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| Title | Chemotherapy-Induced IL34 Enhances Immunosuppression by Tumor-Associated Macrophages and Mediates Survival of Chemoresistant Lung Cancer Cells |
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## Supplementary materials and methods

### Primary lung adenocarcinoma cells

A surgical specimen was obtained from a patient (female, 73 years old) who was diagnosed with lung adenocarcinoma (mixed subtypes) and underwent pulmonary lobe resection at the Department of Cardiovascular and Thoracic Surgery, Hokkaido University Hospital in 2015. The patient did not undergo any preoperative chemotherapy or radiotherapy. The resected lung cancer tissues (~1.0 $cm^3$ without necrosis) were put into ice-cold RPMI-1640 supplemented with 10% fetal bovine serum (HyClone, GE Healthcare), 0.1 mM non-essential amino acids (Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco) and 0.03% glutamine (Gibco), and transported immediately to the lab. After the removal of blood clots, the sample was rinsed with sterile PBS and cut into small fragments (~1 $mm^3$). The resulting suspension was then treated with 1% collagenase (Sigma) in a gently shaking water bath for 1 hour at 37°C. After passed through a 45 μm cell strainer, the resulting cell suspension was washed 3 times with sterilized PBS, and obtained cells were cultured in RPMI-1640 medium as mentioned above. The growth state of live cells was observed microscopically, and cells were passaged more than 5 times before using in further experiments. The clinical protocols for this study were approved by the committees in the Institutional Review Board of Hokkaido University Hospital (Approval No. 014-0274).

## Establishment of A549 cell line deficient for IL-34

A549-DR cells were transfected using Fugene 6 with IL-34 gRNA vector or scramble control (Mock) accompanied with a functional cassette containing GFP and puromycin resistant gene. GFP will be under the native promoter of IL-34 after genome integration, while puromycin resistant gene is under PGK promoter. GFP expression was confirmed by fluorescence microscope. Genome PCR was performed to verify the integration of the functional cassette using GFP primers as indicated by the manufacturer. After puromycin selection, IL-34 production was confirmed by ELISA (Biolegend, Catalog No. 439604).

## Cell viability assay

Cells were seeded at a density of $5 \times 10^3$ cells per well into 96-well plates 24 hours before stimulation. Following 24 hours treatment with drugs at the indicated concentrations, cell viability was determined using MTT assay. In some experiments, cells were pretreated with an inhibitor for S6K (PF4708671, Sigma-Aldrich, 160 nM), ERK (U0126, Sigma-Aldrich, 80 nM) or AKT (LY294002, CST, 10 μM) 1 hour before stimulation. All assays were performed in triplicate.

## Immunosuppression assay

Autologous human $CD4^+$ or $CD8^+$ T cells were isolated from PBMC of healthy donors using $CD4^+$ T cell isolation kit (Miltenyi Biotec 130-091-155) or $CD8^+$ T cell isolation kit (Miltenyi Biotec 130-045-201), respectively. $CD4^+$ or $CD8^+$ T cells were cultured in the presence of 3 μg/ml of α-CD3 antibody (eBioscience 16-0037-85) and 1 μg/ml of α-CD28 antibody (Biolegend 302914). Stimulated $CD4^+$ or

$CD8^+$ T cells were then co-cultured with macrophages differentiated in the presence of tumor supernatants as indicated at a ratio of 5:1. Supernatants were collected after 72 hours and used to measure levels of IFNγ production using ELISA Max Deluxe Set Human IFNγ kit (Biolegend 430104) according to the manufacture's protocol.

## Flow cytometry analysis

Single cell suspensions were stained with fluorescent antibodies and run on FACS canto II (BD Biosciences) and then analyzed using FlowJo software. For tumor samples obtained from *in vivo* humanized mouse model, single cell suspensions from tumors were prepared and stained for FACS analysis. In some experiments, $CD68^+CD163^+$ cells were isolated by FACS ARIA II from tumor single cell suspensions and used to collect total RNA. Fluorescent antibodies were purchased as follows: CD3 (Biolegend, Catalog No. 300311), CD4 (Biolegend, Catalog No. 357405), CD8 (Biolegend, Catalog No. 300905), CD11b (Biolegend, Catalog No. 301305), CD14 (Biolegend, Catalog No. 325603), CD68 (Biolegend, Catalog No. 333805), CD80 (Biolegend, Catalog No. 305219), CD86 (Biolegend, Catalog No. 305411), CD107a (Biolegend, Catalog No. 328623), CD163 (Biolegend, Catalog No. 333609), CD274 (Biolegend, Catalog No. 329707), HLA-A,B,C (Biolegend, Catalog No. 311409) and HLA-DR (Biolegend, Catalog No. 307609). For intracellular staining, cells were treated with Brefeldin A (Biolegend, Catalog No. 420601) for 4 hours, fixed and permeabilized (BD Cytofix/Cytoperm, Catalog No. 554714), and then stained with an antibody against Granzyme B (Biolegend, Catalog No. 515407). In other experiments, cells were fixed and permeabilized using True-Nuclear™ Transcription Factor Buffer Set (Biolegend, Catalog No. 424401) and then stained with an antibody against FoxP3 (Biolegend, Catalog No. 320115) or T-bet (Biolegend, Catalog No. 644807). All antibodies were used according to manufacturer's instruction.

### Quantitative real-time PCR

Total RNAs were collected from samples using RNeasy Mini Kit (Qiagen) and reversely transcribed to single strand complementary DNA using ReverTra Ace qPCR RT kit (Toyobo Co. Osaka, Japan). The quantification of targeted genes expression levels was performed by real-time quantitative polymerase chain reaction (RT-qPCR) using Fast SYBR Green Master Mix on a StepOne Plus real-time PCR (Applied Biosystems). Gene expression level was normalized to *Actin*. According to the supplier's recommendation, RT-qPCR was carried out in triplicate. Primers sequences are available upon request.

## Western blot

Cell lysates were applied to SDS-PAGE, and western blot transfer was done on PVDF membranes (Millipore) using a wet western blot system (Bio-Rad). Primary antibodies were purchased as follow: phospho IKKβ (CST, Catalog No. 2697), total IKKβ (CST, Catalog No. 2370), phospho p65 (CST, Catalog No. 3033), total p65 (CST, Catalog No. 8242), phospho AKT (CST, Catalog No. 4060), total

AKT (CST, Catalog No. 9272), total CSF1R (CST, Catalog No. E7S2S), phospho CSF1R (Y708) (CST, Catalog No. D5F4Y), phospho CSF1R (Y723) (CST, Catalog No. 3155), phospho STAT1 (CST, Catalog No. 9167), phospho STAT3 (CST, Catalog No. 4093), phospho STAT6 (CST, Catalog No. 9361), IRF4 (CST, Catalog No. 4964), IRF5 (CST, Catalog No. 13496), phospho C/EBPα (CST, Catalog No. 2844), total C/EBPα (CST, Catalog No. 8178), phospho C/EBPβ (CST, Catalog No. 3084) and Actin (Biolegend, Catalog No. 622102). As a secondary antibody, horseradish peroxidase (HRP) conjugated Ab was used for detection by Chemiluminescence (Super signal West Femto, Thermo Scientific) or ECL prime (GE health care). Blots were acquired with Image Quant, and quantified using Image J.

## Prognoscan analysis

The relationship between IL-34 expression levels and overall survival was evaluated in lung cancer patients by the minimum *P*-value approach using the PrognoScan database (Mizuno et al., 2009). Briefly, patients were divided into two groups according to IL-34 expression levels in tumors, and the risk differences of any two groups were then calculated by the log-rank test.