Journal for ImmunoTherapy of Cancer

New soluble CSF-1R-dimeric mutein with enhanced trapping of both CSF-1 and IL-34 reduces suppressive tumor-associated macrophages in pleural mesothelioma

Noémie Joalland,[1,2] Agnès Quéméner,[2,3] Sophie Deshayes,[2,3] Romain Humeau,[1,2] Mike Maillasson,[2,3,4] Héloïse LeBihan,[1,2] Apolline Salama,[1,2] Judith Fresquet,[2,3] Séverine Remy,[1] Erwan Mortier ,[2,3,4] Christophe Blanquart,[2,3] Carole Guillonneau,[1,2] Ignacio Anegon [1,2]

To cite: Joalland N, Quéméner A, Deshayes S, *et al*. New soluble CSF-1R-dimeric mutein with enhanced trapping of both CSF-1 and IL-34 reduces suppressive tumor-associated macrophages in pleural mesothelioma. *Journal for ImmunoTherapy of Cancer* 2025;**13**:e010112. doi:10.1136/jitc-2024-010112

► Additional supplemental material is published online only. To view, please visit the journal online (https://doi.org/10.1136/jitc-2024-010112).

EM, CB, CG and IA contributed equally.

Accepted 18 February 2025

For numbered affiliations see end of article.

Correspondence to
Dr Ignacio Anegon; ianegon@nantes.inserm.fr

Dr Carole Guillonneau; carole.guillonneau@univ-nantes.fr

ABSTRACT

Background Colony stimulating factor-1 receptor (CSF-1R) and its ligands CSF-1 and interleukin (IL)-34 have tumorigenic effects through both induction of suppressive macrophages, and survival/proliferation of tumor cells. In addition, the IL-34 tumorigenic effect can also be mediated by its other receptors, protein-tyrosine phosphatase zeta, Syndecan-1 (CD138) and triggering receptor expressed on myeloid cells 2. Small tyrosine kinase inhibitors are used to block CSF-1R signaling but lack specificity. Neutralizing anti-CSF-1 and/or IL-34 antibodies have been proposed, but their effects are limited. Thus, there is a need for a more specific and yet integrative approach.

Methods A human mutated form of the extracellular portion of CSF-1R was in silico modelized to trap both IL-34 and CSF-1 with higher affinity than the wild-type CSF-1R by replacing the methionine residue at position 149 with a Lysine ($_{M149K}$). The extracellular portion of the mutated CSF-1R M149K was dimerized using the immunoglobulin Fc sequence of a silenced human IgG1 (sCSF-1R$_{M149K}$-Fc). Signaling through CSF-1R, survival of monocytes and differentiation of suppressive macrophages were analyzed using pleural mesothelioma patient's samples and mesothelioma/macrophage spheroids in vitro and in vivo in the presence of sCSF-1R$_{M149K}$-Fc or sCSF-1R-Fc wild type control (sCSF-1R$_{WT}$-Fc).

Results We defined that the D1 to D5 domains of the extracellular portion of CSF-1R were required for efficient binding to IL-34 and CSF-1. The mutein sCSF-1R$_{M149K}$-Fc trapped with higher affinity than sCSF-1R$_{WT}$-Fc both CSF-1 and IL-34 added in culture and naturally produced in mesothelioma pleural effusions. sCSF-1R$_{M149K}$-Fc inhibited CSF-1R signaling, survival and differentiation of human suppressive macrophage in vitro and in vivo induced by pleural mesothelioma cells. Neutralization of IL-34 and CSF-1 by sCSF-1R$_{M149K}$-Fc also resulted in higher killing of pleural mesothelioma cells by a tumor-specific CD8$^+$ T cell clone in mesothelioma/macrophage spheroids.

Conclusions sCSF-1R$_{M149K}$-Fc efficiently traps both CSF-1 and IL-34 and inhibits CSF-1R signaling, monocyte survival and suppressive macrophage differentiation induced by pleural mesothelioma cells producing CSF-1 and IL-34, as well as restores cytotoxic T-cell responses. sCSF-1R$_{M149K}$-Fc has therapeutic potential vs other therapies under development targeting single components of this complex cytokine pathway involved in cancer.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The differentiation of suppressive macrophages by colony stimulating factor-1 (CSF-1) and interleukin (IL)-34 has deleterious effects in different cancers. Blocking of CSF-1 and IL-34 interactions with their membrane receptors (CSF-1R for both cytokines and protein-tyrosine phosphatase zeta, Syndecan-1 and triggering receptor expressed on myeloid cells 2 for IL-34) has therapeutic potential in cancer, but current therapeutic strategies have limitations.

WHAT THIS STUDY ADDS

⇒ We generated a new soluble human CSF1-R protein bearing a mutation providing an increased affinity for both CSF-1 and IL-34 and dimerized by fusion to a silenced human immunoglobulin Fc domain (sCSF-1R$_{M149K}$-Fc). As compared with the wild-type CSF-1R$_{WT}$-Fc, it blocked more efficiently CSF-1R signaling and monocyte viability induced by both CSF-1 and IL-34, in vitro and in vivo. sCSF-1R$_{M149K}$-Fc also more efficiently inhibited monocyte survival induced by mesothelioma pleural effusions and in three-dimensional mesothelioma/macrophage spheroids in vitro and in vivo.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ sCSF-1R$_{M149K}$-Fc has the potential to become a therapeutic molecule in cancers with involvement of tumor-associated suppressive macrophages.

INTRODUCTION

Colony stimulating factor-1 receptor (CSF-1R) (CD115) is a tyrosine kinase receptor

leading to activation of the PI3K/Akt and MAPK pathways following ligand binding.[1] CSF-1R is the receptor of both CSF-1 and interleukin (IL)-34.[2] CSF-1 has as unique receptor CSF-1R, while IL-34 has two other receptors identified so far, protein-tyrosine phosphatase zeta (PTPzeta) as well as the very recently discovered triggering receptor expressed on myeloid cells 2 (TREM2), and one co-receptor, Syndecan-1 (SDC-1, CD138).[3] CSF-1R is expressed by cells of the monocytic/macrophage cell lineage and is a major pathway of differentiation of monocytes from bone marrow precursors, as well as of monocytes into tissue macrophages.[4] IL-34 through its other receptors also has effects on other cell types, both normal and cancerous.[2] CSF-1 and IL-34 are expressed by different normal and cancerous cell types.[2]

CSF-1R, IL-34 and CSF-1 play an important role in cancer, mostly favoring cancer development, through different autocrine and paracrine effects, particularly development of suppressive tumor-associated macrophages (TAMs).[5–8]

Several reports have shown that various types of cancer express IL-34 and demonstrated a positive correlation between IL-34 and poor prognosis.[2 5–7] IL-34 produced by colon, gastric and hepatic tumorous cells favors metastasis and tumor progression by an autocrine effect.[9] Further, IL-34 expression by tumorous cells has been shown to induce resistance to chemotherapy in colorectal[10] and lung[11] cancer. In other cancers, such as in osteosarcoma, IL-34 promotes tumor progression and metastatic processes by favoring angiogenesis and suppressive TAM recruitment.[12]

The presence of CSF-1 in the tumor microenvironment favors a range of solid tumors including breast, melanoma, pancreatic, bladder,[8] certain leukemias[13] and tenosynovial giant cell tumor (TGCT).[14] CSF-1 is produced by different tumor cells, such as melanoma[15] or TGCT and also by fibroblasts present in the tumorous microenvironment, inducing suppressive TAMs.[16]

Additionally, chemotherapy and radiotherapy can induce the expression of CSF-1 and/or IL-34 by tumor cells inducing suppressive TAMs.[17] Specifically in pleural mesothelioma, both IL-34 and CSF-1 are produced by tumorous cells and contribute to creating an immunosuppressive environment through TAM suppressive induction that not only inhibits $CD8^+$ T cells cytotoxic responses against tumorous cells, but also induces resistance of tumorous cells to chemotherapy.[7 18]

Inhibition or blocking of CSF-1R, IL-34 or CSF-1 independently has been shown to reduce tumorous development in several preclinical animal models and in some clinical conditions, but have also shown limited clinical outcomes in several tumors.[19 20] It has been shown in preclinical animal models of cancer expressing IL-34 that a combination of IL-34 inhibition and therapies such as immune-checkpoint inhibitors,[21 22] radiotherapy or chemotherapy[23 24] may improve the therapeutic effect of these existing therapies. The effect of eliminating IL-34 with neutralizing antibodies or using IL-34-deficient tumorous cell lines in these models showed that the mechanism of action was autocrine in certain tumor models[11] and paracrine in others, mainly through infiltrating suppressive TAMs.[23 24] Blocking CSF-1 in combination with chemotherapy in patients with breast cancer did not ameliorate clinical outcomes.[20] High levels of CSF-1 predicted absence of response to checkpoint inhibitors.[25]

Blocking of either IL-34 or CSF-1 has the drawback of sparing either cytokine and thus not inhibiting the final pathway of cell activation through the CSF-1R. Inhibition of CSF-1R has been addressed using small molecule tyrosine kinase inhibitors[19 26–28] or blocking antibodies.[15 29 30] In preclinical models and early-phase clinical trials, responses to immune checkpoint inhibitors, chemotherapy or radiotherapy were partially ameliorated on blockade of CSF-1R using tyrosine kinase inhibitors or anti-CSF-1R blocking antibodies and the effect was through inhibition of TAM suppressive macrophages.[21 31–36] Nevertheless, at least certain tyrosine kinase inhibitors, such as PLX3397 (pexidartinib), inhibit tyrosine kinase receptors other than CSF-1R that are involved in immune responses[27 28] and when used in patients with colorectal cancer associated with anti-programmed death-ligand 1 (PD-L1) did not show improved clinical responses, potentially due to inhibition of FLT3 and dendritic cell development.[37] Blocking of CSF-1R in mice using a monoclonal antibody depleted a subpopulation of monocytes and tissue resident macrophages including those infiltrating pleural mesothelioma tumorous cells, but did not block inflammatory responses or even responses against the tumor.[38] Unfortunately, anti-CSF-1R blocking antibodies have limitations in reaching solid tumors and showed secondary effects.[29] Tyrosine kinase inhibitors and anti-CSF-1R antibodies, while blocking the actions of CSF-1 and IL-34 on CSF-1R, do not block the effect of IL-34 on its other receptors. These encouraging results on the inhibition of CSF-1R, IL-34 or CSF-1 indicate that this therapeutic strategy has considerable potential, but suffers from limitations and side effects, thus new molecules are needed.

This manuscript describes a strategy based on the development of a novel soluble dimeric form of the extracellular portion of human CSF-1R to avoid the binding of both CSF-1 and IL-34 to CSF-1R and of IL-34 to its other receptors. The affinity of this CSF-1R molecule for CSF-1 and IL-34 was increased by mutating the extracellular binding domain at a single key position involved in IL-34 and CSF-1 interaction with CSF-1R ($_{M149K}$). The mutated CSF-1R sequences were fused to a silenced human IgG1 Fc sequence (sCSF-$1R_{M149K}$-Fc) to dimerize the molecule and to increase the half-life in vivo. This mutated molecule neutralized in vitro both recombinant, as well as CSF-1 and IL-34 present in the supernatant of pleural mesothelioma cell lines and pleural effusions from patients with pleural mesothelioma more efficiently than the dimeric wild-type form of the CSF-1R (sCSF-$1R_{WT}$-Fc) or of the blocking anti-IL-34 and CSF-1 monoclonal antibodies (mAbs) alone or combined. sCSF-$1R_{M149K}$-Fc was

superior to sCSF-1R_{WT}-Fc to block monocyte survival and suppressive TAM differentiation in in vitro mesothelioma multicellular tumor spheroid (MCTS) and to increase antitumor T-cell cytotoxicity.

MATERIALS AND METHODS

Reagents

Recombinant human monomeric CSF-1R domains D1 to D3 (aa 1 to 290, 10161-H08H1) and D1 to D5 (aa 1 to 512, 10161-H08H), rabbit anti-TREM2 (11084-R016) were purchased from Sino Biological (Düsseldorfer, Germany). Recombinant human CSF-1R-Fc (329-MR-100), Syndecan-1 (2780-SD) and granulocyte macrophage colony stimulating factor (215 GM) were obtained from Bio-Techne (Rennes, France). Recombinant human CSF-1 (130-096-491) was from Miltenyi (Bergisch Carlsbad, Germany) and recombinant IL-34 was from ProteoGenix (Schiltigheim, France). Recombinant human TREM2 (9256-T2-50), human anti-IL-34 (MAB5265), human anti-CSF-1 (MAB216), control isotype (MAB002), phycoerythrin (PE)-conjugated anti-CSF-1R (FAB329P) antibodies, human IL-34 Quantikine ELISA (D3400) and human CSF-1 Quantikine ELISA (DMC00B) were obtained from R&D Systems (Bio-Techne, Rennes, France). PE-rabbit anti-human TREM2 antibody (ANR-018-PE) and PE-rabbit IgG isotype control (RIC-001-PE) were from Alomone labs (Jerusalem, Israël). PE-rabbit anti-human PTPzeta (bs-11327R-PE) was from Bioss Antibodies (Woburn, Massachusetts, USA). GW2580 CSF-1R tyrosine kinase inhibitor (SML1047) was from Sigma Aldrich (Saint-Louis, USA). PE-mouse anti-human CD138 (561704) antibody, PE-mouse IgG isotype control (550617), APC-Cy7 CD45 antibody (557833), FITC-CD14 antibody (555397), AF647-CD163 antibody (562669), BD Cytofix Fixation Buffer (554655), BD Phosflow Perm Buffer III (558050) and Fc Block (564765) were from BD Biosciences (Le pont de Claix, France). Rabbit anti-phospho-Akt (Ser473, 4060) or rabbit anti-phospho-ERK1/2 (Thr202/Tyr204, 4370) primary antibodies were purchased from Cell Signaling (Danvers, Massachusetts, USA). Alexa Fluor 647 (AF647)-conjugated goat anti-rabbit IgG (A-21245) was from Life Technologies (Carlsbad, California, USA). Anti-CD45-APC was from BioLegend (clone 304037) and anti-CD163-Alexa Fluor 488 from eBioscience (clone eBioGHI/61) Monocytes Isolation Kit (130-117-337) and Human Tumor Dissociation Kit (130-095-929) were purchased from Miltenyi (Bergisch Carlsbad, Germany). Viability dyes efluor-450 (FVD450) (65-0863-14) and efluor506 (FDV506) (00-8333-56), Fixation/Permeabilization Buffer Kit (00-5223-56 and 005 123–43) and Permeabilization Buffer 10X (00-8333-56) were from eBioscience (Thermo Fischer Scientific, Waltham, Massachusetts, USA). LPS (L4391) was purchased from Sigma (Saint-Quentin Fallavier, France) and Trypsin (TrypLE, 12 605–010) was from Gibco (Life Technologies, Carlsbad, California, USA). CellTiter-Glo Assay was purchased from Promega (Wisconsin, USA).

Cell lines

Macrophage human THP-1 and mouse WM266.4 cells were cultured respectively in Roswell Park Memorial Institute (RPMI) 1640 medium (21875–034, Gibco, Life Technologies, Carlsbad, California, USA) or Dulbecco's Modified Eagle Medium (DMEM) (21969–035, Gibco, Life Technologies, Carlsbad, California, USA) supplemented with decomplemented-fetal bovine serum (FBS) 10% (16141–079, Gibco), L-Glutamine 2 mM (25030–123, Gibco), 100 UI/mL Penicillin, 100 g/mL Streptomycin (15140–122, Gibco).

CSF-1R muteins predictions

Prediction of the free energy of mutation (ΔΔGmut) of some residues of the extracellular portion of human CSF-1R located in the interface with IL-34 or CSF-1 was performed. The ΔΔGmut at pH 7.4 was calculated for the substitutions of some residues of CSF-1R, using the method of Spassov and Yan[39] implemented under Discovery Studio (Dassault Systèmes BIOVIA Release 2017, San Diego) in the protocol "Calculate Mutation Energy (Binding)". The input data were the atomic coordinates of IL-34/CSF-1R and CSF-1/CSF-1R complexes (PDB codes 4DKD and 4WRL, respectively).

IL-34 and CSF-1R recombinant protein productions

Recombinant human IL-34 (aa 19 to 242) was produced in CHO mammalian cells, purified by ProteoGenix (France) through an HisTag affinity column and purity was controlled by SEC-HPLC (>90%). Dimeric D1-D3 and D1-D5 wild-type ($_{WT}$) or mutated at position 149 ($_{M149K}$) CSF-1R-Fc proteins were produced in CHO cells, purified using an Fc affinity column by Biotem (France) and purity was controlled by SEC-HPLC (>95%). The human IgG1 Fc domain used to dimerize CSF-1R was mutated (LALA-ASPS mutations)[40 41] to abolish interactions with FcgR and complement activation while allowing interaction with FcRn and prolonging the half-life of the molecule.

Surface plasmon resonance analysis

The biosensor used in this study was a Biacore T200 instrument (GE HealthCare, Limonest, France). CM5 Research Grade Sensor Chips (carboxymethyl-dextran surface) and HBS-EP (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 0.005% (v/v) surfactant P20, 3 mM EDTA) running buffer were also purchased from GE HealthCare. Recombinant sCSF-1R_{WT}-Fc and mutated sCSF-1R_{M149K}-Fc were coupled at 1154 and 1172 RU, respectively, on carboxymethyl-dextran surface of a CM5 Chip following the standard amine coupling protocol. IL-34 and CSF-1 recombinant proteins were diluted in HBS-EP at concentrations ranging from 12.5 to 200 nM and injected over the chip-immobilized with sCSF-1R_{WT}-Fc or $_{M149K}$ in a single cycle kinetics mode. The flow rate was set-up at 30 µL×min^{-1} and association and dissociation were allowed for 2 and 10 min, respectively. A 10 mM NaOH solution was injected over the chip

for 30 s for regeneration between each cycle. Rmax value (RU), k on ($M^{-1}\cdot s^{-1}$), k off (s^{-1}), and KD (M) were calculated from kinetic sensorgrams using the Two State Reaction model adapted for SCK analysis with "Biacore T200 Biaevaluation Software 3.1".

ELISA for inhibition of binding of IL-34 to CSF-1R-Fc, Syndecan-1 and TREM2 with sCSF-1R$_{WT}$-Fc or sCSF-1R$_{M149K}$-Fc

CSF-1R-Fc, Syndecan-1 or anti-TREM2 (0.25, 0.5 µg/mL and 1/500, respectively) were plated in a MaxiSorp 96-well microplate and incubated overnight at 4°C. THP-1 and WM266.4 cells (5,000 and 25,000 cells/well, respectively) were plated in a MaxiSorp 96-well microplate and dried overnight at 37°C in a drying oven. After a 2-hour saturation step with phosphate-buffered saline (PBS)/bovine serum albumin (BSA) 1% at room temperature, followed by a 2-hour incubation step with TREM2 (10 nM) in the case of TREM2, increasing concentrations of sCSF-1R$_{WT}$-Fc and sCSF-1R$_{M149K}$-Fc pre-incubated with biotinylated IL-34 (1 or 3 nM) were added to the plate and incubated for 2 hours at room temperature. Revelation was then performed using horseradish peroxidase (HRP-conjugated streptavidin.

CSF-1R signaling pathways analysis

THP-1 cells were plated in 96-well round-bottom culture plate at 10^5 cells/well in 100 µL RPMI 1640 (Gibco). The cells were rested for 2 hours, then IL-34 or CSF-1 was added at 4 nM with or without sCSF-1R-Fc or blocking antibodies against IL-34, CSF-1 or control isotype. THP-1 cells were fixed 3 min later using BD Cytofix Fixation Buffer, then permeabilized with BD Phosflow Perm Buffer III and stained with rabbit anti-phospho-Akt (Ser473, 4060, Cell Signaling, Danvers, Massachusetts, USA) or rabbit anti-phospho-ERK1/2 primary antibodies. Revelation was performed by a secondary staining with an AF647-goat anti-rabbit IgG antibody, fluorescence was recorded on a Canto II (BD Biosciences), mean fluorescence intensity analyzed on FlowJo (BD Biosciences) and results normalized against no cytokine condition (negative control) and IL-34/CSF-1 alone condition (positive control) as follows: % Akt pathway activation = ((experimental (MFI) – no cytokine MFI) / (cytokine alone MFI – no cytokine MFI)) × 100.

Monocyte isolation from healthy donors

Peripheral blood from healthy volunteers was obtained at the Etablissement Français du Sang (EFS, Nantes, France). After Ficoll density-gradient centrifugation (Ficoll-Plaque Plus, GE HealthCare, Little Chalfont, UK), peripheral blood mononuclear cells were recovered and CD14+ monocytes isolated by immunomagnetic negative sorting following manufacturer's recommendations of the Classical Monocytes Isolation Kit (130-117-337) from Miltenyi. Monocytes were used both immediately or frozen at −150°C for later use.

Monocyte viability assay

Sorted monocytes were cultured 3 days in 200 µL RPMI 1640 medium (Gibco) supplemented with decomplemented-FBS 10% (Gibco), L-Glutamine 1% (Gibco), 100 UI/mL Penicillin, 100 g/mL Streptomycin (Gibco), non-essential amino acid 1% (11140–035, Gibco), Sodium Pyruvate 1% (11360–039, Gibco), in 96-well flat-bottom culture plate. Monocyte culture medium was supplemented with 4 nM of CSF-1, IL-34, sCSF-1R variants and anti-CSF-1 and anti-IL-34 blocking antibodies described above. Pleural effusions from patients with pleural mesothelioma were diluted by half in the monocyte's culture medium to a final volume of 200 µL and sCSF-1R variants as well as anti-CSF-1 and anti-IL-34 blocking antibodies at the concentrations described in figures. 3 days later, monocytes were harvested, stained with fixable viability FVD450 and fluorescence was acquired on a Canto II (BD Biosciences). Results were analyzed with FlowJo (BD Biosciences), expressed as percentage of live cells (FVD450 negative cells) and compared with no cytokine condition (negative control) and IL-34 or CSF-1 alone condition (positive control).

Multicellular tumor spheroids

Meso13 and Meso34 mesothelioma cell lines, previously described in[42] were cultured in 96-well low adherence culture plate (174929, Thermo Fischer Scientific) at 2.10^4 cells/well in 200 µL of half conditioned medium/half fresh complete RPMI medium. Conditioned medium was obtained as follows: 10^6 Meso13 or/Meso34 cells were cultured 48 hours in 15 mL of complete RPMI medium in 75 cm^2 culture flasks. Monocytes were added at a cellular ratio of 2/1 Meso34/monocytes. sCSF-1R$_{WT}$-Fc, sCSF-1R$_{M149K}$-Fc and IL-34 or CSF-1 neutralizing antibodies were also added at a final concentration of 50 nM. For dissociation, replicated MCTSs were pooled and dissociated with trypsin for 30 min at 37°C before performing macrophage phenotyping on a spectral flow cytometer.

Spectral flow cytometry

Macrophages or dissociated spheroids were first stained with fixable FDV506, then Fc receptors were blocked using Fc Block and extracellular staining was performed. Then, cells were fixed and permeabilized using Fixation/Permeabilization Buffer Kit and intracellular staining was realized in Permeabilization Buffer. Antibodies used for this multicolor panel are listed in online supplemental table 1. Fluorescence acquisition was made with an Aurora Cytometer (Cytek Biosciences, San Diego, California, USA) and results were analyzed with FlowJo X software (BD Biosciences) and OMIQ software (OMIQ, Boston, Massachusetts, USA).

Cytokine quantification

IL-34 and CSF-1 were quantified in monocytes, Meso13 or Meso34 culture supernatant using Human IL-34 Quantikine ELISA and Human CSF-1 Quantikine ELISA following manufacturer's recommendations.

CD8+ T-cell clone cytotoxicity assay

Meso34 cells transfected to stably express nanoluciferase[7] were used in MCTS realized as described above, with a final number of Meso34/monocytes mixed cells of 5.10^3 cells in the presence or absence of sCSF-1R variants and anti-CSF-1 and anti-IL-34 blocking antibodies as described above. After 5 days, HLA-A*0201/MUC1 (950–958)-specific cytotoxic $CD8^+$ T cells,[43] were added at an effector/target ratio of 5/1. After 24 hours, 45 µL of culture supernatant were recovered, 5 µL of coelenterazine at 30 µM (CellTiter-Glo Assay, Promega, Wisconsin, USA) were added before measuring light emission at 480 nm on a Mithras LB 940 microplate analyzer (Berthold Technologies, Bad Wildbad, Germany). A total lysis control was obtained by incubation of MCTS with digitonin 0.1 mg/mL (Promega) for 15 min at 37°C. Cytotoxicity of the $CD8^+$ T cell was expressed as Meso34 lysis calculated as follows: % lysis = ((experimental measure – Meso34 MCTSs alone measure) / (total lysis of Meso34 MCTS measure – Meso34 MCTSs alone measure)) × 100.

Immunofluorescence/immunochemistry staining on MCTS

MCTSs were collected, washed one time in PBS and fixed in paraformaldehyde 4% (Electron Microscopy Sciences) for 1 hour at room temperature (RT). MCTSs were washed once with PBS and permeabilized for 6 hours with PBS containing 2% Triton X-100 at RT under agitation. This solution was removed, and then MCTSs were incubated with a solution of PBS containing 1% BSA, 0.5% Triton X-100, Hoechst 5 µg/mL (Sigma-Aldrich), anti-CD45-APC (1/50) and anti-CD163-Alexa Fluor 488 (1/100) for 72 hours at RT. Two steps of washing were performed with PBS for 24 hours at RT. 24 hours before observations, spheroids were mounted onto 8-well µ-Slide (Ibidi GmbH) pre-coated with 1.75 µg/cm² Cell-Tak cell and tissue adhesive (Corning) and cleared with Rapiclear 1.47 (Sunjin Lab). Finally, spheroids were imaged using a Nikon A1rHD LFOV confocal microscope with a 25×/1.05 oil immersion objective (Nikon Instruments).

RNA sequencing analysis

MCTSs were collected and messenger RNA (mRNA) were extracted using the NucleoSpin RNA kit (Macherey-Nagel). The mRNA quality was analyzed using the Agilent 20100 Bioanalyzer (Agilent) in RNA Nanochips (Agilent). Regarding gene analysis, 3′ sequencing RNA profiling was performed by the GenoBird platform (IRSUN, Nantes, France) using a NovaSeq 6000 (Illumina) and NovaSeq 6000 SP Reagent Kit 100 cycles (Illumina) according to the manufacturer's protocol (NovaSeq 6000 Sequencing System Guide Document #1000000019358v11 Material #20023471, Illumina). The full procedure is described in[44] The raw sequence reads were filtered based on quality using FastQC. Adapted sequences were trimmed off the raw sequence reads using Cutadapt. Reads were then aligned to the reference genome using BWA. Moreover, differential expressions were detected with the DESeq2 Bioconductor package. The sequences were deposited in the GEO database (GSE282239).

Pleural fluids

Pleural effusions from patients (online supplemental table 2) with a suspected pleural mesothelioma were aseptically collected, in accordance with the standards established by the Declaration of Helsinki, by thoracocentesis at the Laënnec Hospital (St-Herblain, France). Samples were centrifuged at 1,000×g in a Heraeus Multifuge for 20 min at +4°C and supernatants were aliquoted and stored at −80°C. Diagnoses were established by both fluid cytology and immunohistochemical staining of pleural biopsies performed by the pathology department at Laënnec Hospital (St-Herblain, France) and then externally confirmed by Mesopath, the French panel of pathology experts for the diagnosis of mesothelioma. All recruited patients had received no prior anti-cancer therapy and gave signed informed consent.

Monocyte survival and suppressive macrophage differentiation in vivo

This procedure in animals was performed according to institutional guidelines (Agreement APAFIS #37895, Regional ethics committee of Pays de la Loire; France). NSG (*NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ*) mice from Charles River Laboratories (Wilmington, Massachusetts, USA) were bred in the humanized rodent platform from the Labex IGO, housed in the animal facility of Nantes University (UTE, SFR F. Bonamy, Nantes, France) and used at 8–12 weeks of age. Meso34 cells were implanted in the right flank of mice at 1.10^6 cells per mouse. When the tumor reached 250/300 mm^3, 10^6 human monocytes were injected into the tumor, with or without $sCSF\text{-}1R_{WT}$-Fc or $sCS\text{-}1R_{M149K}$-Fc (50 nM). 3 days later, tumors were removed, dissociated using the Human Tumor Dissociation Kit (130-095-929, Miltenyi), then stained with viability dye FVD450, APC-Cy7 CD45 antibody, FITC-CD14 antibody, AF647-CD163 antibody and finally analyzed on Canto II (BD Biosciences).

Statistical analysis

All data were expressed as mean±SEM and statistical analyses were performed with GraphPad Prism V.8 (GraphPad Software, Boston, Massachusetts, USA). Statistical tests and significant differences for each figure were mentioned in its legends.

RESULTS

Soluble dimeric CSF-1R efficiently traps both IL-34 and CSF-1 and avoids activation of cell membrane-bound CSF-1R downstream signalization pathways

CSF-1R is a monomeric chain that binds a monomer of CSF-1 or IL-34, but both cytokines exhibit a dimeric form allowing the dimerization of CSF-1R with then effective signal transduction into the cell.[1–3] First, several forms of the extracellular domains (ECD) of CSF-1R were

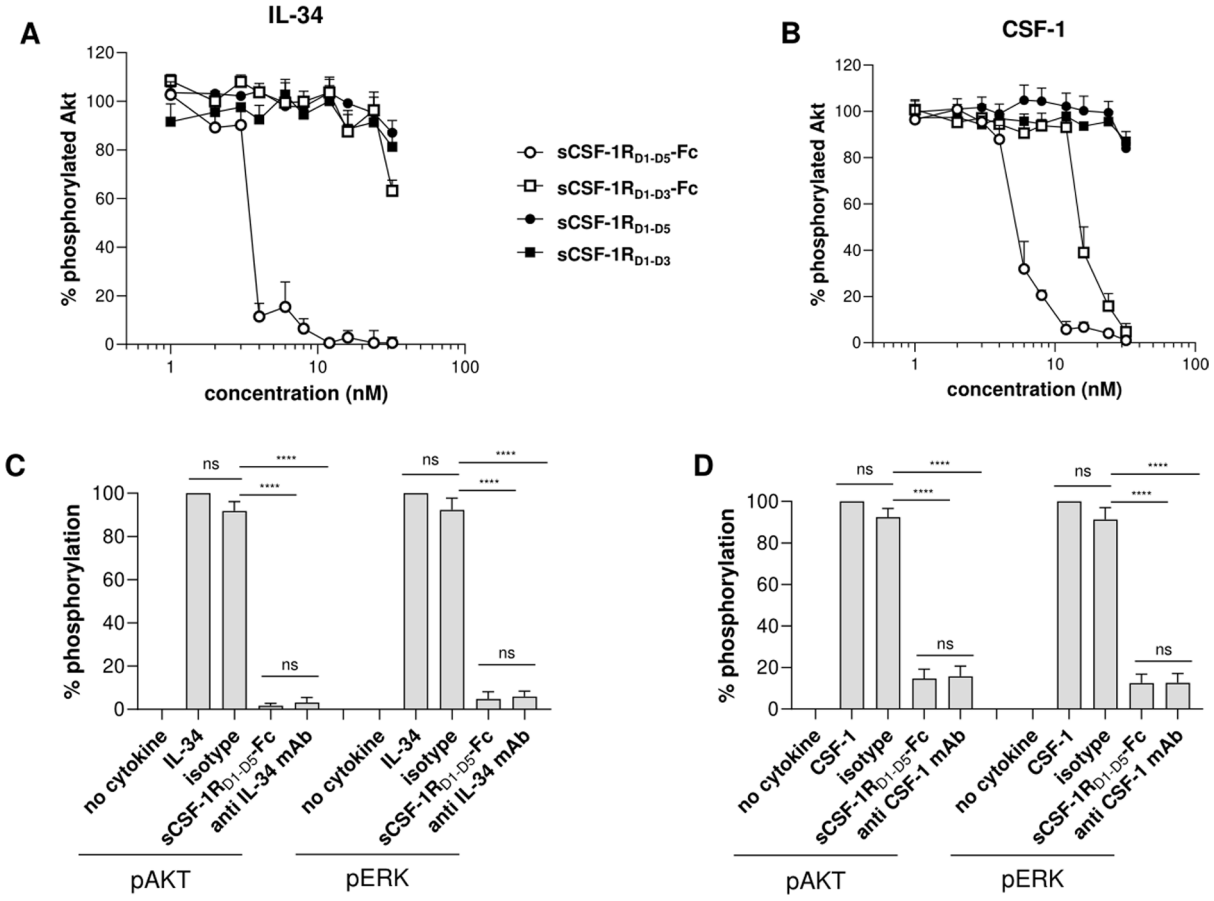

Figure 1 Soluble CSF-1R-Fc is efficient to trap both IL-34 and CSF-1 and avoid CSF-1R downstream signalization pathways. (A–B) Different formats of CSF-1R, D1-D3 versus D1-D5, monomeric or dimeric through an Fc, were tested. Increasing concentration of each form was pre-incubated with IL-34 (A) or CSF-1 (B), then mixed with THP-1 cells to measure the intracellular level of phosphorylated Akt. Results were normalized against no cytokine (0%) and IL-34 or CSF-1 alone (100%) conditions, and expressed as mean±SEM, n=4 experiments. (C–D) sCSF-1R$_{D1-D5}$-Fc efficacy to trap either recombinant IL-34 (C) or recombinant CSF-1 (D) was confirmed, in comparison with respective blocking antibodies, on two CSF-1R pathways, Akt and ERK. Results were normalized against no cytokine condition (0%) and IL-34 or CSF-1 alone (100%), expressed as mean±SEM; n=8 experiments; Tukey's multiple comparisons test; ****p<0.0001. CSF-1R, colony stimulating factor-1 receptor; IL, interleukin.

tested. The N-terminal D1-D3 immunoglobulin structural domains of CSF-1R bind CSF-1 and IL-34, whereas D4-D5 stabilize the ligand.[1–3] ECD sequences were either in a monomeric form (CSF-1R$_{D1-D3}$ and CSF-1R$_{D1-D5}$) or dimerized by fusing them to an immunologically silent human IgG1 Fc fragment (sCSF-1R$_{D1-D3}$-Fc or CSF-1R$_{D1-D5}$-Fc). The Fc portion will also contribute to increasing the molecule's half-life. We assessed the effect of trapping IL-34 (figure 1A) or CSF-1 (figure 1B) in the presence of increasing doses of the different soluble forms of CSF-1R-Fc and by analyzing the downstream pathway Akt phosphorylation of CSF-1R on the monocytic THP-1 cell line. Interestingly, the monomeric forms of sCSF-1R$_{D1-D3}$ or sCSF-1R$_{D1-D5}$, were not capable of inhibiting Akt phosphorylation induced by either IL-34 or CSF-1. On the opposite, the dimeric sCSF-1R$_{D1-D5}$-Fc inhibited the Akt pathway induced by both IL-34 and CSF-1 in a dose-dependent manner (IC50=3.4 nM and 5.6 nM, respectively) (figure 1A,B). In contrast, the dimeric sCSF-1R$_{D1-D3}$-Fc did not inhibit Akt phosphorylation induced by IL-34 and inhibited the one induced by CSF-1 with lower affinity (IC50=15.8 nM) than sCSF-1R$_{D1-D5}$-Fc (figure 1B).

Further analysis of sCSF-1R$_{D1-D5}$-Fc at a molecular ratio of 1/1 with IL-34 (figure 1C) or CSF-1 (figure 1D) showed efficient inhibition of the two major CSF-1R pathways, phosphorylation of Akt and ERK, that was comparable to the inhibition obtained with neutralizing anti-IL-34 or anti-CSF-1 mAb. Based on these results, we selected for further analysis the soluble dimeric CSF-1R$_{D1-D5}$-Fc form of CSF-1R and decided to increase its affinity to more efficiently compete with the cell membrane CSF-1R.

Trapping of both IL-34 and CSF-1 by soluble CSF-1R improved by a single amino acid mutation

Our strategy was to increase the affinity of sCSF-1R-Fc for IL-34 and CSF-1 to improve its efficiency in capturing soluble cytokines. In silico visual observation allowed identification of amino acids common in the interface between IL-34/CSF-1R and CSF-1/CSF-1R (figure 2A,B) and prediction of the free energy of mutation (ΔΔGmut)

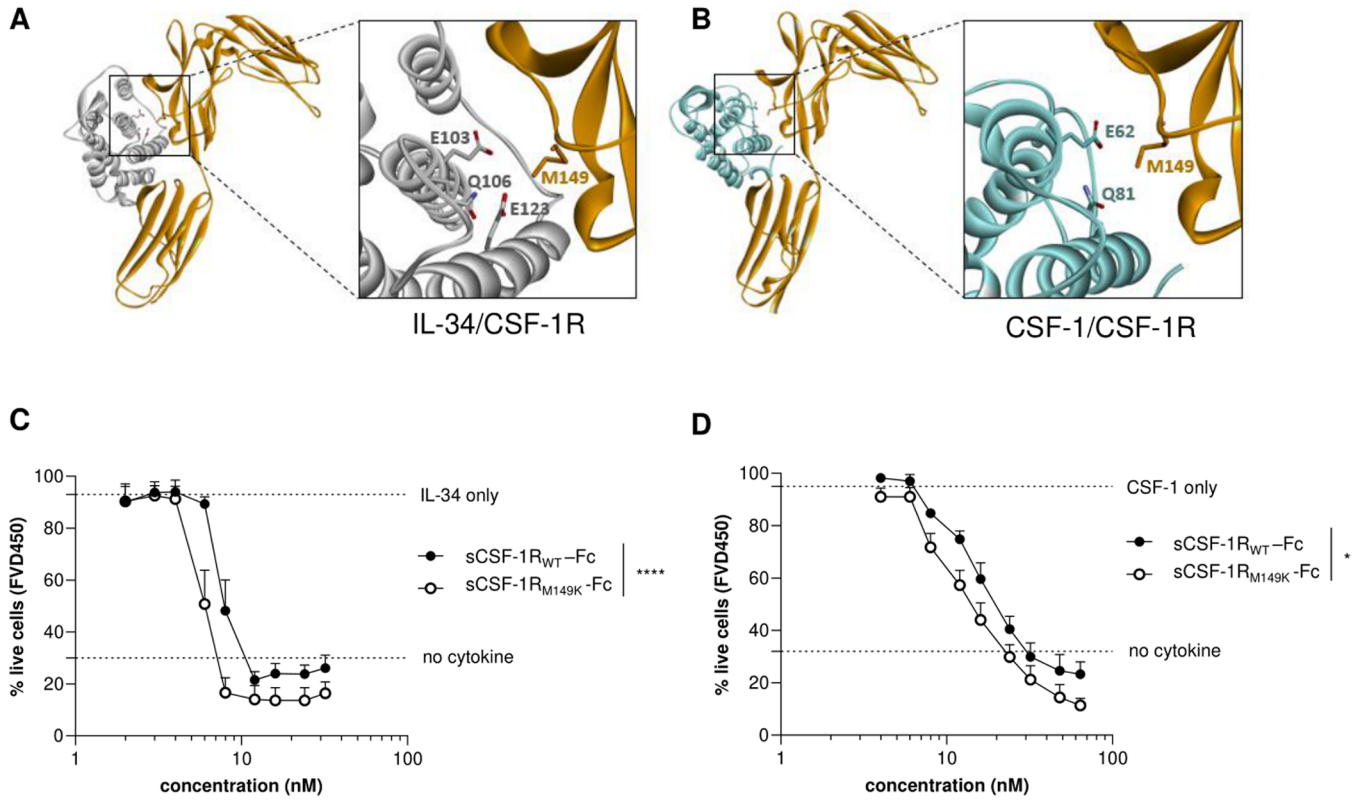

Figure 2 sCSF-1R-Fc efficacy to trap both IL-34 and CSF-1 to inhibit monocyte viability can be improved by a single amino acid mutation. (A–B) Ribbon representations of IL-34/CSF-1R (A, PDB code 4DKD) and CSF-1/CSF-1R (B, PDB code 4WRL) complexes and close views of the interaction sites showing the amino acids in the vicinity of the residue M149 of CSF-1R. (C–D) Monocytes, isolated from human peripheral blood mononuclear cells were cultured 3 days in the presence of 4 nM of IL-34 (C) or 4 nM of CSF-1 (D) with sCSF-1RWT-Fc or sCSF-1RM149K-Fc at different concentrations. Cell viability was analyzed, and results were expressed as % of live cells, mean±SEM, n=6/8 experiments using 3/4 donors. Tukey's multiple comparisons test; *p<0.05 ****p<0.0001. CSF-1R, colony stimulating factor-1 receptor; IL, interleukin.

of these residues, revealed that a methionine (M) located at position 149 of the aa sequence could be substituted by a lysine (K) ($_{M149K}$) to potentially increase the affinity between CSF-1R and its two ligands simultaneously (online supplemental figure 1A,B). The soluble form CSF-1R-Fc mutated in position 149 (sCSF-1R$_{M149K}$-Fc) was successfully produced in CHO cells (online supplemental figure 1C) at a comparable or higher yield than the wild-type form of sCSF-1R-Fc (sCSF-1R$_{WT}$-Fc). Surface plasmon resonance analysis showed that the affinity of the sCSF-1R$_{M149K}$-Fc versus sCSF-1R$_{WT}$-Fc was increased for IL-34, mainly due to a slower dissociation phase (Kd=3 vs 8 nM, respectively) and for CSF-1 (Kd 2 vs 4 nM, respectively) (table 1). The neutralizing capacity of the mutated sCSF-1R$_{M149K}$-Fc was also superior to that of sCSF-1R$_{WT}$-Fc and to neutralizing antibody in a test of inhibition of monocyte viability when cells were incubated with a fixed concentration of IL-34 (figure 2C and online supplemental figure 2Aonline supplemental file 2) or CSF-1 (figure 2D and online supplemental figure 2Bonline supplemental file 2) and increasing concentrations of inhibiting molecule. For both IL-34 and CSF-1, sCSF-1R$_{M149K}$-Fc resulted in a significantly higher neutralizing capacity than that of sCSF-1R$_{WT}$-Fc (IC50s of 8 and 6 nM vs 12 and 16 nM,

Table 1 SPR analysis of IL-34 and CSF-1 when bound to immobilized sCSF-1R$_{WT}$-Fc or sCSF-1R$_{M149K}$-Fc

	ka ($M^{-1}.s^{-1}$)	kd (s^{-1})	KD (M)
IL-34			
sCSF-1$_{WT}$-Fc	6.2 10^4	4.0 10^{-4}	8.1 10^{-9}
sCSF-1$_{M149K-}$Fc	6.3 10^4	1.6 10^{-4}	3.0 10^{-9}
CSF-1			
sCSF-1$_{WT}$-Fc	8.3 10^6	3.1 10^{-2}	3.8 10^{-9}
sCSF-1$_{M149K}$Fc	1.3 10^7	2.9 10^{-2}	2.1 10^{-9}

CSF-1, colony stimulating factor-1; IL, interleukin.

respectively). Monocyte viability in the presence of high concentrations of sCSF-1R$_{M149K}$-Fc and sCSF-1R$_{WT}$-Fc was even lower than in the absence of IL-34 or CSF-1. Analysis of CSF-1 and IL-34 production in the cell culture supernatant showed that monocytes spontaneously produced CSF-1 (online supplemental figure 2C), but not IL-34

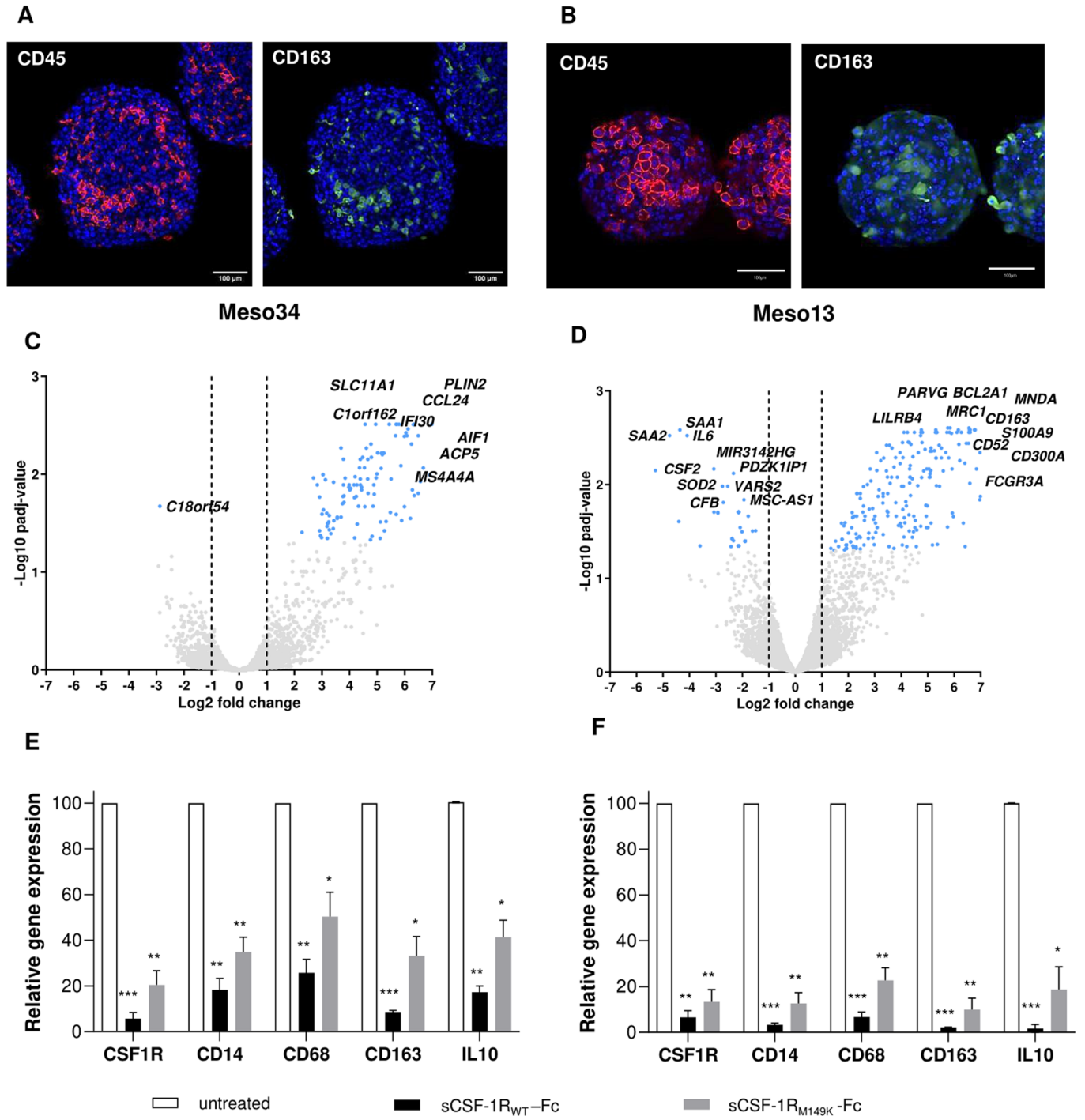

Figure 3 Characterization of Meso13/ and Meso34/monocytes, MCTS and monocytes inhibition by sCSF-1R-Fc. Meso13 and Meso34 cells, as well as monocytes from healthy donors, were seeded in a low adherence 96-round well plate for 5 days. (A–B) Confocal microscopy pictures after labeling macrophages of Meso34/monocytes MCTS (A) or Meso13/monocytes MCTS (B) with anti-CD45 (left panel, magenta) or anti-CD163 (right panel, green) antibodies. Hoechst (blue) was used to label cell nuclei. Scale bar=100 µm. (C–D) Volcano plot of the differentially expressed mRNAs between Meso34 (C) or Meso13 (D) MCTS without versus with monocytes. Blue dots: p<0.05, gray dots: p>0.05. Dotted lines: gene regulated with a log2 fold change ≥1 or ≤−1. (E–F) mRNA were extracted from Meso34/monocytes MCTS (E) or Meso13/monocytes MCTS (F) and the expression of macrophage markers and IL-10 was measured using real-time PCR. Bar graphs represent mean±SEM of 3 independent experiments. T test *p<0.05; **p<0.01 and ***p<0.001. CSF-1R, colony stimulating factor-1 receptor; IL, interleukin; MCTS, multicellular tumor spheroid; mRNA, messenger RNA.

Table 2 List of the 10 most significantly upregulated genes in Meso34/monocytes multicellular tumor spheroid compared with Meso34

Gene	Log2 fold change	P value	Adjusted p value
SPP1	8.22	2.66E–06	4.45E–03
VSIG4	7.41	3.05E–06	4.45E–03
RNASE1	7.17	7.70E–07	4.45E–03
AIF1	6.99	3.06E–06	4.45E–03
C1orf162	6.35	5.79E–07	4.45E–03
MS4A4A	5.80	1.38E–06	4.45E–03
PLIN2	5.72	3.34E–06	4.45E–03
ACP5	5.70	3.18E–06	4.45E–03
CCL24	5.45	1.57E–06	4.45E–03
IFI30	4.96	2.11E–06	4.45E–03

(online supplemental figure 2D). This suggests that the sCSF-1R$_{M149K}$-Fc muteins were able to neutralize this spontaneous production of CSF-1, as confirmed by comparison with neutralizing antibodies (online supplemental figure 2E).

Using an ELISA assay, sCSF-1R$_{M149K}$-Fc also inhibited the binding of IL-34 to coated CSF-1R-Fc, Syndecan-1 and TREM2 with the same efficacy as sCSF-1R$_{WT}$-Fc (online supplemental figure 3A). sCSF-1R$_{M149K}$-Fc also inhibited the binding of IL-34 to THP-1 cells, which express mainly CSF-1R, TREM2, Syndecan-1 and PTPzeta (Online supplemental figure 3B,C). sCSF-1R$_{M149K}$-Fc also inhibited the binding of IL-34 to WM266.4 cells which express mainly Syndecan-1 and PTPzeta (online supplemental figure 3B and D). For recombinant Syndecan-1 and TREM2, as well as for both cell lines, inhibition of binding of IL-34 by sCSF-1R$_{M149K}$-Fc and sCSF-1Rwt-Fc was partial (60–80%) whereas for recombinant CSF-1R was complete.

Altogether, these results demonstrate that sCSF-1R$_{M149K}$-Fc was more efficient than sCSF-1R$_{WT}$-Fc to neutralize recombinant IL-34 and CSF-1 binding to CSF-1R and to inhibit monocyte activation and reduce survival even in basal culture conditions in the absence of added cytokines. The M149K mutation preserved blocking of IL-34 to its other receptors, TREM-2, Syndecan-1 and PTPzeta.

Characterization of suppressive macrophage differentiation in an in vitro three-dimensional model of pleural mesothelioma MCTSs

To evaluate the efficacy of sCSF-1R$_{M149K}$-Fc in cancer, we selected two mesothelioma cell lines, the Meso13 and the Meso34 on the basis of the different mutations they carry, which they are representative of some of the mutations observed in mesothelioma.[45] These cell lines also present different molecular characteristics, and then different overexpressed pathways, illustrating the heterogeneity of PM cells between patients[46 47] (online supplemental figure 4A–C). In addition, Meso13 produced in the culture supernatant higher CSF-1 and lower IL-34 levels than Meso34 (online supplemental figure 4D). Meso34/monocytes and Meso13/monocytes MCTS formed multilayer, structured and compact spheroids (online supplemental figure 4E,F, respectively), where we observed by

Table 3 List of the 10 most significantly upregulated genes in Meso13/monocytes multicellular tumor spheroid compared with Meso13

Gene	Log2 fold change	P value	Adjusted p value
CD300A	6.56	3.99E–07	2.48E–03
LILRB4	5.84	5.80E–07	2.48E–03
CD52	5.76	5.50E–07	2.48E–03
CD163	6.79	2.24E–06	2.61E–03
S100A9	6.76	1.02E–06	2.61E–03
FCGR3A	6.60	2.08E–06	2.61E–03
MNDA	6.58	1.91E–06	2.61E–03
MRC1	6.16	1.23E–06	2.61E–03
PARVG	5.31	1.65E–06	2.61E–03
BCL2A1	4.76	1.64E–06	2.61E–03
RNASE1	6.60	3.92E–06	2.77E–03

immunofluorescence the presence of $CD45^+CD163^+$ macrophages, compatible with suppressive macrophages (figure 3A and B, respectively). Comparison of the differentially expressed genes between Meso34/monocytes and Meso13/monocytes MCTS and Venn diagram representation show that each model expressed specific genes (n=214 for Meso13/monocytes MCTS and n=130 for Meso34/monocytes MCTS) and a set of common genes (n=94) (online supplemental figure 4G).

RNA sequencing (RNA-seq) analysis comparing Meso34/monocytes and Meso34 MCTS showed enrichment for genes expressed by myeloid cells in the former, such as *MS4A4A*, *AIF1*, *CCL24*, *SLC11A1*, *C1orf162* and *1F30* (table 2); genes expressed by macrophages and tumor cells and increases in proliferation and metastasis, such as *ACP5*; and genes expressed ubiquitously including macrophages and tumor cells, such as *PLIN2* (figure 3C and online supplemental table 3). Similarly, RNA-seq analysis comparing Meso13/monocytes and Meso13 MCTS showed enrichment for genes expressed by myeloid cells and particularly by suppressive macrophages in the former, such as *CD163*, and *MRC1* (top 10 genes overexpressed in Meso13/monocytes MCTS compared with Meso13 MCTS) (table 3) (figure 3D). However, here, macrophages seemed different and probably less immunosuppressive given that *CD163* and *MRC1* only appeared in the top 50 genes overexpressed in Meso34/monocytes MCTS compared with Meso34 MCTS (online supplemental table 4). Analysis of cellular functions with enrichment by genes overexpressed in Meso34/monocytes versus Meso34 MCTS showed an enrichment in immune processes with activated macrophages, such as allograft rejection and inflammatory responses in both Meso34/monocytes (online supplemental figure 4H) and Meso13/monocytes (online supplemental figure 4I) MCTS. These results indicate the presence of activated macrophages in mesothelioma MCTSs with a suppressive phenotype (*CD163+MRC1+*).

mRNA levels of macrophage cell membrane markers (CSF-1R, CD68 and CD163 as well as the macrophage marker CD14) in Meso 34/monocytes (figure 3E) and Meso13/monocytes (figure 3F) MCTSs showed a significant decrease in the presence of both $sCSF\text{-}1R_{M149K}$-Fc or $sCSF\text{-}1R_{WT}$-Fc. Similarly, the mRNA levels for IL-10 were decreased in both conditions (figure 3E–F).

$sCSF\text{-}1R_{M149K}$-Fc is more efficient than $sCSF\text{-}1R_{WT}$-Fc to decrease monocyte viability in vitro in a 3D model of mesothelioma MCTSs

Monocyte survival and macrophage differentiation were evaluated in mesothelioma MCTSs in the presence of $sCSF\text{-}1R_{M149K}$-Fc compared with $sCSF\text{-}1R_{WT}$-Fc (figure 4 and

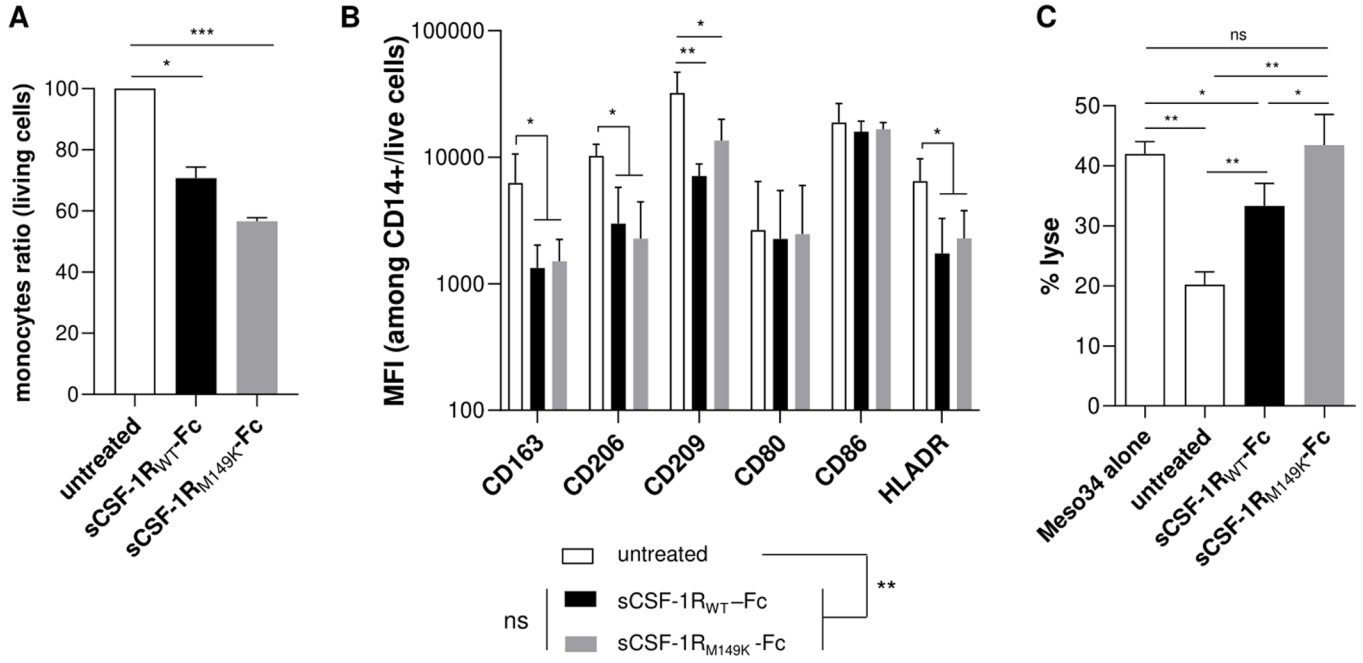

Figure 4 sCSF-1RM149K-Fc is more efficient than sCSF-1RWT-Fc to inhibit immunosuppressive macrophages phenotype and function in Meso34/monocytes MCTS. Meso34 cells, as well as monocytes from healthy donors, were seeded in low adherence 96-round well plate for 5 days. (A) Meso34/monocytes MCTS, treated or not with sCSF-1RWT-Fc or sCSF-1RM149K-Fc, were dissociated and macrophage viability was analyzed. Results were expressed as monocytes ratio compared with the control situation (untreated=100%); mean±SEM; n=8 using five different donors; Friedman multiple comparisons test; *p<0.05 ***p<0.001. (B) Meso34/monocytes MCTS, treated or not with sCSF-1RWT-Fc or sCSF-1RM149K-Fc, were dissociated and expression level (MFI) of differentiated macrophages markers were analyzed among CD14+/live cells; mean±SEM; n=4 using two different donors; Tukey's multiple comparisons test; *p<0.05 **p<0.01. (C) $CD8^+$ T cells from a clone specific for HLA-A*0201/MUC1(950–958) were added to Meso34 HLA-A*0201/monocytes MCTS at effector/target ratio 5/1. 24 hours later, supernatants were recovered, luciferase activity measured and % lysed cells calculated; mean±SEM; n=6 using three donors; Dunn's multiple comparisons test; *p<0,05 **p<0.01. MCTS, multicellular tumor spheroid.

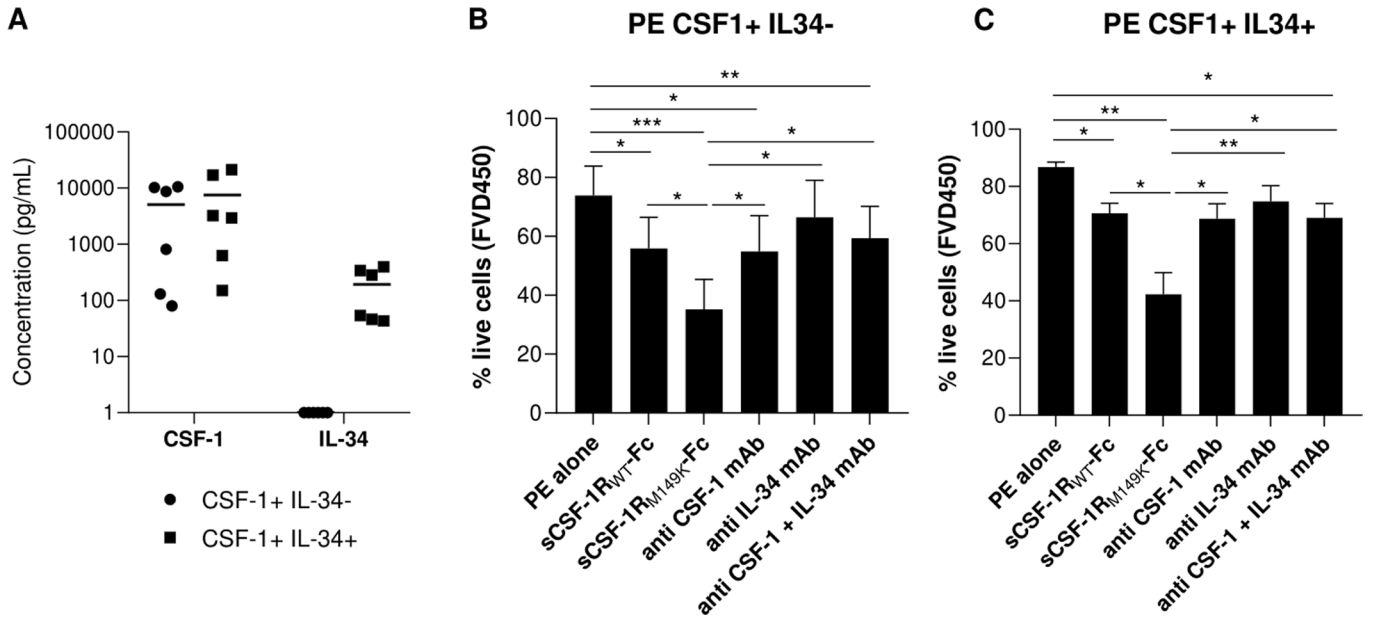

Figure 5 sCSF-1RM149K-Fc was more efficient than sCSF-1RWT-Fc to inhibit CSF-1 and IL-34 present in pleural effusions of patients with mesothelioma. (A) CSF-1 and IL-34 quantification in pleural effusion and classification of pleural effusions based on the production of IL-34: CSF-1$^+$IL-34$^+$ (circle) versus CSF-1$^+$IL-34$^-$ (square). (B–C) Monocytes, sorted from fresh human peripheral blood mononuclear cell, were cultured for 3 days with pleural effusions from patients with pleural mesothelioma, then cell viability was analyzed. Pleural effusions were divided into two categories: CSF-1$^+$IL-34$^-$ (B) and CSF-1$^+$IL-34$^+$ (C) based on the production or not of IL-34. Results were expressed as mean±SEM, n=8 experiments using three donors; Tukey's multiple comparisons test; *p<0.05 **p<0.01 ***p<0.001. CSF-1R, colony stimulating factor-1 receptor; IL, interleukin; mAb, monoclonal antibodies; PE, phycoerythrin.

online supplemental figure 5A–E). The survival of monocyte/macrophages in Meso 34/monocytes (figure 4A) and Meso13/monocytes (online supplemental figure 5D) MCTS was significantly reduced in the presence of sCSF-1R$_{M149K}$-Fc. Moreover, in Meso34/monocytes MCTS, compared with CSF-1 and IL-34 neutralizing antibodies sCSF-1R$_{M149K}$-Fc showed a significant decrease, whereas the decrease with sCSF-1R$_{WT}$-Fc was non-significant (online supplemental figure 5E).

Using spectral flow cytometry, we analyzed the phenotype of surviving macrophages from Meso34/monocytes MCTSs in the presence of sCSF-1R$_{M149K}$-Fc or sCSF-1R$_{WT}$-Fc (online supplemental figure 5A–C). Both molecules significantly decreased markers of immunosuppressive

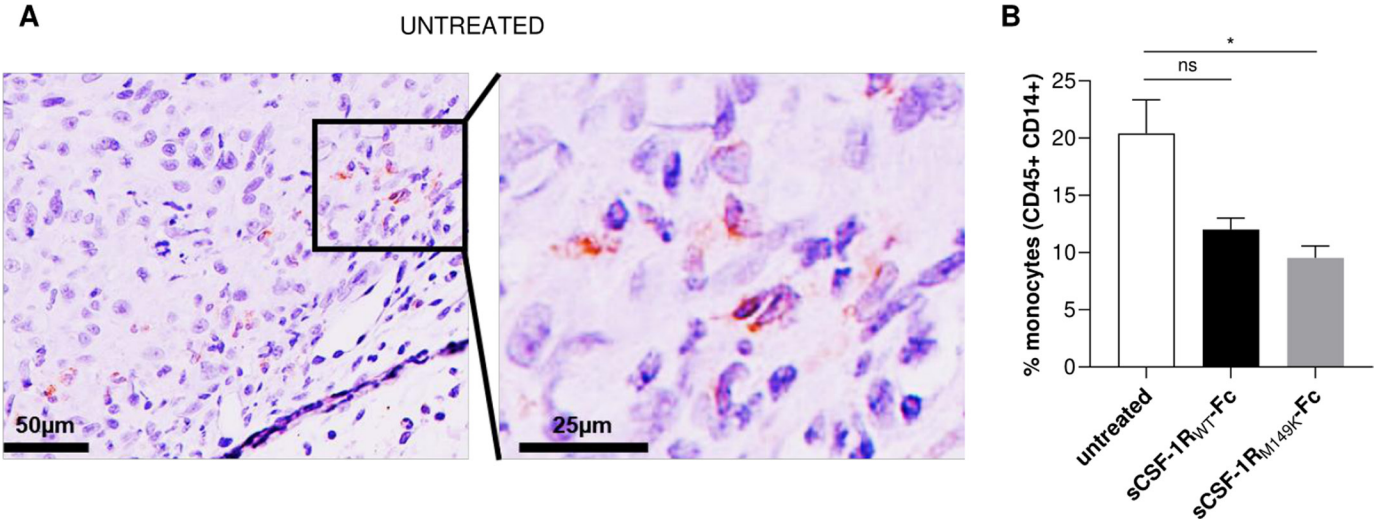

Figure 6 sCSF-1RM149K-Fc significantly inhibits human macrophage survival in Meso34 tumors in vivo. Meso34 cells were subcutaneously implanted in NSG immunodeficient mice. When tumors reached 250 mm^3, monocytes and sCSF-1RWT-Fc or sCSF-1RM149K-Fc were injected into the tumor and 3 days later, tumors were harvested for analyses. (A) Representative immunohistology using an anti-CD68 antibody, showing the presence of human macrophages in untreated tumors. (B) Tumors were recovered, dissociated and the number of living hCD45$^+$CD14$^+$ macrophages was analyzed using flow cytometry. The graphic represents mean±SEM; n=3/4. Paired t-test; *p<0.05.

macrophages, such as CD163, CD206, CD209 and HLA-DR (figure 4B) without affecting other markers of activated macrophages (online supplemental figure 5F). The presence of macrophages in Meso34 MCTS led to the inhibition of approximately 50% of the cytotoxic activity of a $CD8^+$ T-cell clone specific for HLA-A*0201/MUC1 as we previously observed[7] (figure 4C). In the presence of sCSF-$1R_{WT}$-Fc and particularly of sCSF-$1R_{M149K}$-Fc or, the cytotoxic activity of the clone was restored to around 80% and 100%, respectively (figure 4C).

Altogether, these results indicate that sCSF-$1R_{M149K}$-Fc was more efficient than sCSF-$1R_{WT}$-Fc to inhibit survival of monocytes in three-dimensional mesothelioma MCTS and, more importantly, improved CTL activity against mesothelioma cells.

sCSF-$1R_{M149K}$-Fc is more efficient than sCSF-$1R_{WT}$-Fc and neutralizing antibodies to inhibit CSF-1 and IL-34 in pleural effusions of patients with mesothelioma

We then aimed to confirm the results obtained with sCSF-$1R_{M149K}$-Fc using pleural effusions from patients with pleural mesothelioma. We first observed that all mesothelioma pleural effusion contained CSF-1 in variable amounts but not all contained IL-34, as previously observed[7] (figure 5A). We thus divided the pleural effusions into two categories: one group containing only CSF-1 ($CSF\text{-}1^+IL\text{-}34^-$) and a second group containing both ($CSF\text{-}1^+IL\text{-}34^+$). Pleural effusion, from both groups, maintained monocytes survival in vitro and sCSF-$1R_{M149K}$-Fc was superior to sCSF-$1R_{WT}$-Fc in inhibiting their survival (figure 5B–C). Each individual pleural infusion incubated with sCSF-$1R_{WT}$-Fc or sCSF-$1R_{M149K}$-Fc showed reduced monocyte survival (online supplemental figure 6A,B). sCSF-$1R_{M149K}$-Fc was more efficient than sCSF-$1R_{WT}$-Fc to inhibit monocyte survival in every sample (online supplemental figure 6C). Neutralizing anti-CSF-1 or anti-IL-34 mAbs showed only little efficacy to inhibit either CSF-1 or CSF-1 and IL-34 present in pleural effusion (figure 5B–C). Interestingly, sCSF-$1R_{WT}$-Fc showed similar inhibition as the one obtained with the combination of anti-CSF-1 and anti-IL-34 neutralizing mAbs (figure 5B–C) and was less effective than GW2580, a CSF-1R tyrosine kinase inhibitor (online supplemental figure 6DE) whereas sCSF-$1R_{M149K}$-Fc was more efficient to reduce monocyte survival in both groups of pleural effusion compared with neutralizing antibodies (figure 5B–C) and was as efficient as GW2580 (online supplemental figure 6F).

Altogether, these results demonstrate that sCSF-$1R_{M149K}$-Fc was not only superior to sCSF-$1R_{WT}$-Fc, but also to neutralizing anti-CSF-1 and anti-IL-34 antibodies alone or combined to inhibit the activity on monocytes of CSF-1 and IL-34 present in pleural effusion.

sCSF-$1R_{M149K}$-Fc is more efficient than sCSF-$1R_{WT}$-Fc to inhibit monocyte survival in mesothelioma MCTSs in vivo

Meso34 cells were implanted into immunodeficient mice and after tumors reached 250 mm^3 human monocytes were injected into the tumors with sCSF-$1R_{M149K}$-Fc or sCSF-$1R_{WT}$-Fc and monocyte survival in the tumors was then analyzed after 3 days. Immunohistology of explanted Meso34/monocytes MCTS confirmed the presence of CD68+ macrophages in the tumors (figure 6A). Flow cytometry analysis of explanted Meso34/monocytes MCTS showed a decrease in the presence of CD45+CD14+ macrophages in tumors treated with sCSF-$1R_{WT}$-Fc (approximately 30%, non-significant) and with sCSF-$1R_{M149K}$-Fc (approximately 45%, $p<0.05$) (figure 6B) and this was confirmed by immunohistology using the CD68 marker (online supplemental figure 7).

These results demonstrate that sCSF-$1R_{M149K}$-Fc was superior to sCSF-$1R_{WT}$-Fc to inhibit $CD14^+$ monocytes/macrophages survival in vivo.

DISCUSSION

Our results show that sCSF-$1R_{M149K}$-Fc, a soluble dimeric and mutated form of the extracellular binding domain of CSF-1R blocked more efficiently both IL-34 and CSF-1 compared with the wild-type form of sCSF-1R-Fc and thus reduced monocyte survival and suppressive macrophage differentiation in a tumor context. Inhibition of monocyte survival by sCSF-$1R_{M149K}$-Fc was more effective than sCSF-$1R_{WT}$-Fc when using both recombinant IL-34 or CSF-1 but importantly, also when using pleural effusion from patients containing IL-34 and/or CSF-1. Additionally, the decrease of monocyte survival by sCSF-$1R_{M149K}$-Fc using pleural effusion was more efficient than neutralizing anti-CSF-1 and anti-IL-34 antibodies alone or combined. Inhibition of monocyte survival and suppressive differentiation by sCSF-$1R_{M149K}$-Fc was also observed in vitro in a 3D model of pleural mesothelioma MCTSs and in vivo in a xenograft model of pleural mesothelioma in immunodeficient mice.

CSF-1R is expressed by all cells of the monocyte/macrophage lineage, from early bone marrow precursors to all tissue macrophages.[4] Dendritic cells and trophoblast cells also express CSF-1R, expression by other cell types is debated.[4] The unrelated cytokines IL-34 and CSF-1 are the only CSF-1R ligands known.[4] CSF-1R ligation by CSF-1 or IL-34 activates the classical MAPK and PI-3-kinase pathways and the final outcome on monocytes is their survival and differentiation in suppressive macrophages that have anti-inflammatory and pro-angiogenic activities.[1 2]

CSF-1R, IL-34 and CSF-1 play important roles in cancer, mostly favoring cancer development, through different autocrine and paracrine effects, and mostly but not only, through recruitment, survival and differentiation of monocytes into suppressive TAMs.[5–7 9 21] Furthermore, CSF-1 and CSF-1R fusion genes have been identified in certain leukemias[13] and TGCT[14] and are implicated in their tumorigenesis. IL-34 and CSF-1 are overexpressed in many cancers, produced at least in part by tumor cells and associated with overall reduced survival of patients.[48 49] Thus, blockade of the CSF-1R axis has emerged as a promising therapeutic alternative in cancer notably to reduce TAMs formation and consequently

immunosuppression.[19] Blocking antibodies, directed against CSF-1 or CSF-1R, or small molecules to inhibit tyrosine kinase activity of CSF-1R were developed and are currently evaluated in the clinic following interesting antitumor activity observed in preclinical models of cancer.[19] However, the results obtained are for the moment disappointing in solid tumors. Nevertheless, the effects of CSF-1R vary with the tumor and even with the subtype of tumor, as exemplified by glioblastoma in which certain forms were responsive to CSF-1R blockade with tyrosine kinases whereas others showed increased tumorigenesis and others were neutral.[19] Different cancer cells can also express some of the other IL-34 receptors such as PTPzeta and Syndecan-1.[3 50] Thus, the redundancy of IL-34 and CSF-1 for CSF-1R activation, their co-expression is some cancers and the existence of several receptors for IL-34 suggest that inhibition of both cytokines would be more efficient than individual blocking. In contrast to other strategies of blocking CSF-1R, sCSF-1R$_{M149K}$-Fc would also block these other receptors. Neutralizing antibodies against IL-34 and CSF-1 could be an alternative to the use of an sCSF-1R-Fc strategy, but this approach would need to use two antibodies and penetration of antibodies in solid cancers is limited.[29] Moreover, we showed using mesothelioma pleural effusions that sCSF-1R$_{M149K}$-Fc was better than anti-CSF-1 and anti-IL-34 antibodies alone or combined to reduce monocyte viability supporting the interest of our strategy. Inhibition of CSF-1R has also been addressed using small tyrosine kinase inhibitors and they have been approved for the treatment of TGCT.[19 26–28] Nevertheless, tyrosine kinase inhibitors have shown side effects, probably due to inhibition of tyrosine kinase receptors other than the CSF-1R, such as FLT3.[27 28] Furthermore, tyrosine kinase receptors would not inhibit the other receptors of IL-34 (PTPzeta, Syndecan-1 and TREM2) expressed by tumor cells or in its microenvironment and thus this type of inhibitor would have a limited effect. Here, with sCSF-1R$_{M149K}$-Fc, we specifically inhibit IL-34 and CSF-1 binding to all of their receptors, limiting off-target effects as observed with CSF-1R inhibitors and potentially side effects in vivo.

Immunotherapy, particularly the anti-programmed cell death protein-1 (PD-1)/anti-cytotoxic T-lymphocyte associated protein 4 antibodies combination, has profoundly modified the therapeutic care of patients with pleural mesothelioma in recent years and contributed to improving their overall survival.[50 51] Unfortunately, only around 20% of patients really benefit from this treatment and new single or combinatorial approaches are necessary. In a previous work, we showed, using pleural mesothelioma MCTS containing macrophages, that the use of a CSF-1R inhibitor, GW2580, reduces the formation of immunosuppressive TAMs and then restores the cytotoxic activity of a CD8+ T-cell clone towards tumor cells.[7] In this work, we also obtained the same effect using sCSF-1R$_{WT}$-Fc and sCSF-1R$_{M149K}$-Fc with a complete restoration of the CD8+ T-cell clone cytotoxic activity for the latter. Recent work suggests that inhibition of the CSF-1R/CSF-1/IL-34 axis could be an interesting complement to current immunotherapy. Given their role in the formation of TAMs and the impact of TAMs on the antitumor immune response, a combined approach could be effective in overcoming resistance to treatment. In vivo, it was already demonstrated that the inhibition of macrophage formation in pleural mesothelioma improves immunotherapies such as dendritic cell vaccination, IL-2/anti-CD40 and anti-PD-1/anti-PD-L1 approaches.[52–54] Thus, the combination of immunotherapy with sCSF-1R$_{M149K}$-Fc could improve the efficacy of anticancer treatments at least in certain cancers.

Increased IL-34 levels in serum have also been described in several disease states, including cancer and autoimmune diseases (reviewed in[2]). Thus, not only in cancer but also in some autoimmune diseases, the use of sCSF-1R$_{M149K}$-Fc could improve treatments. It should also be considered that in some other autoimmune diseases, organ transplantation and acute graft-versus host disease (GVHD), us and others have shown that IL-34 is tolerogenic through the induction of suppressive macrophages and T regulatory (Treg) cells.[2] CSF-1 elevation has been detected in non-cancerous disease states, such as severe COVID-19, autoimmunity (lupus nephritis, arthritis) and pulmonary fibrosis (reviewed in[19]).

Long-term (3 or 6 weeks) blockade of CSF-1R in adult mice, results in tissue macrophage depletion in several (lung, uterus, brain), but not all tissues as well as in selective depletion of the mature non-classical Ly6C± blood monocytes in mice and increased levels of the immature classical inflammatory Ly6++ subset, resulting in no change in total monocytes.[38 55] Additionally, depletion of macrophages in tumor models did not diminish macrophage recruitment to infection sites and certain T-cell responses were increased.[38] Following depletion, macrophage replenishment in non-lymphoid tissues developed in 1–2 weeks and was mostly dependent in basal conditions on resident macrophages.[56] In inflammatory conditions, macrophages were derived from circulatory monocytes.[56] Related to systemic inhibition of CSF-1R, recent clinical data obtained with pexidartinib (PLX-3397), an inhibitor of CSF-1R kinase activity, have demonstrated a benefit in patients with symptomatic tenosynovial giant cell tumors[57] with manageable side effects, notably liver toxicity. However, pexidartinib is also an inhibitor of c-Kit that could contribute to adverse events. Our molecule is more specific and inhibits only the CSF-1/macrophage colony stimulating factor 1/IL-34 pathways; therefore, we can expect less side effects. Altogether, these results suggest that macrophage depletion for some weeks may not have major deleterious effects. Another therapeutic approach in pleural mesothelioma to avoid systemic effects of macrophage depletion could be the injection of the sCSF-1R$_{M149K}$-Fc molecule into the pleural cavity, as has recently been done using oncolytic herpes virus therapy or chimeric antigen receptor (CAR-T cells.[58 59]

Metabolization of CSF-1 is largely dependent on CSF-1R-mediated endocytosis by tissue macrophages.[38 60] CSF-1R

endocytosis and CSF-1 clearance do not require CSF-1R kinase activity.[61] Thus, CSF-1 levels in serum increased on blocking of CSF-1R in mice and rats with deficiencies in CSF-1R, but not when inhibitors of CSF-1R signaling are used.[62] sCSF-1R-Fc by trapping CSF-1 should not result in increased circulating CSF-1. Our results confirm that cultured monocytes spontaneously produced CSF-1, but not IL-34, and show sCSF-1R$_{M149K}$-Fc inhibited monocyte survival in the absence of added cytokines, suggesting blockade of this endogenously produced CSF-1.

In conclusion, we have developed sCSF-1R$_{M149K}$-Fc, a soluble dimeric and mutated blocker of CSF-1 and IL-34. We have demonstrated its utility in inhibiting the differentiation of immunosuppressive macrophages in 3D mesothelioma spheroids and, in addition, in promoting CD8+ T-cell activity. This molecule has therapeutic potential in at least some cancers, alone or in combination with current immunotherapies, and potentially also in other diseases, such as autoimmune, inflammatory and neurodegenerative diseases, in which CSF-1 and/or IL-34 are implicated and other inhibitors of CSF-1R are used.[19]

Author affiliations
[1]INSERM, Center for Research in Transplantation and Translational Immunology, UMR 1064, Nantes Universite, Nantes, France
[2]LabEx IGO, Nantes Université, Nantes, France
[3]INSERM, UMR 1307, CNRS UMR 6075, Université d'Angers, CRCI2NA, University of Nantes, Nantes, France
[4]INSERM, CNRS, SFR Bonamy, UMS BioCore, Imp@ct Platform, Nantes Université, Centre Hospitalo-Universitaire (CHU) Nantes, Nantes, France

Acknowledgements We thank Fabienne Haspot and Véronique Daguin from the humanized rodent platform from the Labex IGO and the staff of the animal facility of Nantes University for animal husbandry and care. We acknowledge the IBISA MicroPICell facility member of the national infrastructure France-Bioimaging supported by the French national research agency (ANR-10-INBS-04), "CytoCell" flow cytometry and "Imp@ct" molecular interaction facilities (SFR Bonamy and Biogenouest consortium).

Contributors Conceptualization and supervision of the project: IA, CG, EM, CB, NJ. In silico modelling: AQ and EM. Experiments: NJ, AQ, SD, RH, MM, HL, JF, AS, SR. Writing of the original draft of the manuscript: IA and NJ. Review and editing of the manuscript: EM, AQ, CB and CG. Funding acquisition: IA, EM, CB, CG, NJ. IA and CG act as guarantors for this study.

Funding This work was supported by the LabEx IGO program (n° ANR-11-LABX-0016) funded by the «Investment into the Future» French Government program, managed by the National Research Agency (ANR) and by a grant from the « Region Pays de Loire». This work was also supported by the "Ligue contre le Cancer". This work has been supported by the Fondation ARC pour la recherche sur le cancer (ARCPJA2023070006731).

Competing interests NJ, AQ, EM, CG and IA have a patent pending on the sCSF-1RM149K-Fc molecule.

Patient consent for publication Not applicable.

Ethics approval All the collected samples and the associated clinical information were registered in a database (DC-2017-2987) validated by the French ministry of research. This study was approved by an independent local ethical committee (CPP Ouest-IV-Nantes).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. All data are available in the manuscript and materials are available upon reasonable request.

ORCID iDs
Erwan Mortier http://orcid.org/0000-0002-6321-6488
Ignacio Anegon http://orcid.org/0000-0001-8700-5645

REFERENCES

1 Stanley ER, Chitu V. CSF-1 receptor signaling in myeloid cells. *Cold Spring Harb Perspect Biol* 2014;6:a021857.
2 Freuchet A, Salama A, Remy S, *et al*. IL-34 and CSF-1, deciphering similarities and differences at steady state and in diseases. *J Leukoc Biol* 2021;110:771–96.
3 Shang J, Xu Y, Pu S, *et al*. Role of IL-34 and its receptors in inflammatory diseases. *Cytokine* 2023;171.
4 Sehgal A, Irvine KM, Hume DA. Functions of macrophage colony-stimulating factor (CSF1) in development, homeostasis, and tissue repair. *Semin Immunol* 2021;54:101509.
5 Monteleone G, Franzè E, Maresca C, *et al*. Targeted Therapy of Interleukin-34 as a Promising Approach to Overcome Cancer Therapy Resistance. *Cancers (Basel)* 2023;15:971.
6 Baghdadi M, Endo H, Takano A, *et al*. High co-expression of IL-34 and M-CSF correlates with tumor progression and poor survival in lung cancers. *Sci Rep* 2018;8:418.
7 Blondy T, d'Almeida SM, Briolay T, *et al*. Involvement of the M-CSF/IL-34/CSF-1R pathway in malignant pleural mesothelioma. *J Immunother Cancer* 2020;8:e000182.
8 Laoui D, Van Overmeire E, De Baetselier P, *et al*. Functional Relationship between Tumor-Associated Macrophages and Macrophage Colony-Stimulating Factor as Contributors to Cancer Progression. *Front Immunol* 2014;5:489.
9 Zhou S-L, Hu Z-Q, Zhou Z-J, *et al*. miR-28-5p-IL-34-macrophage feedback loop modulates hepatocellular carcinoma metastasis. *Hepatology* 2016;63:1560–75.
10 Franzè E, Dinallo V, Rizzo A, *et al*. Interleukin-34 sustains pro-tumorigenic signals in colon cancer tissue. *Oncotarget* 2018;9:3432–45.
11 Baghdadi M, Wada H, Nakanishi S, *et al*. Chemotherapy-Induced IL34 Enhances Immunosuppression by Tumor-Associated Macrophages and Mediates Survival of Chemoresistant Lung Cancer Cells. *Cancer Res* 2016;76:6030–42.
12 Ségaliny AI, Mohamadi A, Dizier B, *et al*. Interleukin-34 promotes tumor progression and metastatic process in osteosarcoma through induction of angiogenesis and macrophage recruitment. *Int J Cancer* 2015;137:73–85.
13 Schwab C, Roberts K, Boer JM, *et al*. SSBP2-CSF1R is a recurrent fusion in B-lineage acute lymphoblastic leukemia with diverse genetic presentation and variable outcome. *Blood* 2021;137:1835–8.
14 Mejbel H, Siegal GP, Wei S. Intramuscular Tenosynovial Giant Cell Tumor Harboring a Novel *CSF1-CD96* Fusion Transcript. *Int J Surg Pathol* 2022;30:335–8.
15 Neubert NJ, Schmittnaegel M, Bordry N, *et al*. T cell-induced CSF1 promotes melanoma resistance to PD1 blockade: Science Translational Medicine. *Sci Transl Med* 2018;10.
16 Buechler MB, Fu W, Turley SJ. Fibroblast-macrophage reciprocal interactions in health, fibrosis, and cancer. *Immunity* 2021;54:903–15.
17 DeNardo DG, Brennan DJ, Rexhepaj E, *et al*. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discov* 2011;1:54–67.
18 Chéné A-L, d'Almeida S, Blondy T, *et al*. Pleural Effusions from Patients with Mesothelioma Induce Recruitment of Monocytes and Their Differentiation into M2 Macrophages. *J Thorac Oncol* 2016;11:1765–73.
19 Wen J, Wang S, Guo R, *et al*. CSF1R inhibitors are emerging immunotherapeutic drugs for cancer treatment. *Eur J Med Chem* 2023;245:114884.

20 Kuemmel S, Campone M, Loirat D, *et al*. A Randomized Phase II Study of Anti-CSF1 Monoclonal Antibody Lacnotuzumab (MCS110) Combined with Gemcitabine and Carboplatin in Advanced Triple-Negative Breast Cancer. *Clin Cancer Res* 2022;28:106–15.
21 Hama N, Kobayashi T, Han N, *et al*. Interleukin-34 Limits the Therapeutic Effects of Immune Checkpoint Blockade. *iScience* 2020;23:101584.
22 Han N, Jang HY, Hama N, *et al*. An optimized protocol for patient-derived xenograft in humanized mice to evaluate the role of IL-34 in immunotherapeutic resistance. *STAR Protoc* 2021;2:100460.
23 Han N, Wada H, Kobayashi T, *et al*. A mechanism of IL-34-induced resistance against cytotoxic anti-cancer therapies such as radiation by X-ray and chemotherapy by Oxaliplatin. *Oncoimmunology* 2023;12:2238499.
24 Nakajima S, Mimura K, Kaneta A, *et al*. Radiation-Induced Remodeling of the Tumor Microenvironment Through Tumor Cell-Intrinsic Expression of cGAS-STING in Esophageal Squamous Cell Carcinoma. *Int J Radiat Oncol Biol Phys* 2023;115:957–71.
25 Takam Kamga P, Mayenga M, Sebane L, *et al*. Colony stimulating factor-1 (CSF-1) signalling is predictive of response to immune checkpoint inhibitors in advanced non-small cell lung cancer. *Lung Cancer (Auckl)* 2024;188:107447.
26 Strachan DC, Ruffell B, Oei Y, *et al*. CSF1R inhibition delays cervical and mammary tumor growth in murine models by attenuating the turnover of tumor-associated macrophages and enhancing infiltration by $CD8^+$ T cells. *Oncoimmunology* 2013;2:e26968.
27 Tap WD, Wainberg ZA, Anthony SP, *et al*. Structure-Guided Blockade of CSF1R Kinase in Tenosynovial Giant-Cell Tumor. *N Engl J Med* 2015;373:428–37.
28 Lee J-H, Chen TW-W, Hsu C-H, *et al*. A phase I study of pexidartinib, a colony-stimulating factor 1 receptor inhibitor, in Asian patients with advanced solid tumors. *Invest New Drugs* 2020;38:99–110.
29 Cruz E, Kayser V. Monoclonal antibody therapy of solid tumors: clinical limitations and novel strategies to enhance treatment efficacy
30 Dowlati A, Harvey RD, Carvajal RD, *et al*. LY3022855, an anti-colony stimulating factor-1 receptor (CSF-1R) monoclonal antibody, in patients with advanced solid tumors refractory to standard therapy: phase 1 dose-escalation trial. *Invest New Drugs* 2021;39:1057–71.
31 Olson OC, Kim H, Quail DF, *et al*. Tumor-Associated Macrophages Suppress the Cytotoxic Activity of Antimitotic Agents. *Cell Rep* 2017;19:101–13.
32 Akkari L, Bowman RL, Tessier J, *et al*. Dynamic changes in glioma macrophage populations after radiotherapy reveal CSF-1R inhibition as a strategy to overcome resistance. *Sci Transl Med* 2020;12:eaaw7843.
33 Wesolowski R, Sharma N, Reebel L, *et al*. Phase Ib study of the combination of pexidartinib (PLX3397), a CSF-1R inhibitor, and paclitaxel in patients with advanced solid tumors. *Ther Adv Med Oncol* 2019;11.
34 Shi G, Yang Q, Zhang Y, *et al*. Modulating the Tumor Microenvironment via Oncolytic Viruses and CSF-1R Inhibition Synergistically Enhances Anti-PD-1 Immunotherapy. *Mol Ther* 2019;27:244–60.
35 Zhu Y, Knolhoff BL, Meyer MA, *et al*. CSF1/CSF1R blockade reprograms tumor-infiltrating macrophages and improves response to T-cell checkpoint immunotherapy in pancreatic cancer models. *Cancer Res* 2014;74:5057–69.
36 Chen T-W, Hung W-Z, Chiang S-F, *et al*. Dual inhibition of TGFβ signaling and CSF1/CSF1R reprograms tumor-infiltrating macrophages and improves response to chemotherapy via suppressing PD-L1. *Cancer Lett* 2022;543:215795.
37 Voissière A, Gomez-Roca C, Chabaud S, *et al*. The CSF-1R inhibitor pexidartinib affects FLT3-dependent DC differentiation and may antagonize durvalumab effect in patients with advanced cancers. *Sci Transl Med* 2024;16:eadd1834.
38 MacDonald KPA, Palmer JS, Cronau S, *et al*. An antibody against the colony-stimulating factor 1 receptor depletes the resident subset of monocytes and tissue- and tumor-associated macrophages but does not inhibit inflammation. *Blood* 2010;116:3955–63.
39 Spassov VZ, Yan L. pH-selective mutagenesis of protein-protein interfaces: in silico design of therapeutic antibodies with prolonged half-life. *Proteins* 2013;81:704–14.
40 Wines BD, Powell MS, Parren PW, *et al*. The IgG Fc contains distinct Fc receptor (FcR) binding sites: the leukocyte receptors Fc gamma RI and Fc gamma RIIa bind to a region in the Fc distinct from that recognized by neonatal FcR and protein A. *J Immunol* 2000;164:5313–8.
41 Vafa O, Gilliland GL, Brezski RJ, *et al*. An engineered Fc variant of an IgG eliminates all immune effector functions via structural perturbations. *Methods* 2014;65:114–26.
42 Gueugnon F, Leclercq S, Blanquart C, *et al*. Identification of novel markers for the diagnosis of malignant pleural mesothelioma. *Am J Pathol* 2011;178:1033–42.
43 Roulois D, Blanquart C, Panterne C, *et al*. Downregulation of MUC1 expression and its recognition by CD8+ T cells on the surface of malignant pleural mesothelioma cells treated with HDACi. *Eur J Immunol* 2012;42:783–9.
44 Charpentier E, Cornec M, Dumont S, *et al*. 3' rna sequencing for robust and low-cost gene expression profiling. *Protocol Exchange* [Preprint] 2021.
45 Blanquart C, Jaurand M-C, Jean D. The Biology of Malignant Mesothelioma and the Relevance of Preclinical Models. *Front Oncol* 2020;10:388.
46 Mangiante L, Alcala N, Sexton-Oates A, *et al*. Multiomic analysis of malignant pleural mesothelioma identifies molecular axes and specialized tumor profiles driving intertumor heterogeneity. *Nat Genet* 2023;55:607–18.
47 Giotti B, Dolasia K, Zhao W, *et al*. Single-Cell View of Tumor Microenvironment Gradients in Pleural Mesothelioma. *Cancer Discov* 2024;14:2262–78.
48 Otsuka R, Wada H, Seino K. IL-34, the rationale for its expression in physiological and pathological conditions. *Semin Immunol* 2021;54:101517.
49 Yi L, Gai Y, Chen Z, *et al*. Macrophage colony-stimulating factor and its role in the tumor microenvironment: novel therapeutic avenues and mechanistic insights. *Front Oncol* 2024;14:1358750.
50 Scherpereel A, Antonia S, Bautista Y, *et al*. First-line nivolumab plus ipilimumab versus chemotherapy for the treatment of unresectable malignant pleural mesothelioma: patient-reported outcomes in CheckMate 743. *Lung Cancer (Auckl)* 2022;167:8–16.
51 Peters S, Scherpereel A, Cornelissen R, *et al*. First-line nivolumab plus ipilimumab versus chemotherapy in patients with unresectable malignant pleural mesothelioma: 3-year outcomes from CheckMate 743. *Ann Oncol* 2022;33:488–99.
52 Magkouta SF, Vaitsi PC, Pappas AG, *et al*. CSF1/CSF1R Axis Blockade Limits Mesothelioma and Enhances Efficiency of Anti-PDL1 Immunotherapy. *Cancers (Basel)* 2021;13:2546.
53 Duong L, Radley-Crabb HG, Gardner JK, *et al*. Macrophage Depletion in Elderly Mice Improves Response to Tumor Immunotherapy, Increases Anti-tumor T Cell Activity and Reduces Treatment-Induced Cachexia. *Front Genet* 2018;9:526.
54 Dammeijer F, Lievense LA, Kaijen-Lambers ME, *et al*. Depletion of Tumor-Associated Macrophages with a CSF-1R Kinase Inhibitor Enhances Antitumor Immunity and Survival Induced by DC Immunotherapy. *Cancer Immunol Res* 2017;5:535–46.
55 Sauter KA, Pridans C, Sehgal A, *et al*. Pleiotropic effects of extended blockade of CSF1R signaling in adult mice. *J Leukoc Biol* 2014;96:265–74.
56 Hashimoto D, Chow A, Noizat C, *et al*. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity* 2013;38:792–804.
57 Gelderblom H, de Sande M van. Pexidartinib: first approved systemic therapy for patients with tenosynovial giant cell tumor. *Future Oncol* 2020;16:2345–56.
58 Danson SJ, Conner J, Edwards JG, *et al*. Oncolytic herpesvirus therapy for mesothelioma - A phase I/IIa trial of intrapleural administration of HSV1716. *Lung Cancer (Auckl)* 2020;150:145–51.
59 Ghosn M, Cheema W, Zhu A, *et al*. Image-guided interventional radiological delivery of chimeric antigen receptor (CAR) T cells for pleural malignancies in a phase I/II clinical trial. *Lung Cancer (Auckl)* 2022;165:1–9.
60 Bartocci A, Mastrogiannis DS, Migliorati G, *et al*. Macrophages specifically regulate the concentration of their own growth factor in the circulation. *Proc Natl Acad Sci U S A* 1987;84:6179–83.
61 Irvine KM, Burns CJ, Wilks AF, *et al*. A CSF-1 receptor kinase inhibitor targets effector functions and inhibits pro-inflammatory cytokine production from murine macrophage populations. *FASEB J* 2006;20:1921–3.
62 Bissinger S, Hage C, Wagner V, *et al*. Macrophage depletion induces edema through release of matrix-degrading proteases and proteoglycan deposition. *Sci Transl Med* 2021;13:eabd4550.