockdown clones were cultured for 36 hours, the amount of sIL-6R in supernatant was determined by ELISA. ADAM10/17-knockdown clones that produced lower sIL-6R were selected. Knockdown of *Adam10/17* expression were confirmed by real time-PCR. The selected ADAM10/17-knockdown MCA-OVA clones (8×105)were inoculated subcutaneously.

**Western blotting**

 siRNA-transfected CD4+ T cells that were differentiated under Th1 condition (in the presence of IL-12) in the presence or absence of IL-6/sIL-6R *in vitro* for 3 days, were lysed with the lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Tris, pH7.4, 2 mM EDTA, 10% glycerol, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate, 25 mM -glycerophosphate, 10 mM NaF, and a protease inhibitor tablet (Roche)). Cell lysates were separated on 10% SDS-PAGE under the reducing condition, and then western blotting were carried out with antibodies specific to mouse STAT3 or -actin (Cell Signaling Technology).

**Supplementary figure legends**

**Supplementary figure 1.** Macrophages and MDSC produce sIL-6R in tumor-bearing mice. **A**, Splenocytes from tumor-free or MCA-OVA-bearing mice were stimulated with PMA/ionomycin for 36 hours in the presence of TAPI-0. The concentration of sIL-6R in culture supernatant was determined by ELISA. **B**, Indicated cell fractions were isolated from tumor-free or tumor-bearing mice. TACE activity in each cell fraction or MCA-OVA was measured.**C-E**, Indicated cell fractions were isolated　from spleen and LNs of tumor-free or MCA-OVA-bearing mice. and cultured for 36 hours in the absence (C) or presence of PMA/ionomycin (D). The concentration of sIL-6R in culture supernatant was determined. The indicated mRNA expression was measured by real-time PCR (E). Shown are relative values to *Gapdh* expression. **F and G**, MCA-OVA-bearing mice were injected with anti-Ly6G or anti-Gr-1 Ab (E) or clodronate (Clod; F). Four days after treatment, sIL-6R concentration in serum was measured. The values represent mean ± SEM with n = 4-7 /group; \* *p* < 0.05, \*\* *p* < 0.01, \*\*\* *p* < 0.001. The data are representative of 2 or more independent experiments with similar results.

**Supplementary figure 2.** Tumor cell-derived sIL-6R does not play a major role in attenuating Th1 differentiation in tumor-bearing mice. **A,** ADAM10/17 double knockdown MCA-OVA clones (#1 and #2) were established by the transfection of short hairpin RNAs specific for ADAM10 and ADAM17. These tumor cells were cultured with or without TAPI-0, an inhibitor of TACE, for 36 hours. The concentrations of sIL-6R in the culture supernatants were determined by ELISA. **B,** Quantitation of sIL-6R in serum from tumor-bearing mice 5 and 10 days after inoculation of indicated MCA-OVA cells. **C**,Mice were inoculated with ADAM10/17-knockdown MCA-OVA or mock control tumor cells. Seven days later, tumor-bearing mice were transferred with OT-II cells, and immunized with OVA-IIp-pulsed DC. Six days after immunization, donor OT-II cells were harvested from spleens and LNs. The number of donor OT-II cells and the frequencies of IFN-γ+ cells re-stimulated with OVA-IIp-pulsed DC were determined. Similar results were obtained from the mice that were inoculated with the other clone #2 of ADAM-knockdown MCA-OVA. The values represent mean ± SEM withn = 5 mice per group. \*\*\* *p* < 0.001. NS, not significant.

**Supplementary figure 3.** Myeloid cell-derived sIL-6R in tumor-bearing mice modulates CD4+ T-cell property. **A-C**, As in Fig. 1, MCA-OVA-bearing LysM-Cre+/- x IL-6Rfl/fl (IL-6R mKO) or littermate Cre-/- x IL-6Rfl/fl (WT) mice were transferred with OT-II cells, and then immunized with DC/OVA-IIp. IFN-γ+, CXCR3+ (A), LAG-3+, PD-1+, or ICOS+ cells (B) in donor OT-II cells from spleen, or SIINFEKL/ H-2Kb-tetramer+CD44hiCD8+ cells in tumor-draining LNs (C) were determined. **D**, OT-II transfer and immunization with DC/OVA-IIp were performed as in (A) in MO4-bearing WT litter or IL-6R mKO mice of the pulmonary metastatic model. Six days after immunization, metastatic tumors (lung) were harvested, and analyzed for IFN-γ+ OT-II cells or OVA-tetramer+CD44hiCD8+ cells. The values represent mean withn = 3-5 mice per group. **E,** MCA-OVA–bearing WT or IFN-γ KO mice were immunized with DC that were pulsed with H-2Kb-binding SIINFEKL peptide (OVA-Ip) together with sgp130 administration. Six days later, tumor-draining LNs were harvested, and analyzed for the proportion of OVA-specific CD8+ T cells with the OVA-tetramer. Representative dot plots (left) and the absolute number of OVA-tetramer+CD44hiCD8+ T cells (right) are shown. **F and G**, Polyclonal CD4+ T cells were stimulated with anti-CD3/CD28 Abs in the presence of IL-12 together with IL-6/sIL-6R *in vitro*. siRNA against *Stat3* (#1 and #2) or negative control were transfected after 24 hours of stimulation. STAT3 protein expression (F), and mRNA expression of *Stat3* and *c-maf* (G) were assessed 48 hours after transfection. \* *p* < 0.05, \*\* *p* < 0.01, \*\*\* *p* < 0.001. The data are representative out of 3 independent experiments.

**Supplementary figure 4.** Comparative analysis in immunological parameters from cancer patients and healthy donors. **A,** Surface IL-6R expression on human CD4+ T cells from PBMC of HNT patients or HD were analyzed *ex vivo* (right) or after *in vitro* stimulation with anti-CD3/CD28 Abs for 24 hours (left). n = 5-7. Individual values along with the mean are shown. \* *p* < 0.05.**B**, The levels of IL-6 and sIL-6R in plasma, the frequency of CD14+CD16- classical monocytes and their surface IL-6R expression, and the frequency of CD4+ T cells and their c-Maf expression in PBMC from 25 samples from HD and 48 samples collected from 22 HNT patients along with the vaccination were used in this analysis. The structural equation models with covariates are represented by the path diagram, and each estimate and *p* value from all HNT patients (underlined) and HD (bold) are shown.The effect of age and times of peptides vaccine received were adjusted according to Fig. 6D.