PNAS
www.pnas.org

Supplementary Information for

CCRL2 promotes anti-tumor T cell immunity via amplifying TLR4-mediated immunostimulatory macrophage activation

Wei Yin, Yihong Li, Yan Song, Chao Wu, Yu Chen, Jiarui Zhang, Yuli Lin, Ying Miao, Changdong Lin, Dapeng Yan, Jianfeng Chen and Rui He

Rui He
Email: ruihe@fudan.edu.cn

This PDF file includes:

SI methods
Figures S1 to S7
Legends for Figures S1 to S7
SI References

SI Methods

Cell lines

B16F10, OVA−B16F10, 4T1, LLC and CT26 cells were cultured in complete RPMI-1640 medium, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco, Grand Island, NY). HEK 293T and human melanoma cell line A2058 and A375 were cultured in complete DMEM medium, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. THP1 derived macrophages were induced with 300 ng/μl PMA (Sigma) for 24 hrs in complete RPMI-1640 medium. All cell lines were obtained from the American Type Culture Collection (ATCC) and routinely tested for Mycoplasma. To establish chemerin-overexpressing cell lines, B16F10 cells were transfected with pCDH1 vector expressing Rarres2 gene that encodes chemerin.

The Cancer Genome Atlas datasets

TCGA datasets were obtained from https://portal.gdc.cancer.gov. Expression values of each gene were converted to TPM and expression values of CCRL2 were classified as low and high based on the median expression. Gene Set Enrichment Analyses were performed using the MSigDB Hallmark, GO and KEGG gene sets. The CIBERSORT, an algorithm to evaluate hematopoietic cell distribution from tumor RNA mixtures, was used to analyze the differential immune cell infiltration distributions. The correlation of expression levels between CCRL2 and CD80, CD86, HLA-DRA, HLA-DRB1 as well as MRC1 in melanoma was analyzed by using TCGA datasets. t-SNE plot of CCRL2 expression in melanoma infiltrating immune cells was generated from the data of Titosh I et al. and Livnat Jerby-Arnon et al via Single Cell Portal (https://singlecell.broadinstitute.org/).

Preparation of bone marrow derived macrophage (BMDM)

To generate BMDM, single-cell suspension of bone marrow aspiration from mouse femurs was obtained and placed in complete RPMI-1640 medium supplemented with 20 ng/ml M-CSF (Peprotech) for 7-day culture. On days 3 and 6, the media containing nonadherent cells were replaced with a fresh medium with 20 ng/ml M-CSF. On day 7, the adherent BMDM were harvested for further study. The purity of BMDM was determined by flow cytometry using F4/80 antibody and more than 90% of cells were F4/80 positive.

In vitro stimulation of BMDM

To prepare conditioned media (CM), tumor cells were grown starting from 2×10^6 in RPMI-1640 complete medium in 75 cm2 BD Falcon flasks at 37°C. When the cells reached 30% confluence, the RPMI-1640 complete medium was aspirated, the RPMI-1640 complete medium (10 ml) was added. CM was collected when the cells reached 100% confluence and centrifuged at 1,500 rpm for 5 min followed by filtering through a 22 μm filter. For detection of CCRL2, BMDM from different genotypes were stimulated with different stimuli, including LPS (100 ng/ml, Sigma), CM from different tumor cell lines, LPS (100 ng/ml) together with IFN-γ (20 ng/ml) or IL-4 (20 ng/ml, Peprotech), and LPS in medium with pH6.8 and pH7.4, respectively, for indicated time. For macrophage polarization, WT and *Ccrl2*$^{-/-}$ BMDM were treated with IFN-γ (20 ng/ml) and LPS (100 ng/ml) or IL-4 (20 ng/ml) for 12 hrs.

Murine model

Mice anesthetized with intraperitoneal (i.p.) injection of pentobarbital sodium were shaved and inoculated subcutaneously (s.c.) into the right flanks with B16F10 cells or OVA-B16F10 cells (5×10^5 cells in 100 μl PBS). For co-injection assay, BMDM were s.c. co-injected with B16F10 cells at ratio of 1:1. Tumor sizes were measured every

2 days using calipers starting from day 6 post inoculation. The tumor volume was calculated using the following formula: V= (larger diameter) × (smaller diameter)^2/2. For generation of bone marrow (BM) chimeras, WT and *Ccrl2*$^{-/-}$mice were sublethally irradiated (9 Gy), followed by intravenous transplantation of 2 × 10^6 bone marrow cells from *Ccrl2*$^{-/-}$ and WT donors, respectively. Mice were sacrificed at indicated time points and tumors were collected for further study.

Flow cytometry

For preparation of single-cell suspension for flow cytometry, tumor tissues were cut into pieces and digested by Collagenase IV (1 mg/ml, Sigma) and DNAase I (20 U/ ml, Sigma) for 20 mins in a 37°C shaking incubator (180 rpm), and filtered through a 100 μm nylon mesh. After blocking the Fc-receptors with anti-mouse CD16/32 (93), the following fluorochrome-labelled anti-mouse antibodies were used: CD45 (30-F11), Gr-1 (RB6-8C5), F4/80 (BM8), CD11c (N418), Ly6C (HK1.4), CD80 (16-10A1), MHCII (M5/114.15.2), CD40 (3/23), CD206 (C068C2), CD4 (GK1.5), CD8 (53-6.7), CD3 (17A2), CD11c (N418), TLR4 (MTS510) PD-1 (135206) (all from Biolegend), CCRL2 (BZ2E3), NK1.1 (PK136) (all from BD Biosciences), CD11b (M1/70), CD86 (GL1), MHCI (AF6-88.5.5.3) (all from Invitrogen). For intracellular staining, cells were stimulated with cell stimulation cocktail (Cat#423303, Biolegend) for 5 hrs followed by cell surface staining of CD8 or CD4, and then cells were fixed and permeabilized using the intracellular staining kit (Cat#560409 BD) followed by intracellular staining of IFN-γ (XMG1.2), Granzyme B (GB11) (all from Biolegend), and Ki67 (SolA15, Invitrogen). Samples were acquired by FACS BD celesta (BD Biosciences) and analyzed by FLOWJO 7.6.1 software (FlowJo, LLC). MFI intensity of each molecules were calculated by geometric arithmetic mean of fluorescence intensity.

Enzyme-linked immunosorbent assay (ELISA)

Tumor samples were minced and mechanically homogenized in phosphate-buffered saline containing protease inhibitor (Cat#04693159001, Roche). The supernatants were harvested after centrifuging at 13 800 g for 10 mins. IFN-γ protein levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (DuoSet; R&D Systems) according to the manufacturer's instructions, and normalized to the total protein concentration determined by BCA assay (Sigma, St Louis, MO). Chemerin levels in the serum of WT and Ccrl2$^{-/-}$ mice or supernatants from chemerin-overexpressing B16F10 cells were measured using an ELISA specific for murine chemerin (R&D Systems) according to the manufacturer's protocol.

Immunofluorescence (IF)

After antigen retrieval, human melanoma tumor sections were incubated with the antibodies against human CCRL2 (1:200, Cat#DF8643, Affinity Biosciences), CD68 (1:100, Cat#GB13067-M-2 Servicebio) overnight at 4°C, followed by incubating with rabbit-specific and mouse-specific secondary antibodies conjugated with AlexaFluor 488 (Cat#GB21303, Servicebio) and 594 labeled (Cat#GB21301, Servicebio) for 1 hr. The images were taken and analyzed by confocal microscope (NIKON ECLIPSE C1).

In vitro OVA stimulation

Single-cell suspensions of OVA-B16F10 tumor tissues from WT and *Ccrl2*$^{-/-}$ mice on day 8 post inoculation were prepared as described previously followed by stimulation with OVA (100μg/ml, CAT#A5503, Sigma-Aldrich) for 3 days.

Co-culture assay of BMDM and OT1 splenocytes

OT1 mice were i.p. immunized with 100 mg of OVA adsorbed to 2 mg of an aqueous solution of alum (Cat#77161, Thermo Fisher) on days 0 and 7. On day 14, mice were sacrificed and spleens were harvested to generate single-cell suspension. WT or *Ccrl2*$^{-/-}$ BMDM were treated with IFN-γ (20 ng/ml,) and LPS (100 ng/ml) for 24 hrs followed by pulsing with 10 nM specific SIINFEKL ($OVA_{257-264}$) (Cat#S7951, Sigma-Aldrich) for 4 hrs. After extensive washing, BMDM were cocultured with splenocytes of OVA-sensitized OT1 mice at a ratio of 1:5 for 72 hrs (1).

Quantitative PCR (qPCR)

Total RNA of in vitro-stimulated BMDM or tumor tissues was extracted using TRIzol reagent (Invitrogen) and transcribed into cDNA by PrimeScript RT Master Mix (TaKaRa). Quantitative PCR (qPCR) was performed using the Power SYBR Green Master kit (TaKaRa) with LightCycer 480 II (Roche). The relative expression of target gene was calculated using the 2-ΔΔC(t) method. Fold change of target gene expression was calculated by normalization to the control group. The primer sequences of all genes for PCR are listed as following: Sequence (5' → 3)', *Cxcl10*: F- CCAAGTGCTGCCGTCATTTTC, R-GGCTCGCAGGGATGATTTCAA; *Il12b*: F-TGGTTTGCCATCGTTTTGCTG, R-ACAGGTGAGGTTCACTGTTTCT; *Arg1*: F- CAGAAGAATGGAAGAGTCAG, R-CAGATATGCAGGGAGTCACC; *Il1b*: F-GCAACTGTTCCTGAACTCAACT, R-ATCTTTTGGGGTCCGTCAACT; *Nos2*: F- GTTCTCAGCCCAACAATACAAGA, R- GTGGACGGGTCGATGTCAC; *Il6*: F-CCAAGAGGTGAGTGCTTCCC, R- CTGTTGTTCAGACTCTCTCCCT; *Tnfa*: F- TCCTTTTGGGCATCATCTTCC, R- TTTGTAGTGGATCGTGCCTCG; *Tlr4*: F- ATGGCATGGCTTACACCACC, R- GAGGCCAATTTTGTCTCCACA; *Retnla*: F- CCAATCCAGCTAACTATCCCTCC, R- ACCCAGTAGCAGTCATCCCA; *Chil3*: F-CTGAATGAAGGAGCCACTGA, R- AGCCACTGAGCCTTCAACTT; *β-actin*: F- CCAGCCTTCCTTCTTGGGTATG, R- TGTGTTGGCATAGAGGTCTTTACG; *CCRL2*: F- AGCGATGAGGCAGAGCAATG, R-GGACACCGATCACAAACACAG; *GAPDH*: F- GGAGCGAGATCCCTCCAAAAT, R-GGCTGTTGTCATACTTCTCATGG.

Western Blotting

BMDM were solubilized in RIPA buffer (Cat#P0013B Beyotime Biotechnology) containing protease and phosphatase inhibitors (Roche). Target protein was electrophorized on Bio-Rad precast gradient gels and electroblotted onto PVDF membranes. Proteins were detected by incubation with 1:1,000 dilutions of each primary antibody, including anti-phosphorylated p65 (93H1), anti-phosphorylated STAT1 (58D6), anti-phosphorylated STAT6 (D8S9Y) (all from Cell Signaling Technology), anti-MyD88 (EPR21824, Abcam), anti-TLR4 (3G9A4, Proteintech) followed by incubating with goat anti-rabbit-HRP (Cat#7074, Cell Signaling Technology) second antibodies. Blots were developed using ECL plus reagents (MeilunBio).

Co-immunoprecipitation (Co-IP)

To construct plasmids expressing CCRL2-HA and TLR4-Flag fusion protein, the full-length gene that encodes murine Ccrl2 or Tlr4 was inserted into the cloning vector pcDNA3.0 and pcDNA3.1, respectively, using the XhoI and BamHI restriction enzyme digestion sites. For anti-Flag immunoprecipitation, HEK 293T cells were collected at 48 hrs after co-transfection with expression vectors for TLR4-Flag and CCRL2-HA and lysed in NP40 (MeilunBio) for 30 mins on ice. Supernatants were incubated with anti-Flag M2 affinity gel beads (Sigma-Aldrich) overnight at

4°C. Protein was eluted by 100°C for 10 min and prepared for western blotting analysis. Primary antibody rabbit anti-HA (1:1000, Cat#SAB4300603, Sigma-Aldrich) and anti-Flag antibody (1:1000, Cat#F7425, Sigma-Aldrich) were used for detect CCRL2 and TLR4, respectively. For detecting the interaction between TLR4 and CCRL2 in BMDM, WT BMDM were treated with B16-CM for 4 and 12 hrs and then lysed in NP40. Supernatants were incubated with Protein G-Agarose beads (CAT#05015979001, Roche) and anti-TLR4 antibody (1:200, Cat#NB100-56723, Novus) or IgG (2ug, Cat#A7016, Beyotime Biotechnology) overnight at 4°C. Primary antibody rabbit anti-TLR4 (1:1000, Cat#19811-I-AP, Proteintech), anti-CCRL2 antibody (1:1000, Cat#DF8643, Affinity Biosciences) were used for western blotting analysis.

NanoLuc® Binary Technology (NanoBit)

NanoBiT, a two-subunit system based on NanoLuc luciferase, is used for intracellular detection of protein: protein interactions. Large BiT (LgBiT; 17.6kDa) and Small BiT (SmBiT; 11 amino acids) subunits are fused to proteins of interest, and when expressed, the PPI brings the subunits into closeproximity to form a functional enzyme that generates a bright, luminescent signal. The full-length gene that encodes murine CCRL2, TLR4, murine Ccrl2, Tlr4, was inserted into the cloning vector LgBIT and SmBIT (Promega), respectively, using the XhoI and BglII restriction enzyme digestion sites. HEK 293T were plated in24-well plates to reach 60% to 80% confluence on the day of transfection. On day 2, plasmids were transfected using Lipo2000 Transfection Reagent (Thermo). Forty-eight hrs post-transfection, Nano-Glo live cell substrate (Promega) was added to the cells at a 1:20 dilution. Immediately after addition, luminescence was detected using Envision-CBCF (Perkin Elmer).

Fig. S1

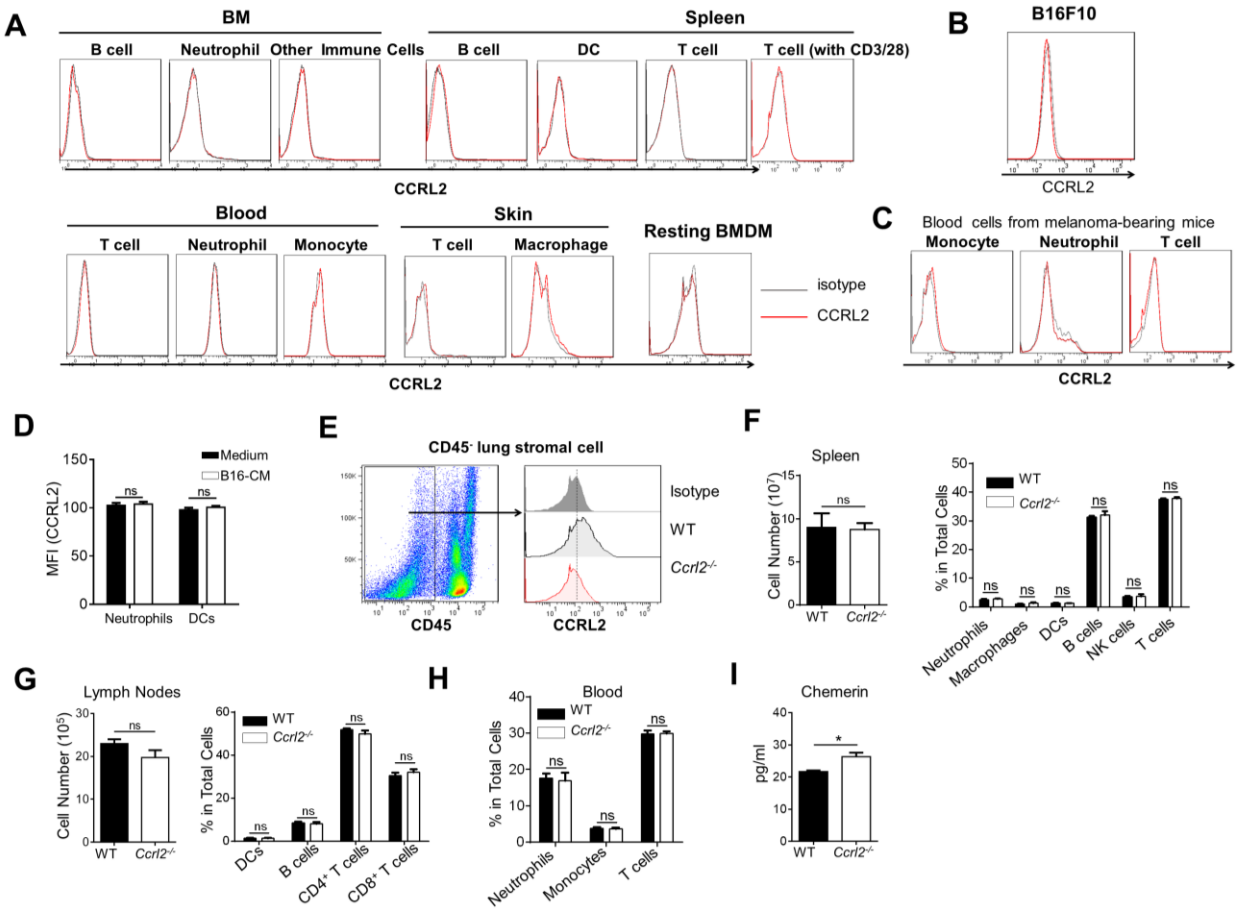

Fig. S1. CCRL2 expression and the phenotype of *Ccrl2*$^{-/-}$ mice.

(A-B) Representative histograms showing CCRL2 expression in myeloid cells and lymphocytes from bone marrow (BM), spleen, blood, skin, and resting BMDM of naive WT mice (A), B16F10 cells (B) and myeloid cells and lymphocytes in blood from melanoma-bearing mice (C). (D) Mean Fluorescence Intensity (MFI) of CCRL2 expression was determined in WT neutrophils and DCs that were stimulated with condition media (CM) of B16F10 cells (B16-CM) or culture media as control for 24 hrs. (E) Representative histograms showing CCRL2 expression in lung stromal cells of WT and *Ccrl2*$^{-/-}$ mice. (F-H) The numbers of total cells and frequencies of different types of immune cells in spleens (F), lymph nodes (G) and in peripheral blood (H) in naïve WT and *Ccrl2*$^{-/-}$ mice. (I) The concentrations of serum chemerin in WT and *Ccrl2*$^{-/-}$ mice determined by ELISA. In B-C, data represent mean ± SEM of triplicate wells from a representative of three independent experiments. In D-H, data represent mean ± SEM (n = 3).

Fig. S2

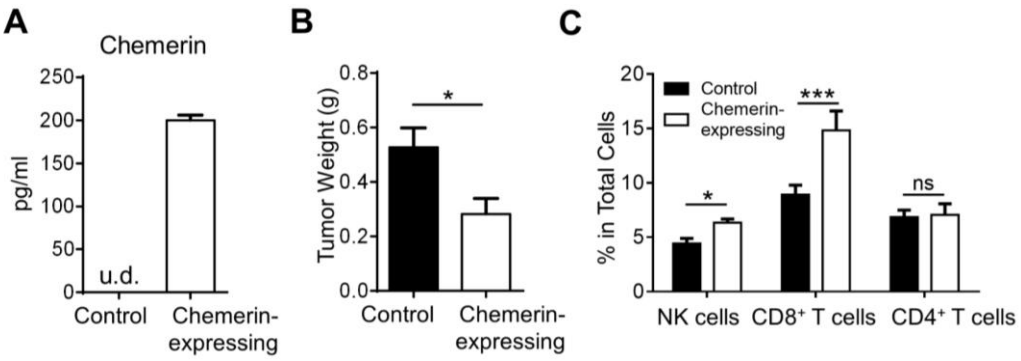

Fig. S2. Chemerin overexpression inhibits melanoma growth in *Ccrl2*$^{-/-}$ mice.

(A) The concentrations of chemerin in cultures of B16F10 cells transfected with control vector or chemerin-expressing vector. (B) Tumor weight of *Ccrl2*$^{-/-}$ mice on day 14 following inoculation with control or chemerin-expressing B16F10 cells. (C) Average percentages of different types of lymphocytes in tumors. Data represent mean ± SEM of triplicate wells from a representative of three independent experiments in (A). Data represent mean ± SEM (n = 5-7) and similar results were obtained from two independent experiments in (B-C).

Fig. S3

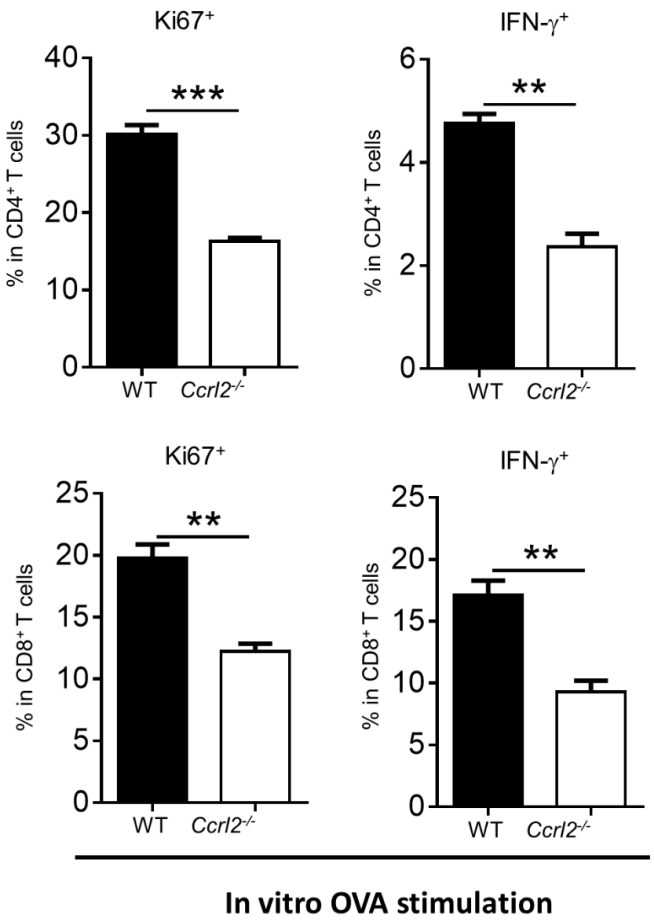

Fig. S3. Chemerin overexpression inhibits melanoma growth in Ccrl2$^{-/-}$ mice
The average percentages of Ki67^{+} and IFN-γ^{+} cells in CD4^{+} T or CD8^{+} T in the single-cell suspensions of OVA-B16 tumors tissues obtained from WT and *Ccrl2*$^{-/-}$ mice on day 8 followed by OVA stimulation in vitro for 3 days. Data represent mean ± SEM (n = 6).

Fig. S4

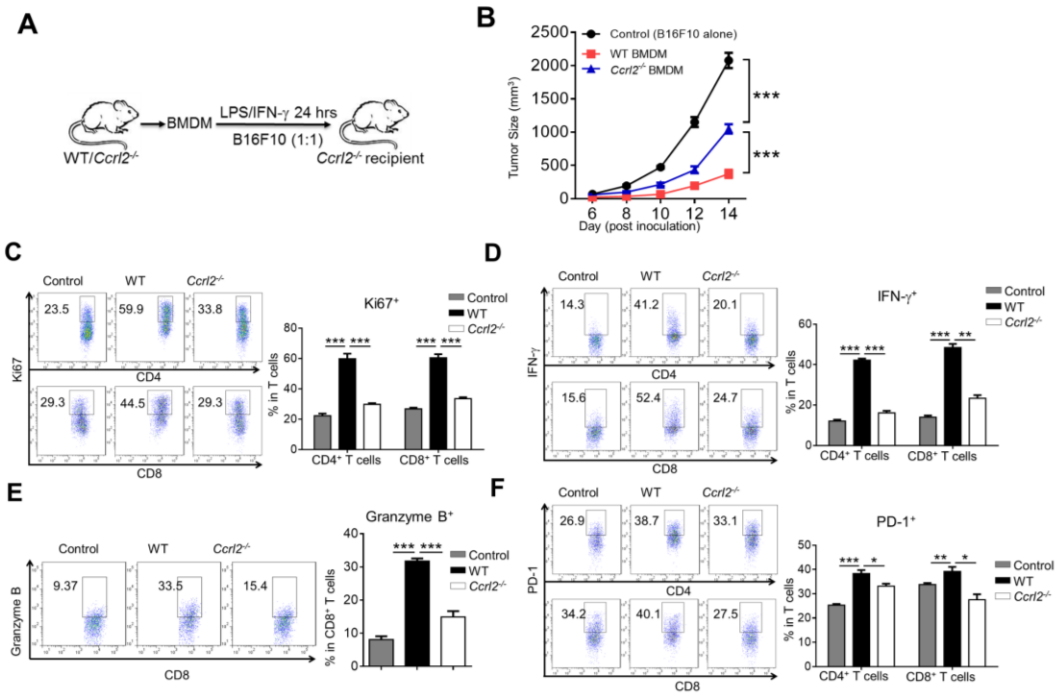

Fig. S4. Co-injection of LPS/IFN-γ-activated *Ccrl2*$^{-/-}$ BMDM causes increased B16 tumor growth and reduced anti-tumor T cell responses

(A) Schematic protocol for s.c co-injection model. (B) Tumor growth curve. (C-D, F) CD4^{+} and CD8^{+} T cells were gated and examined for expression of Ki67 (C), IFN-γ (D) and PD-1 (F). (E) Expression of Granzyme B. Data represent mean ± SEM (n=5-6) and similar results were obtained from 2 independent experiments.

Fig. S5

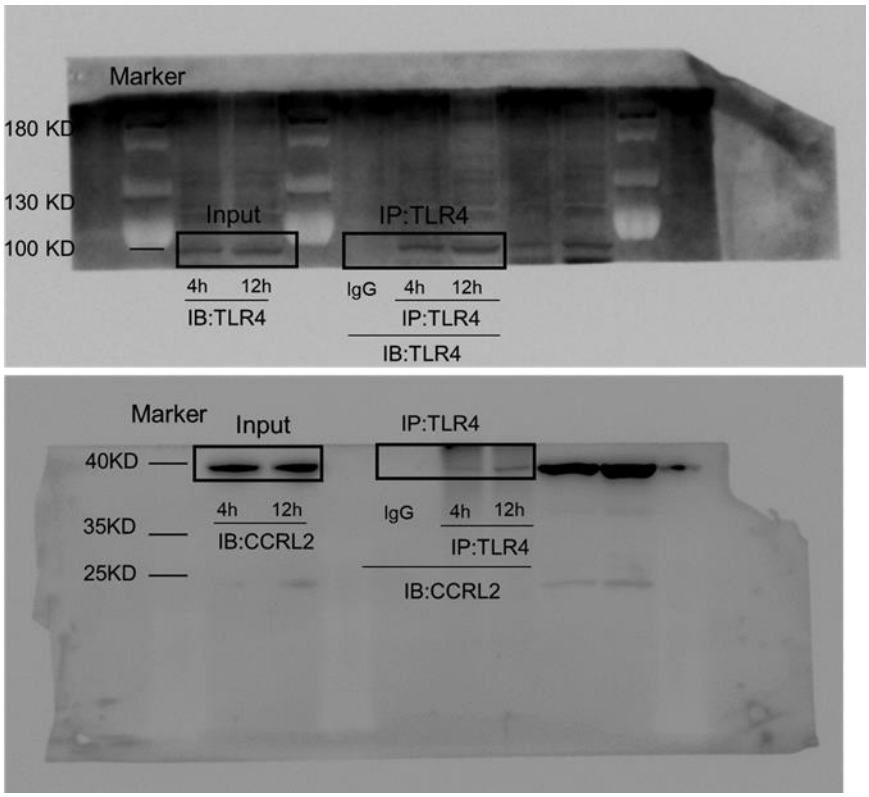

Fig. S5. The original whole photo image used for Figure 7C

Fig. S6

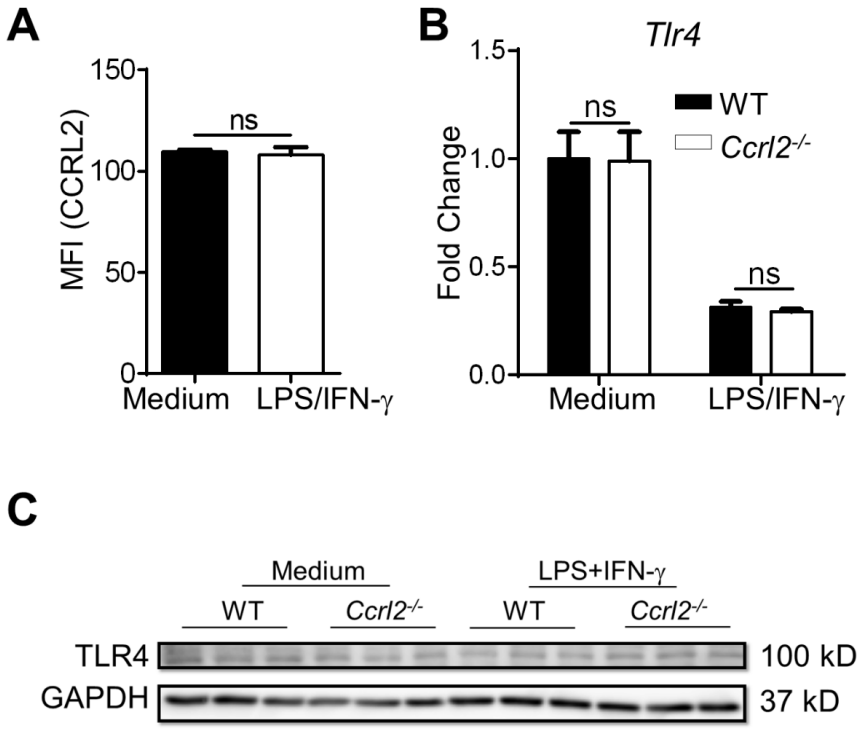

Fig. S6. Lack of CCRL2 has no effect on total TLR4 expression

(A) MFI of CCRL2 expression was determined in WT BMDMs that were stimulated with LPS/IFN-γ for 1 hr. (B-C) qPCR analysis of CCRL2 mRNA expression (B) and representative western blots showing TLR4 levels (C) in WT and *Ccrl2*$^{-/-}$ BMDM that were stimulated with LPS/IFN-γ. Columns and error bars represent of triplicate wells from a representative of two independent experiments.

Fig. S7

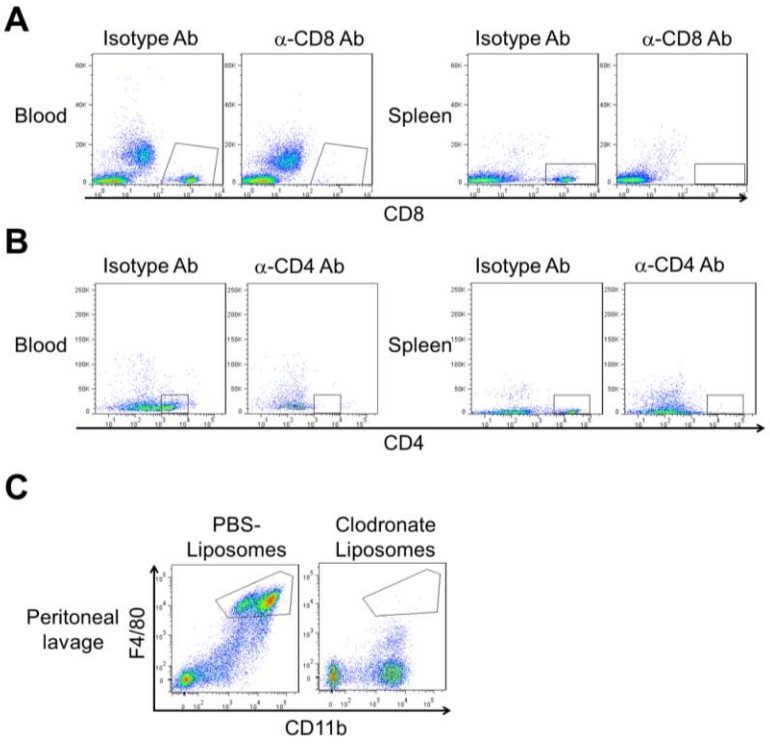

Fig. S7. The efficiencies of cell depletion in vivo
(A-B) Representative flow cytometry dot plots of $CD8^+$ T (A) and $CD4^+$ T (B) cells in the blood and spleen of mice treated with neutralizing anti-CD8 antibody or anti-CD4 antibody and isotype control antibody used as control. (C) Representative flow cytometry dot plots of macrophages in the peritoneal lavage from clodronate-liposomes or PBS liposomes-treated mice.

SI References

1. Li C*, et al.* (2015) ADAP and SKAP55 deficiency suppresses PD-1 expression in CD8+ cytotoxic T lymphocytes for enhanced anti-tumor immunotherapy. *EMBO Mol Med* 7(6):754-769.