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Title	IL6 modulates the immune status of the tumor microenvironment to facilitate metastatic colonization of colorectal cancer cells
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Supplemental Figure Legends

Supplementary Figure S1. Metastatic colonization of colon cancer cells in the lungs of *Il6*$^{-/-}$ mice. GFP-transfected CT26 murine colon cancer cells (2×10^5) were intravenously inoculated into wild-type (*Il6*$^{+/+}$) and *Il6*$^{-/-}$ BALB/c mice (day 0). **A,** HE staining of lung tissue was performed at day 21. Representative micrographs are shown. Bars in the pictures represent 500 μm. **B,** Ratios of tumor area relative to total lung tissue area were calculated by ImageJ software. Means and SDs of the data based on the HE-stained sections from four independent mice are shown. *$P < 0.05$ by Student's t-test. **C,** Metastatic colonization in the lung was evaluated with an *in vivo* imaging system at day 21. Representative images of normal lung and GFP-expressing CT26 cell-bearing lungs are shown. **D,** Photon flux ratios were determined from images of lung metastatic colonization model mice (n = 4, two independent experiments). Means and SDs of the data from four independent mice are shown. *$P < 0.05$ by Student's t-test. **E,** The survival rate of lung metastatic colonization model mice was monitored for 30 days and the percentage survival rate (*Il6*$^{+/+}$, n = 15; *Il6*$^{-/-}$, n = 15) is shown. *$P < 0.05$ by the log-rank test.

Supplementary Figure S2. Metastatic colonization of breast cancer cells in the livers of *Il6*$^{-/-}$ mice. GFP-transfected 4T1 murine breast cancer cells (2×10^5) were

intrasplenically inoculated into wild-type (*Il6*$^{+/+}$) and *Il6*$^{-/-}$ BALB/c mice (day 0). **A,** HE staining of liver tissues was performed at day 14. Representative micrographs are shown. Bars in the pictures represent 500 μm. **B,** Ratios of tumor area relative to total liver tissue area were calculated by ImageJ software. Means and SDs of the data based on the HE-stained sections from four independent mice are shown. *$P < 0.05$ by Student's t-test. **C,** Metastatic colonization in the livers was evaluated with an *in vivo* imaging system at day 14. Representative images of GFP-expressing 4T1 cell-bearing livers are shown. **D,** Photon flux ratios were determined from images of liver metastatic colonization model mice (n = 4, three independent experiments). Means and SDs of the data from four independent mice are shown. *$P < 0.05$ by Student's t-test. **E,** Survival rate of metastatic colonization model mice was monitored for 25 days and the percentage survival rate (*Il6*$^{+/+}$, n = 20; *Il6*$^{-/-}$, n = 22) is shown. *$P < 0.05$ by the log-rank test.

Supplementary Figure S3. Effect of *in vivo* administration of anti-IL-6 mAb into the metastatic colonization model using *Il6*$^{+/+}$ mice. Control IgG or anti-IL-6 mAb (200 mg/mouse) was injected intraperitoneally into *Il6*$^{+/+}$ mice one day before CT26 cell inoculation. GFP-transfected CT26 cells (2×10^5) were intrasplenically inoculated into *Il6*$^{+/+}$ mice (day 0). Control IgG or anti-IL-6 mAb was injected intraperitoneally into

Il6$^{+/+}$ mice at day 3 and then every 4 days thereafter. **A,** HE staining of liver tissue was performed at day 14. Representative micrographs are shown. Bars in the images represent 500 μm. **B,** Ratios of tumor area relative to total liver tissue area were calculated by ImageJ software. Means and SDs of the data based on the HE-stained sections from four independent mice are shown. *$P < 0.05$ by Student's *t*-test. **C,** Metastatic colonization in the liver was evaluated with an *in vivo* imaging system at day 14. Representative images of GFP-expressing CT26 cell-bearing liver tissue are shown. **D,** Photon flux ratios were determined from images of liver metastatic colonization model mice (n = 4, two independent experiments). Means and SDs of the data from four independent mice are shown. *$P < 0.05$ by Student's *t*-test. **E,** Survival of control IgG- or anti-IL-6 mAb-injected *Il6*$^{+/+}$ mice was monitored for 40 days after inoculation. Percentage survival rate (control IgG, n = 16; anti-IL-6 mAb, n = 15) is shown. *$P < 0.05$ by the log-rank test.

Supplementary Figure S4. *Il6* expression levels in whole cells and CT26 cells isolated from the liver tissues of metastatic colonization model mice. GFP-transfected CT26 murine colon cancer cells (2×10^5) were intrasplenically inoculated into wild-type (*Il6*$^{+/+}$) and *Il6*$^{-/-}$ BALB/c mice (day 0). Whole cells and GFP-expressing CT26 cells were collected from the liver tissue of *Il6*$^{+/+}$ and *Il6*$^{-/-}$ mice at day 14 and

expression levels of *Il6* and *Actb* were evaluated by quantitative PCR analysis. Normalized *Il6* expression levels against *Actb* in each cell type were calculated and means and SDs (n=4, two independent experiments) are indicated. $*P < 0.05$ by Dunnett's post-test.

Supplementary Figure S5. Involvement of IL-6 production by colon cancer cells in a liver metastatic colonization model. Knockout of the *Il6* gene in CT26 cells was performed by the CRISPR/Cas9 system. **A,** *Il6* expression levels relative to *Actb* in established *Il6* gene-knockout CT26 cells (CT26-*Il6*KO) and mock control CT26 cells (CT26-mock) were evaluated (n = 4, two independent experiments). $*P < 0.05$ by Student's t-test. **B,** Viability and proliferation of CT26-mock and CT26-*Il6*KO cells were evaluated by MTT assay at 24 and 48 h (n = 4, two independent experiments). Representative data are shown. $*P < 0.05$ by Student's t-test. **C,** CT26-mock and CT26-*Il6*KO cells (2×10^5) were intrasplenically inoculated into $Il6^{+/+}$ BALB/c mice (day 0). HE staining of liver tissues was performed at 14 days after inoculation (two independent experiments). Representative micrographs are shown. Bars in the pictures represent 500 μm. **D,** Ratios of tumor area relative to total liver tissue area were calculated. Means and SDs of the data based on the HE-stained sections from

four independent mice are shown. *$P < 0.05$ by Student's t-test. Means and SDs are shown (**A**, **B** and **D**).

Supplementary Figure S6. Effect of IL-12 and CD8$^+$ effector T cells on metastatic colonization in the livers of *Il6*$^{+/+}$ mice. GFP-transfected CT26 cells (2×10^5) were intrasplenically inoculated into control liposome-, clodronate liposome-, control IgG-, anti-CD8 mAb, or anti-IL-12 mAb-treated *Il6*$^{+/+}$ mice (day 0). **A** and **D,** HE staining of liver tissue was performed at 14 days after inoculation. Representative micrographs are shown. Bars represent 500 μm. **B,** and **E,** Metastatic colonization of GFP-expressing CT26 cells in the liver were evaluated with an *in vivo* imaging system at day 14. Representative images are shown. **C,** and **F,** Photon flux ratios were evaluated from the images (n = 4–6, two independent experiments) and the means and SDs are shown. *$P < 0.05$ by Student's t-test (**C**) and Dunnett's post-test (**F**).

Supplementary Figure S7. Reduction in metastatic colonization by depletion of CD4$^+$ T cells in the livers of *Il6*$^{-/-}$ and *Il6*$^{+/+}$ mice *in vivo*. GFP-transfected CT26 cells (2×10^5) were intrasplenically inoculated into control IgG- or anti-CD4 mAb (GK1.5, 200 mg/mouse)-treated *Il6*$^{-/-}$ mice and *Il6*$^{+/+}$ mice (day 0). Four to six *Il6*$^{-/-}$ mice and *Il6*$^{+/+}$ mice were used in three independent experiments. **A** and **D,** HE staining of liver tissues

was performed at 14 days after inoculation. Representative micrographs are shown. Bars in the pictures represent 500 μm. **B** and **E,** Metastatic colonization was evaluated with an *in vivo* imaging system at day 14. Representative images of GFP-expressing CT26 cell-bearing livers are shown. **C** and **F,** Photon flux ratios were evaluated from the images of liver metastasis model mice (n = 4, three independent experiments) and the means and SDs are shown. *$P < 0.05$ by Student's t-test. **G,** Whole cells were collected from liver tissues of control IgG- or anti-CD4-mAb-treated CT26 tumor-bearing *Il6*$^{-/-}$ mice and *Il6*$^{+/+}$ mice at day 14. Surface expression levels of CD11c, I-Ad, CD44, CD62L, and CD8 on 7-AAD^{-}CD45^{+} cells were evaluated by flow cytometry. The representative data for CD11c^{high}I-Ad^{high} mature DCs and CD44^{+}CD62L^{-}CD8^{+} effector memory phenotype killer T cells in the liver tissues of *Il6*$^{+/+}$ mice were determined by flow cytometry at day 14. Percentages relative to CD45^{+} cells were calculated and the means and SDs (n = 6, three independent experiments) are shown. *$P < 0.05$ by Student's t-test. **H,** Perforin levels in CD8^{+} T cells of liver tissues from *Il6*$^{+/+}$ mice were determined by flow cytometry at day 14. ΔMFIs of perforin relative to each isotype control were calculated. Means and SDs (n = 4, two independent experiments) are shown. *$P < 0.05$ by Student's t-test.

Supplementary Figure S8. Effect of *in vivo* administration of anti-PD-L1 mAb into the metastatic colonization model using *Il6*$^{+/+}$ mice. GFP-transfected CT26 cells (2×10^5) were intrasplenically inoculated into *Il6*$^{+/+}$ mice (day 0). Control IgG or anti-PD-L1 mAb (200 mg/mouse) was injected intraperitoneally into *Il6*$^{+/+}$ mice at day 5 and then every 4 days thereafter. **A,** HE staining of liver tissues was performed to evaluate CD11c and CD3 levels at 14 days after inoculation. Representative micrographs are shown. Bars in the pictures represent 500 μm. **B,** Metastatic colonization images of GFP-expressing CT26 cells in the liver were evaluated with an *in vivo* imaging system at day 14. **C,** Photon flux ratios were evaluated from the images and the means and SDs (n = 4, three independent experiments) are shown. *$P < 0.05$ by Student's *t*-test. **D,** IHC staining of liver tissues was performed to evaluate CD11c and CD3 levels using anti-CD11c and anti-CD3 antibodies at day 14. Representative micrographs are shown. Bars represent 200 μm. **E,** Whole cells were collected from liver tissues of control IgG- or anti-PD-L1-mAb-treated CT26 tumor-bearing *Il6*$^{+/+}$ mice at day 14. Surface expression levels of CD11c, I-Ad, CD44, CD62L, and CD8 on 7-AAD$^-$CD45$^+$ cells were evaluated by flow cytometry. The representative data for CD11c^{high}I-Ad^{high} mature DCs and CD44$^+$CD62L$^-$CD8$^+$ effector memory phenotype killer T cells in the livers of mice were determined by flow cytometry at day 14. Percentages relative to CD45$^+$ cells were calculated and the means and SDs (n = 4, two independent experiments) are shown.

*$P < 0.05$ by Student's t-test. **F,** Survival of control IgG- or anti-PD-L1 mAb-injected *Il6*$^{+/+}$ mice was monitored for 40 days after inoculation. Percentage survival rate (control IgG; n = 23, anti-PD-L1 mAb; n = 24) is shown. *$P < 0.05$ by the log-rank test.

Supplementary Figure S9. Correlation between IL-6 and PD-L1 expression in liver metastasis and DFS of CRC patients. IHC of liver metastasis of colorectal cancer patients (n = 57) was conducted to evaluate IL-6 and PD-L1 levels using anti-IL-6 and anti-PD-L1 antibodies, respectively. **A,** Representative micrographs of tumor tissues from IL-6-positive and -negative and PD-L1-positive and -negative patients are shown. Bars in the images represent 100 μm. **B,** Kaplan-Meier estimates of DFS for 57 CRC patients stratified into two groups: IL-6-negative (22 patients) or IL-6-positive (35 patients); and PD-L1-negative (31 patients) or PD-L1-positive (26 patients).