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Original Research Article

A blocking antibody against canine CSF-1R maturated by limited CDR mutagenesis

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Abstract

CSF-1R is a receptor mostly associated with the mononuclear phagocytic system. However, its expression within tumors has been linked with poor prognosis in both humans and dogs. Accordingly, several reports have demonstrated the beneficial effects of blocking CSF-1R in model systems of cancer. In this study, we generated a monoclonal antibody that could block CSF-1R in dogs as the first step to develop an anticancer drug for this species. Initially, an antibody was raised by the hybridoma methodology against the fragment responsible for receptor dimerization. mAb3.1, one of the resulting hybridoma clones, was able to bind macrophages in fixed tissues and was shown to inhibit cells of the mononuclear phagocytic line. Nevertheless, mAb 3.1 could not bind to some glycoforms of the receptor in its native form, while also demonstrating cross-reactivity with other proteins. To enhance binding properties of the mAb, five amino acids of the complementarity-determining region 2 of the variable heavy chain of mAb3.1 were mutated by PCR, and the variant scFv clones were screened by phage display. The selected scFv clones demonstrated improved binding to the native receptor as well as increased anti-macrophage activity. The resulting scFv antibody fragment presented here has the potential for use in cancer patients and in inflammatory diseases. Furthermore, this work provides insights into the use of such restricted mutations in antibody engineering.

Statement of Significance:

- **A mAb was created against canine CSF-1R by hybridoma; however, it could not bind to some glycoforms of CSF-1R;**
- **The Vh CDR 2 of the mAb was mutated, and the variant scFv clones were screened by phage display. The restricted CDR 2-specific mutations increased antibody specificity.**

KEYWORDS: CDR 2; directed evolution; mAb; CSF-1R; dog

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INTRODUCTION

CSF-1R expression and tumor-associated macrophages (TAMs) are connected with poor prognosis in several cancer types [1, 2]. The presence of TAMs is correlated with increased proteinase expression, leading to tissue invasion [3]. TAMs also assist cancer cells in penetrating blood vessels [4]; they are linked to tumoral immunosuppression and are associated with reduced responses to chemotherapy [5]. Cancer cells recruit TAMs and establish a paracrine loop of stimulation. In this interaction, the tumor secretes CSF-1, which stimulates the macrophages, and in response TAMs produce EGF, which induces cancer cell growth [6]. Because of its significance in the pathogenic progression of cancer, CSF-1R has become an attractive target for drug development.

Several antibodies have been raised against human and murine CSF-1R [7–9] or against the CSF-1 [3]. Clinical trials for breast and prostatic human cancer have been conducted with an inhibitory anti-CSF-1R (https://www.clinicaltrials.gov/ct2/show/study/NCT02265536), and several studies have been published analyzing the effects of anti-CSF-1R mAbs in cancer models [10–13]. However, none of the previously described antibodies targets the canine receptor. Conversely, antibodies that were designed to identify canine macrophages through other receptors have been raised, although none has been assessed for their ability to block macrophage function or survival [14, 15].

As a clinical tool, antibodies that target the CSF-1R are more suitable than antibodies that target its ligands, since these have short circulation half-lives. Also, CSF-1R has two ligands, CSF-1 and IL-34, which are structurally unrelated, therefore requiring different antibodies to block both [16, 17]. Although both IL-34 and CSF-1 share a binding site on the surface of CSF-1R, there are differences in the receptor contact points for binding both ligands, which overlap, but are not identical [16, 18]. Additionally, both of these cytokines have dissimilar spatiotemporal expression patterns, making the targeting of both difficult [19].

Two major strategies exist for blocking CSF-1R using a mAb approach. The antibody can either inhibit ligand binding to its receptor or it can inhibit receptor dimerization. These effects can be performed by direct binding of the antibody to the receptor regions responsible for ligand recognition/dimerization or through stabilization of a folded conformation of the receptor [20]. Stable dimerization is essential for CSF-1R signaling [21]. Blocking dimerization impedes signaling through both IL-34 and CSF-1 and should fully stop CSF-1R signaling [16, 18].

The aim of this study was to identify an antibody that could block the function of canine CSF-1R with the intent of ultimately being able to use it to treat canine cancer patients. For this, mAb 3.1 was generated against the dimerization region of the receptor. Although it showed promising staining patterns in fixed tissue and demonstrated some inhibitory activity against macrophages, it could only bind to restricted glycoforms of the native CSF-1R. A small library of semi-synthetic antibodies based on mAb 3.1 was created by restricted mutations in the complementarity-determining region 2 (CDR2) from the antibody variable heavy chain. A scFv with novel binding properties in relation to mAb 3.1 was raised and characterized. The capacity of such restricted mutations in altering antibody functionality is discussed.

MATERIALS AND METHODS

Antigen production and hybridoma technique

The canine CSF-1R dimerization region (from the start codon, nucleotides 1028 to 1306, accession XM_546306, National Center for Biotechnology Information) was cloned in pTriex1.1 (Millipore) and was expressed in BL21 (DE3) *Escherichia coli*. The dimerization domain was cleared from bacteria by sonication under denaturing conditions (8 M urea). Purification was performed by affinity chromatography with Ni-NTA resin (Qiagen). The eluates were dialyzed against PBS after purification with a stepwise reduction of urea.

Mice were immunized with the dimerization region of CSF-1R every 28 days (at 50 μg/dose) using Freund's adjuvant in a total of four doses. The best clones were selected by dot blots and ELISA. Immunizations and hybridoma production were performed by the Regional Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Brno, Czech Republic. Antibody isotype was determined using a rapid lateral flow kit (IsoQuick, Sigma).

Immunofluorescence

For histological stains, tissue samples were fixed in PBS + 4% paraformaldehyde. The samples were then immersed in 20% and then 40% sucrose. Sections of 6 μm were used. Sections were blocked in 10% goat serum and were permeabilized by the addition of PBS + 0.2% Triton X-100. Sections were incubated overnight at 4°C with hybridoma cell culture supernatants. Mouse anti-human CD163 AM3K (TransGenic Inc.) and mouse anti-canine CD11b (AbD Serotec) were used for comparison where stated. Isotype control was mouse IgG_1 (Abcam). Antibodies were diluted in blocking buffer. Goat anti-mouse Alexa 488 fluorescent secondary antibody (Invitrogen) was used. Slides were mounted with VECTASHIELD Mounting Media with 4´,6-Diamidine-2´-phenylindole dihydrochloride (Vector Labs).

Real-time cell proliferation assay

To assess the functionality of the antibodies in blocking macrophage survival in culture, real-time cell proliferation assays were performed. 3×10^4 canine bone marrow macrophages were used. Cells were harvested as described elsewhere [22]. Macrophages were grown in the presence of rhCSF-1 (20 ng/ml, Invitrogen). Cells were placed on an xCELLigence Real-Time Cell Analyzer (Roche) 96-well plate in 100 μl of Rosewell Park Memorial Institute culture medium. The plate was scanned to measure the initial numbers of cells in each well, and then purified mAb 3.1 treatment was added. The small-molecule inhibitor of CSF-1R, GW2580, was used as a control. Negative controls received equivalent concentrations of murine serum IgG (Sigma) or DMSO (for comparison to GW2580). rhCSF-1

and antibodies were replaced during the experiment. Cells were then maintained at 37°C and 5% atmospheric CO_2 for the duration of the experiment. Cell proliferation was assessed every 15 min.

Inhibition of feline osteoclasts

To further analyze the inhibitory properties of mAb 3.1, feline osteoclasts were targeted with the antibody. Feline bone marrow cells were differentiated for 10 days in rhRANKL (30 ng/ml, R&D Systems) and rhCSF-1 (10 ng/ml, R&D Systems) in low adherence tissue culture plates (Corning) (kindly provided by Seungmee Lee (Roslin Institute)). Anti-CSF-1R mAb 3.1 hybridoma supernatant or an irrelevant anti-CCR2 hybridoma supernatant was added to the cells at 1/6 of the total culture volume. One control well received no CSF-1 after the initial differentiation of the cells. The culture medium and supplements were renewed at 48 h. Cells were lysed on day 4 using 1 ml/well of lysis buffer 15 (R&D Systems, Apoptosis Array Kit) for 30 min at 4°C. The protein mixture was assessed by western blotting. Membranes were probed with anti-pAkt (Ser 473) and total Akt (both Cell Signaling). Antibodies were used sequentially on the same membrane, after stripping the nitrocellulose membrane using Restore PLUS Western Blot Stripping Buffer (Thermo Scientific). Secondary antibody was swine anti-rabbit HRP-conjugated.

Flow cytometry

Canine blood was collected from animals from the Edinburgh Dog and Cat Home, euthanized for humane reasons. Leucocytes were isolated from whole blood using Ficoll-Paque, 1.077, (Sigma). Peripheral blood mononuclear cells (PBMCs) were stained with purified mAb or hybridoma supernatant (from 30 min to overnight, at 4°C or room temperature) or with scFv (40 min at 4°C). An anti-swine CSF-1R mAb was used as control (a kind gift from Lindsey Waddell, Roslin Institute). The anti-swine mAb had no inhibitory capacity against the receptor. For mAb, a goat anti-mouse secondary antibody was used, Alexa 488-conjugated (Invitrogen). For scFv, an anti-His secondary (Invitrogen) and a goat anti-mouse tertiary, Alexa 488-conjugated (Invitrogen) were used. 7-AAD (eBioscience) or Sytox blue (Invitrogen) were added 5 min before the analysis for assessment of cell viability by flow cytometry. Cells were analyzed on a FACScalibur flow cytometer (Becton Dickinson). Data were analyzed using Summit 4.1 software (Dako).

Immunoprecipitation

mAbs (21 μg) in sodium phosphate buffer (pH 7.0) were bound to Protein G beads (GE Healthcare). Unbound mAbs were removed by centrifuging and washing the beads with sodium phosphate buffer. HEK293T cells were transiently transfected to express the extracellular region of CSF-1R [23]. One milliliter of the HEK supernatant was incubated with the protein G + mAbs. The resin was washed 5 × with sodium phosphate buffer. The resin was incubated with 30 μl PBS + reducing sample buffer. This was incubated at 95°C for 5 min and was spun to precipitate the resin. The supernatant was loaded into a 10% SDS-PAGE gel. The western blot nitrocellulose membrane was probed with mouse anti-His tag (Invitrogen) and with goat anti-mouse, HRP-conjugated (Dako). *N*-linked oligosaccharides were removed from the extracellular region of CSF-1R using PNGase F (Promega). The purified extracellular region (50 μg in 12 μl of 0.5 M sodium phosphate buffer, pH 7.5) was mixed with 1 μl SDS (5%) and 1 μl DL-Dithiothreitol (DTT) (1 M). The sample was denatured at 95°C for 5 min and was cooled at room temperature for 5 min. Sodium phosphate buffer, NP-40 (10%) and PNGase F (2 μl each) were added to the mixture. This was incubated at 37°C for 2 h. Samples were resolved in a 10% SDS-PAGE gel and were analyzed by Ponceau S staining of the proteins transferred to a nitrocellulose membrane.

Site-directed CDR mutagenesis

The cloning of the antibody variable region was conducted according to [24]. CDR mutagenesis of the sequence corresponding to five amino acids was performed by PCR using specific oligonucleotides. Mutagenesis primers were designed to bind to the regions around CDR2 of the Vh chain (Fig. 1). Within the CDRs, the mutagenesis primers contained areas of degenerate sequences that coded for all possible amino acids and one stop codon (amber codon, TAG), using the degenerate sequence 'NNK'. The primers contained restriction sites for insertion into the phagemid pSEX81 (Progen). This phagemid was ligated with the normal VL chain from mAb 3.1 and the mutated Vh chain.

Phage display—phage library biopanning

For the identification of the most suitable clones from the phage libraries, these were screened against the extracellular region of the canine CSF-1R, expressed by HEK293T cells. Methods were according to [25]. Immunotubes (Greiner) were coated overnight at 4°C with 0.5 ml of PBS containing 25 μg (rounds 1 and 2 of panning) or 12.5 μg (further rounds) of the CSF-1R protein. The tube was then blocked with 2% milk. Bound phage was eluted with trypsin (10 mg/ml, Gibco). ELISA for testing the phage is described below. Bovine serum albumin (BSA) was used as a control target protein in separate wells. Mouse anti-M13 antibody (1:1 000, GE Healthcare) was used to detect phage particles bound to the plate. Rabbit anti-mouse IgG, HRP-conjugated (1:2 000, Dako), was used as the tertiary antibody. HRP activity was detected using TMB substrate (Millipore). Negative control wells received only the secondary and tertiary antibodies.

Expressing the scFv independently from the phage

The variable regions of the monoclonal antibodies identified from the mutated library were amplified by PCR and were ligated into the pOPE101 plasmid (Progen). These clones were characterized by standard Sanger sequencing (Dundee Sequencing). For scFv production, the bacterial culture was induced with 500 μM Isopropyl β- d-1-thiogalactopyranosid (Sigma) at OD_{600nm} = 0.5,

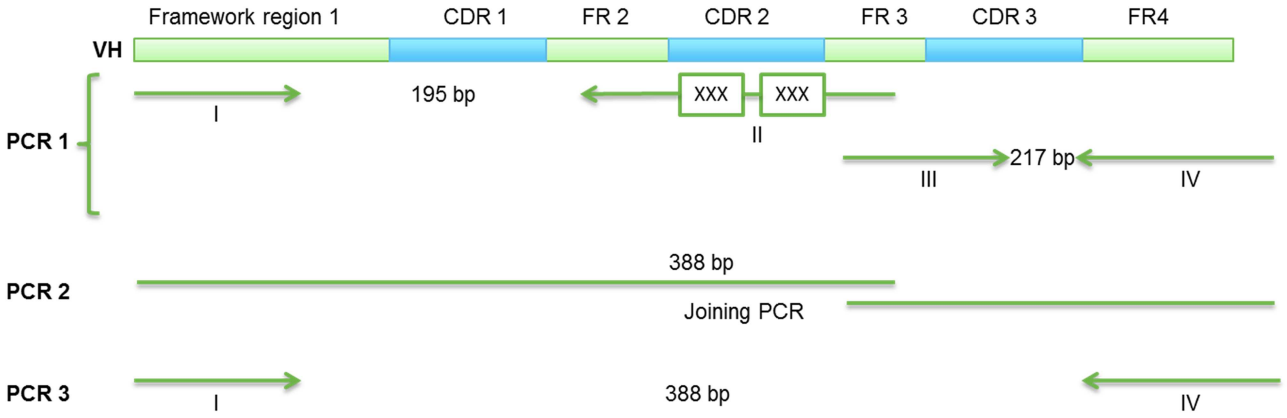

Figure 1. PCR strategy for the mutagenesis of mAb 3.1. The arrows represent primers. The PCR product sizes are indicated in base pairs (bp). The figure is not to scale. The boxes within primer II show the points where mutations were inserted in each region. Several PCR steps were required for the full assembly of each of the variable regions, shown as PCR 1, PCR 2 and PCR 3. The lines in PCR 2 indicate the products from PCR 1.

at 37°C. The soluble protein fraction was removed by bacterial lysis using a mild buffer (200 mM Tris–HCl; 20% sucrose; 1 mM EDTA, pH 8.0). The insoluble remaining pellet was then resuspended (in 10% of the initial culture volume) in a denaturing buffer (50 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA, 8 M urea, pH 8.0; 10 mM DTT was added immediately before using the buffer). The pellet was solubilized in this buffer overnight at 4°C. The scFv were then refolded by dialysis with a stepwise reduction in the concentration of urea in 1.5 l of the same buffer. After dialysis, the soluble scFv was affinity purified with a Ni-NTA resin (Qiagen). The refolded scFv was dialyzed thrice against PBS to remove imidazole. Protein concentration was measured with a NanoDrop spectrophotometer (Thermo Scientific) at 280 nm.

Endpoint proliferation assay

For the assessment of scFv inhibitory activity, an endpoint proliferation assay was used. Cells were treated with either a scFv, the parental mAb 3.1 (at the same concentration) or PBS, as the vehicle control for the scFv. 3×10^4 canine bone marrow macrophages or NIH/3T3 were plated in 100 µl of DMEM medium (Gibco) in individual wells of black opaque 96-well plates (Greiner). The purified scFv, mAb 3.1 and/or rhCSF-1 (100 ng/ml, Invitrogen) were added to the culture at the same time as the cells. After 48 h, viable cells were quantified using CellTiter-Glo, Promega.

ELISA for scFv specificity

An ELISA assay was used to compare antibody specificity before and after mutagenesis. MaxiSorp 96-well plates (Nunc) were coated with 400 ng/well of either canine recombinant CSF-1R extracellular region or BSA in 50 µl of PBS. After blocking, purified mAb 3.1 (12 or 30 µg) or scFv were added to the wells. Secondary antibody (for the mAb) was a rabbit anti-mouse HRP (1:2 000, Dako). The scFv was detected with an anti-c-Myc tag, HRP-conjugated (1:10 000, Bethyl Laboratories). Negative controls received only the secondary anti-mouse or anti-cMyc antibodies. The reaction was developed with TMB Ultra (Fisher). Absorbance was read at 450 nm (Victor3, Perkin Elmer).

Inhibition of CSF-1 binding

The ability of the mutated antibody to block CSF-1 binding was tested. A swine CSF-1-Fc fusion was used, coupled to Alexa Fluor 648 (a kind gift from Anna Raper, The Roslin Institute) [26–28]. Canine peripheral blood monocytes or bone marrow–derived macrophages (50 000 cells) were blocked with either 40 or 10 µg of scFv for 1 h at 37°C. Next, CSF-1-Fc was added to the cells at the same conditions. Finally, cells were washed with PBS and were read in a flow cytometer. Controls consisted of either cells stained with CSF-1-Fc in the presence of the scFv elution buffer or of cells stained with an anti-canine MHC class II antibody after addition of scFv (40 µg) instead of the CSF-1-Fc stain (Bio-Rad MCA1044F, FITC-coupled, 1:50). The experiment was performed three times independently.

Statistical analyses

The real-time proliferation assays were assessed using repeated-measures two-way ANOVA with either Fisher or Holm–Sidak *post hoc* tests. The proliferation assay using purified scFv was analyzed using one-way ANOVA. For all tests, $P < 0.05$. Assumption tests were performed on Minitab 16 (Minitab Inc.). Statistical tests and graphs were made on GraphPad Prism 5 (GraphPad Inc.).

RESULTS

Hybridoma clones

Several clones were obtained by hybridoma fusion and mAb 3.1 was selected, among others, by dot blot and ELISA assays (not shown) and was therefore analyzed for its binding characteristics and binding properties. mAb 3.1 was of the IgG_1 isotype.

mAb 3.1 could stain macrophages in fixed canine tissues

A range of canine tissues was stained with the hybridoma supernatant of mAb 3.1 by immunofluorescence. mAb 3.1 bound to cells in all tissues tested: duodenum, lymph node

and spleen. Commercial control antibodies against CD163 and CD11b showed similar staining patterns to mAb 3.1 (Fig. 2A, B, E). In the duodenum, mAb 3.1$^+$ cells were present around the duodenal glands and infiltrating the villi, as were CD163$^+$ cells (Fig. 2C, D). In the spleen, mAb 3.1$^+$ cells surrounded the white pulp, as did CD11b$^+$ cells (Fig. 2F). In the lymph node, mAb 3.1$^+$ cells were mostly seen in the medullary cords and the paracortical areas but also close to the subcapsular space and the trabeculae (not shown).

mAb 3.1 had an inhibitory effect on canine primary macrophage cultures

The capacity of the anti-CSF1-1R mAb 3.1 to block the proliferation of canine macrophages was analyzed with a real-time proliferation assay. The proliferation curve of mAb 3.1-treated macrophages was different from the isotype control at 1.25 µg/well (Holm–Sidak *post hoc* test) and showed a trend of significant activity at 3.75 and 7.5 µg/well (differences were only significant with a Fisher *post hoc* test). GW2580, a small-molecule inhibitor of CSF-1R, was used as an inhibition control and was able to reduce macrophage survival at 20 µM in relation to its DMSO vehicle control (Fig. 3A).

mAb 3.1 inhibited osteoclast survival

Osteoclasts have a distinct morphology, forming a tight 'tiled' structure in cell culture. Therefore, apoptosis is easy to identify in culture, as this tiled pattern is then broken [29]. The feline and the canine CSF-1R have 82% homology when only the extracellular region is considered—this is the region targeted by mAb 3.1. When mAb 3.1 was used on feline osteoclasts, it induced phenotypical alterations that indicate cellular contraction and membrane blebbing. The use of a control antibody (anti-CCR2 mAb) or the removal of CSF-1 after the cells that had differentiated had no effect on cellular morphology and, in these cases, the cells maintained a 'tiled' pattern (Fig. 3B). The CSF-1R signaling pathways were evaluated in osteoclasts by western blotting. While total Akt was stable between treatments, pAkt was reduced in mAb 3.1-treated cells (Fig. 3C).

mAb 3.1 could not recognize some glycoforms of CSF-1R

Despite the aforementioned properties of mAb 3.1, it could not stain peripheral blood mononuclear cells (PBMCs) from dogs, regardless of staining time (from 30 min to overnight) or whether purified antibodies or hybridoma supernatants were used. The positive control, an anti-swine CSF-1R, bound almost exclusively to canine monocytes, showing little background staining of lymphocytes (Fig. 4A).

To determine the reasons of such unexpected results, mAb 3.1 was used in an immunoprecipitation assay with a recombinant CSF-1R extracellular region. mAb 3.1 recovered proteins with the correct molecular weight of CSF-1R, but it is only bound to a narrow range of the glycoforms of CSF-1R (Fig. 4B).

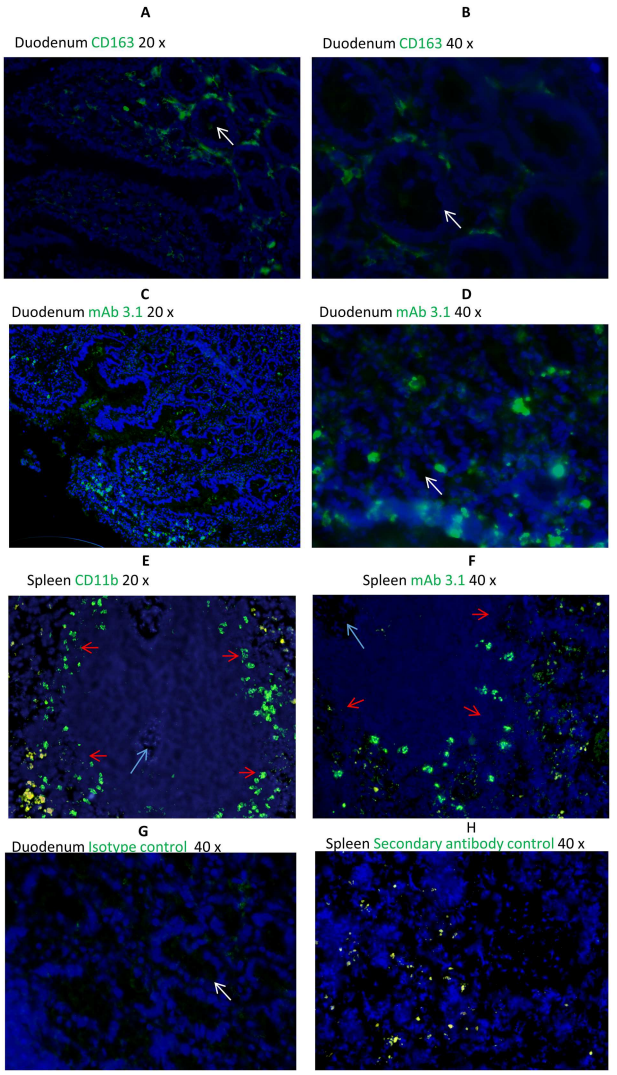

Figure 2. mAb 3.1 stained putative macrophages in several canine tissues. (**A**) Duodenal villi and (**B**) duodenal glands stained with anti-CD163 (1:75, TransGenic Inc.), used as a positive control. (**C**) Duodenum stained with hybridoma supernatant containing mAb 3.1 (60 µg/ml) showing villi in profile. (**D**) mAb 3.1 stain, showing duodenal glands. (**E**) CD11b (1:10, AbD Serotec) stained spleen, used as a positive control. (**F**) Spleen stained with hybridoma supernatant containing mAb 3.1. (**G**) Isotype control IgG1k for the duodenal stain (80 µg/ml, Abcam). (**H**) Secondary antibody control for the spleen stain (1:300 goat anti-mouse, Invitrogen). Notice the similar distribution of cells stained with the controls (**A**, **B**, **E**) and mAb 3.1 (**C**, **D**, **F**). Secondary antibody was goat anti-mouse Alexa 488 (1:300, Invitrogen). The nuclear stain is in blue (DAPI). The white arrows indicate duodenal glands (**A**–**D**); the yellow arrows indicate the central arteriole (**E**, **F**). The red arrows indicate the limits of the white pulp. '20 ×' and '40 ×' indicate the magnification used to acquire the images. The yellow puncta in the secondary control stain of the spleen are believed to be lipofuscin granules due to their fluorescence patterns [50].

Selection of CDR2 mutants yielded hydrophilic and glycine-rich scFv

The CDR2 of mAb 3.1 was mutated by PCR, and nine phage display–selected mutated scFv were sequenced and compared to the original mAb 3.1, from which the mutated semi-synthetic antibody library was originated.

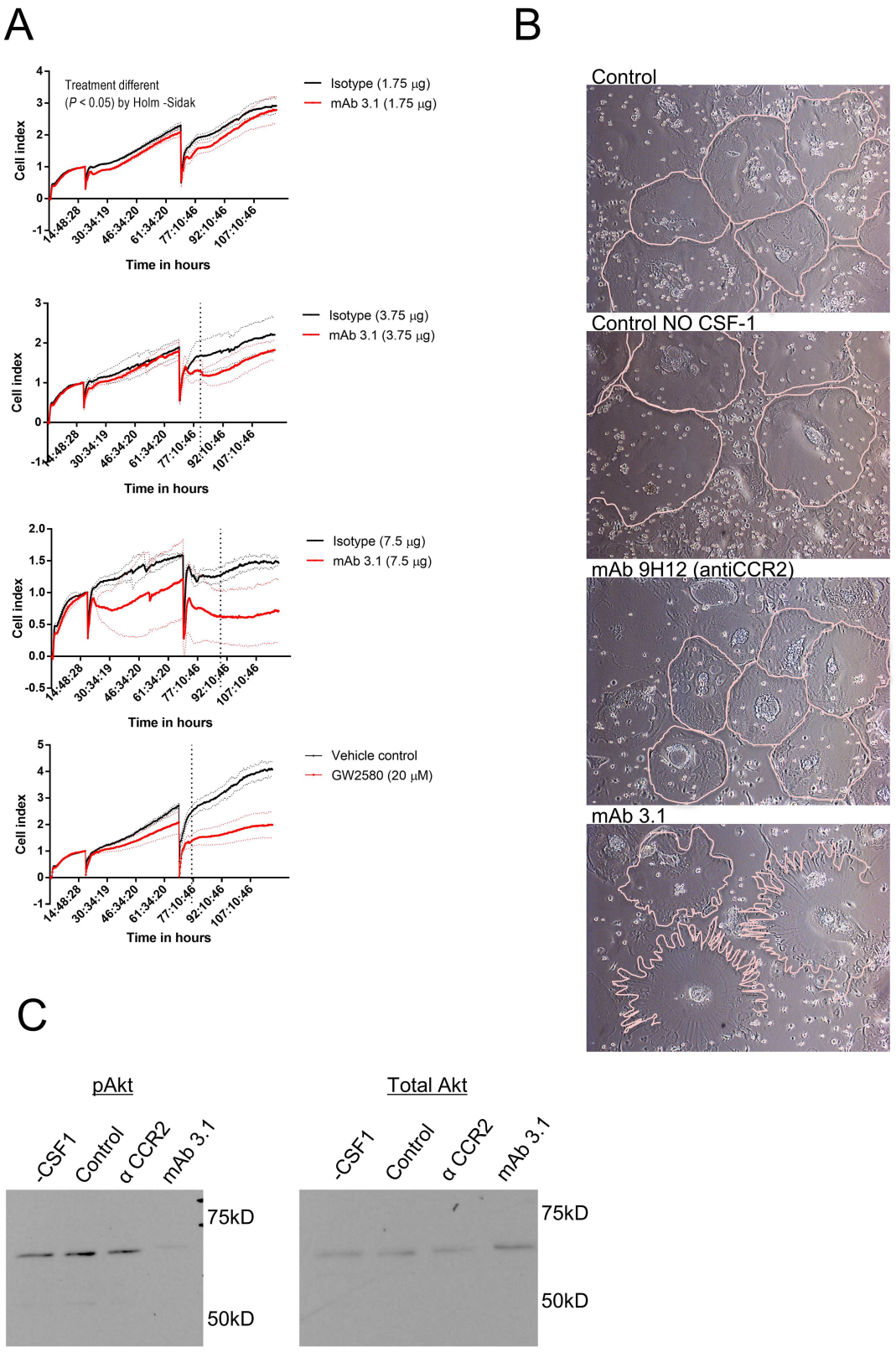

Figure 3. mAb 3.1 inhibited cellular proliferation. (**A**) Effect of mAb 3.1 in proliferation assay of canine BMDM. Cells (in 100 µl medium) were grown in rhCSF-1 (20 ng/ml, Invitrogen) and treated with the purified mAb (at 1.2, 3.75 or 7.5 µg) or GW2580 (20 µM), as a control. The negative controls received equivalent concentrations of mouse serum IgG antibody (Sigma) or DMSO (vehicle control for GW2580). Data was collected every 15 min. Treatments were added at 17 h. Data were also normalized at that point. The abrupt drops in the graphs indicate when the plate was removed from the xCelligence equipment for change of medium/treatment. Results are shown as means of four wells (two wells at 7.5 µg) ± SD for each time point. Results were analyzed by repeated measures two-way ANOVA with a Holm–Sidak or a Fisher (3.75 and 7.5 µg) post-test. The vertical dotted lines indicate when

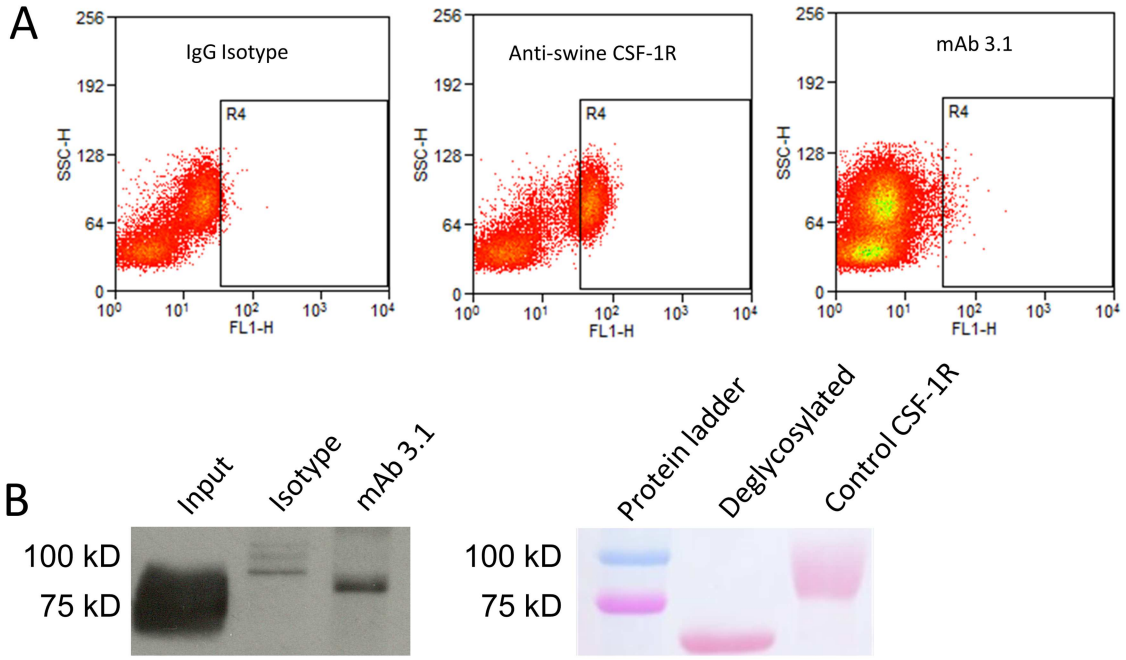

Figure 4. mAb 3.1 did not stain canine PBMCs. (**A**) Live cells stained with mAb 3.1 (10 µg/ml) or mouse anti-swine CSF-1R (25 µg/ml, Lindsey Waddell, Roslin Institute). Cell viability was assessed using Sytox blue (Invitrogen). Fluorescence intensity is shown on the *x*-axis and cell granularity on the *y*-axis. Secondary antibody was goat anti-mouse Alexa 488 (1:800, Invitrogen). (**B**) Immunoprecipitation of CSF-1R with mAb 3.1. Purified murine mAb 3.1 or isotype control (21 µg) were coupled to protein G beads (75 µl, GE Healthcare). Unbound antibodies were removed by washing. Medium from transiently transfected HEK293T cells expressing CSF-1R extracellular region was added to the beads and incubated overnight at 4°C. Unbound proteins were removed by washing. The resin was incubated with reducing sample buffer at 95°C for 5 min. The mixture was centrifuged, and the supernatant was resolved by SDS-PAGE. His-tagged proteins were identified by western blotting (anti-His at 1:500, Invitrogen). Secondary antibody was anti-mouse, HRP-conjugated (Dako, 1:1 000). (**C**) The recombinant extracellular region of CSF-1R was glycosylated, which induced a broad band pattern on the SDS-PAGE. This is evidence that mAb3.1 bound only to selected glycoforms. The recombinant protein was digested with PNGase F and the resulting difference in molecular size was evaluated by SDS-PAGE separation of the deglycosylated and control CSF-1R.

The selected mutants coincided in the selection of proline and glycine residues and increased solubility. Four of the nine sequenced scFv had increased charge (Table 1).

A mutated version of mAb 3.1—a scFv—showed novel functions

The biological activity of the mutated version of mAb 3.1, a scFv, against bone marrow-derived macrophages (BMDM) was tested in a proliferation assay. The murine fibroblast NIH/3T3 cell line was used as a control to assess if the effect of the scFv was indiscriminate or dependent on any bacterial contaminant remaining from the purification process. Whereas mAb 3.1 marginally reduced BMDM proliferation at the concentration used, the scFv reduced BMDM survival even when compared to mAb 3.1. Also, the scFv did not impair NIH/3T3 survival (Fig. 5A).

In an ELISA assay, the scFv demonstrated reduced binding to two control proteins compared to the parental mAb, BSA and soybean trypsin inhibitor (Fig. 5M).

The scFv was tested by flow cytometry for binding to canine peripheral blood mononuclear cells (PBMC). The scFv bound exclusively to live canine monocytes, but not to lymphocytes or granulocytes. Staining with the scFv was greatly reduced if the cells were previously fixed in paraformaldehyde (Fig. 5C).

scFv inhibited CSF-1 association to canine phagocytes

Stable CSF-1 interaction with its receptor depends on CSF-1R dimerization, which 'locks' the ligand within the dimer, while the complex is directed toward internalization [21]. mAb 3.1 was generated against the CSF-1R dimerization region; so, it was tested whether its mutated form, the scFv, could inhibit CSF-1 binding by blocking receptor dimerization. Cells were stained with a fluorescent CSF-1 following scFv blockade. The scFv could inhibit CSF-1 binding to canine PBMC and BMDM in a dose-dependent manner. The scFv did not affect other receptors, as it could

statistical differences appear. (**B**) mAb 3.1 induced apoptosis of feline osteoclasts. Bright field images of feline osteoclasts grown with rhCSF-1 (10 ng/ml, R&D Systems) and rhRANKL (30 ng/ml, R&D Systems) treated with hybridoma culture supernatant containing mAb 3.1 (1/6 of medium volume in osteoclast culture) for 4 days and comparisons with anti-CCR2 hybridoma culture supernatant (similar dose), untreated and no CSF-1 controls. Cellular membranes were manually outlined to highlight the differences between the groups. Pictures are representative of two wells for the negative controls and mAb 3.1 treated cells, and of a single well for the anti-CCR2 treated cells. (**C**) mAb 3.1 reduced pAkt in feline osteoclasts. Proteins from the lysed osteoclasts from (**B**) (20 µg/well) were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. The same membrane was probed with anti-pAkt and anti-total (at 1:1 000, Cell Signalling). Secondary antibody was swine anti-rabbit Ig, HRP-conjugated (1:1 700, Dako).

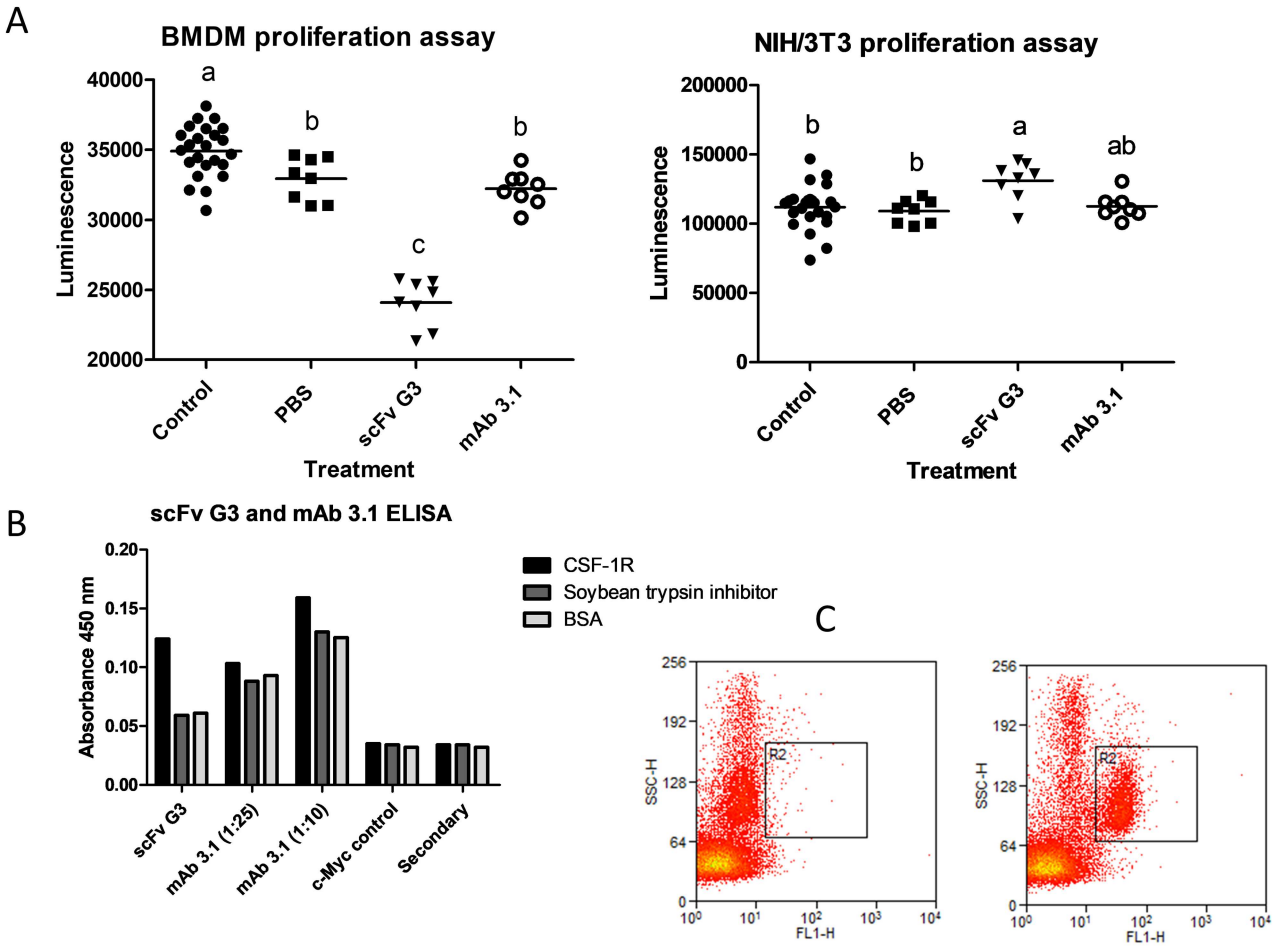

Figure 5. The scFv had different binding properties compared to the parental mAb 3.1. (**A**) Canine BMDM were grown with rhCSF-1 (20 ng/ml, Invitrogen) and treated with 10 µl of scFv or the parental mAb 3.1 (both at 0.3 mg/ml). PBS was used as vehicle control for the scFv. The murine fibroblast NIH/3T3 cell line was used as a control to assess if bacterial products remaining from the purification had toxic effects. These cells received the same treatments as the BMDM (but no CSF-1). Cells were incubated with the treatments for 60 h. The treatment had no effect on NIH cells. Proliferation results as measured by luciferase activity (CellTiter-Glo, Promega). Results are the means of at least eight wells. Data representative of three independent experiments. Data analyzed by ANOVA. Statistically, significant differences are indicated by differing letters above each group. (**B**) The refolded scFv displayed improved binding characteristics when compared to the parent mAb 3.1. The purified and refolded scFv and the murine mAb 3.1 were tested by ELISA in wells coated either with the canine extracellular region of CSF-1R, BSA or soybean trypsin inhibitor (Sigma) (all at 400 ng/well in 50 µl of PBS). scFv had reduced binding to the control proteins when compared to mAb 3.1. scFv was detected with an anti-c-Myc tag, HRP-conjugated (1:10 000, Bethyl Laboratories). mAb 3.1 was detected with an anti-mouse Ig HRP-conjugated antibody (1:2 000, Dako). Negative controls received only the secondary anti-mouse or anti-cMyc antibodies. Result representative of three independent assays. (**C**) Refolded scFv bound to canine monocytes. Fixed (1% PFA, left) or live (right) canine peripheral blood mononuclear cells were stained with 50 µl of urea-refolded scFv for 40 min on ice. After washing cells, bound scFv was detected using mouse anti-His tag (1:700, Invitrogen) and goat anti-mouse, Alexa 488-conjugated (1:800, Invitrogen). Cell viability was assessed with 7-AAD (eBioscience). In this graph, the cellular populations with an SSC-H above ~128 are granulocytes; between ~64 and 128 are monocytes; under ~64 are lymphocytes.

not interfere with the staining of cells with an anti-canine MHC class II antibody (Fig. 6).

DISCUSSION

The tumor microenvironment and the infiltrated immune cells are believed to be potential targets in cancer. The immune components of the tumor bulk are not only tolerant to the cancer cells but can also in fact promote their growth [30, 31]. This work demonstrates the characterization of a monoclonal antibody, mAb 3.1, with potential to inhibit macrophage survival and proliferation by blocking the CSF-1R. These antibodies were created with the intent of affecting the population of tumor-associated macrophages (TAMs), which majorly exhibit pro-tumoral activity [32].

mAb 3.1 could identify cells within normal fixed canine tissues that are compatible with the spatial distribution of macrophages [33]. In the spleen, there is a population of CSF-1R$^{bright+}$ cells surrounding the lymphoid follicles of the spleen [7], which could correspond to the cells stained by mAb 3.1. When the mAb was used to stain lymph nodes, the cells of the medullary cords were preferentially marked, with increased intensity compared to other areas of the organ. This subpopulation of cells is also known to

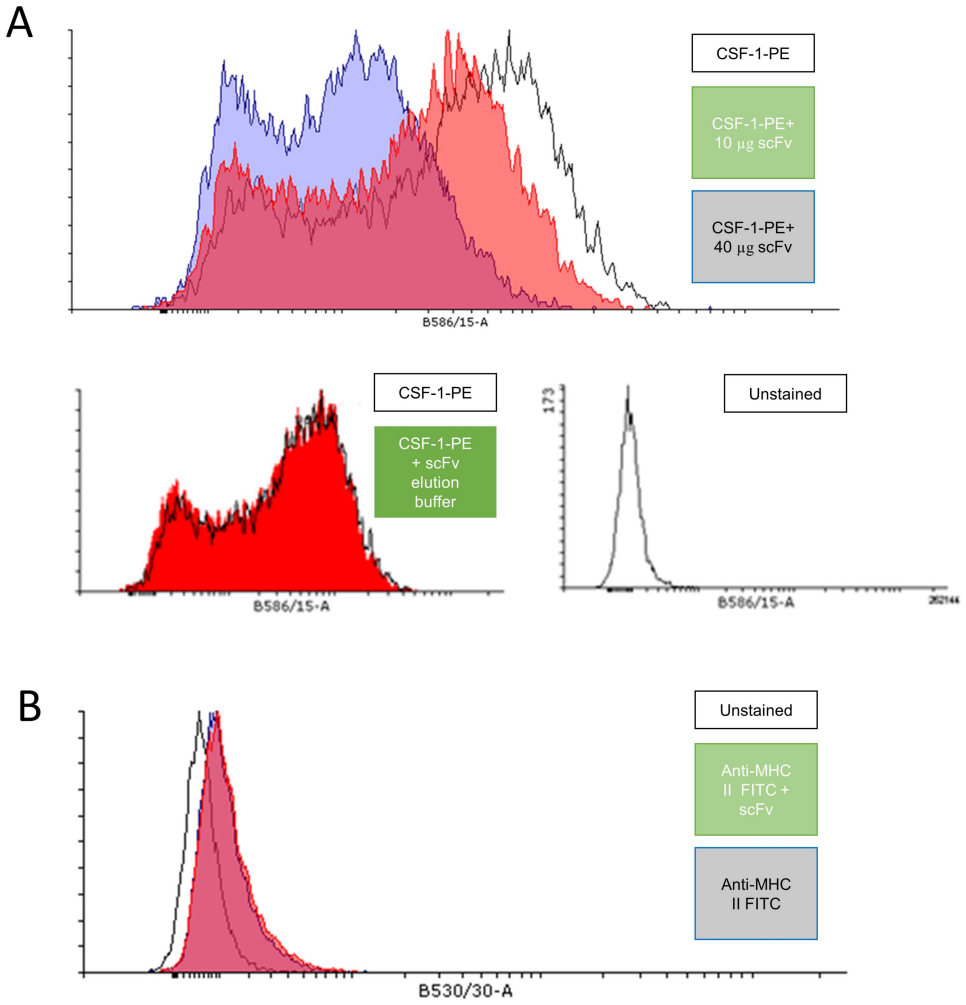

Figure 6. The scFv reduced binding of CSF-1 to canine BMDM and PBMC. (**A**) Cells were blocked with two concentrations of scFv for 1 h (10 or 40 µg), followed by staining with a fluorescent swine recombinant CSF-1-Fc for 1 h. Increasing concentrations of the antibody fragments reduced CSF-1 binding to the cells. (**B**) The scFv did not interfere with the staining of these cells with a commercial anti-MHC class II antibody. Results shown are representative of the assays conducted with BMDM.

highly express the CSF-1R [33]. mAb 3.1 stained cells in the same tissue compartments as other markers such as CD163 and CD11b, which confirms that those cells are likely macrophages [34–36].

mAb 3.1 was raised using the dimerization domain of CSF-1R as an antigen. Blocking receptor dimerization has been shown to be an effective means to impede receptor function [37]. Probably through this mechanism, mAb 3.1 could inhibit BMDM proliferation. Although mAb 3.1 seemed consistent in reducing cellular proliferation, only high concentrations of the mAb were able to affect macrophage replication (~12.5 µg/ml); published CSF-1R-blocking antibodies are effective in the range of 0.1–1 µg/ml [8]. This indicates that the interaction of mAb 3.1 with CSF-1R is weak.

Nevertheless, the effects of mAb 3.1 on cell survival were confirmed using feline osteoclasts. The inhibition assay with osteoclasts was performed because these cells have a distinct morphology, forming a tight 'tiled' structure. When cells undergo apoptosis, they naturally retract. Detecting this occurrence on osteoclasts is simple because of the characteristic arrangement of the cells in culture [29]. Apoptosis was induced using mAb 3.1 but not a control anti-CCR2 monoclonal. When the molecular pathways were analyzed, it could be seen that only mAb 3.1 induced a reduction in the phosphorylation of Akt, which is important for cell survival mediated through CSF-1R [38].

Despite the aforementioned properties of mAb 3.1, it could not stain fresh peripheral blood monocytes from dogs. The reduced capacity to bind to the native CSF-1R may be due to differences in conformation between the antigen used to create mAb 3.1 and the native protein. mAb 3.1 was produced by immunizing mice with a small recombinant protein (dimerization region of the receptor) produced in bacteria. The different protein conformation due to bacterial origin could explain the binding of mAb 3.1 to fixed tissues but not to the native soluble protein expressed on monocytes. Also, in immunoprecipitation, mAb 3.1 bound only to a narrow range of the glycoforms of CSF-1R, reinforcing the hypothesis that the bacterial origin

Table 1. Mutated CDR2 regions of phage display-selected scFv. The original CDR2 from mAb 3.1 is shaded in gray. The underlined glycine (G) was not mutated to preserve protein conformation, as this residue is important in loop formation in the CDRs. GRAVY, grand average of hydropathicity. Parameters calculated with ExPASy ProtParam tool

Mutated CDR2 region	Charge of the region	GRAVY score
SSGGSM	0	−0.217
QGPGAQ	0	−1.267
ALGGES	−1	+0.083
NCPGGR	+1	−1.317
GTKGST	+1	−1.150
GPNGAQ	0	−1.267
APGGPG	0	−0.433
GTRGPR	+2	−2.017
PHGGTC	0	−0.633
HGRGRV	+2	−1.467

of the antigen used for producing this antibody influenced its binding properties.

To improve the characteristics of this antibody, a semi-synthetic library was produced by mutating the variable region of mAb 3.1. Five amino acids from the CDR2 of the Vh chain were mutated by PCR. Such a reduced span in the mutations was an attempt to improve an existing antibody without greatly altering its fundamental capacities, since mAb 3.1 had shown some capacity to bind to CSF-1R. Evidently, for antibodies, even small alterations of the CDRs are sufficient to alter the binding specificity and affinity [39]. Vh CDR2 was selected for mutation for two reasons. Firstly, Vh CDR3 largely determines antibody specificity [40]; since mAb3.1 could recognize the desired target, mutations were not performed in this region to avoid loss of function. Also, Vh CDR2 has considerable sequence tolerance, so that amino acids can be largely altered with no influence on loop formation. This hypervariable antibody region follows only Vh CDR3 in this aspect [41].

Nevertheless, Vh CDR2 shows some constraints regarding 'viable' primary sequences. To maintain conformation, amino acids in this region must form β-hairpin structures. As determined by Cheng and colleagues, 'the loop residues in the CDR-H2 have moderate preferences for hydrophilic or turn (Gly and Pro) amino acid types' [42]. Indeed, the nine sequenced mutated CDR2 coincided in the prevalence of these amino acids. It is possible that increased solubility was important for the functional gains seen in mutated scFv. In naïve antibody repertoires, Tyr, Ser and Gly combine to confer specificity. This seemed to be the case for the original mAb3.1 Vh CDR2 region, in which 5/6 amino acids in the mutated region were Ser or Gly [43]. However, in activated antibody repertoires, Tyr is often associated with specificity, but it was not enriched following scFv selection in the present study [44]. Increased antibody function following mutations may have been gained via increased solubility. Short-chain hydrophilic residues (including Gly) are enriched in antibody paratopes, and this was unanimously found in the mutated scFv [44]. The lack of Tyr and Ser mutants is an indication of the shortfalls of the parsimonious mutations created in this project. The localization of the mutations seems to have been selected for residues that are critical in maintaining antibody structure, perhaps hampering the appearance of more specific clones.

The mutant library was screened against a recombinant version of the extracellular region of the canine CSF-1R produced in mammalian cells and whose conformation was similar to that of the natural protein. The library was also negatively selected for BSA binding [23]. Thus, it was expected that new clones derived from mAb 3.1 would arise that would be able to bind to the native form of the receptor, and not only to the original bacterially derived immunogen.

The scFv originated from the mutated library showed significant changes in binding compared to the original mAb. The scFv preferentially bound to CSF-1R and had reduced cross-reactivity with control proteins when compared to the parental mAb. This indicates that the mutation of the CDR 2 of the Vh chain of mAb 3.1 was a step toward perfecting the binding properties of the mAb.

The addition of the scFv to macrophage cultures affected BMDM proliferation to a greater extent than the parental mAb 3.1. Confounding factors include contaminant *E. coli* bacterial proteins derived from scFv purification, since affinity chromatography did not completely remove non-specific proteins (not shown). However, the scFv (or the impurities) had no effect over the proliferation of a murine fibroblast cell line (NIH/3T3) that was used as a control. Both macrophages and NIH/3T3 cells express at least some pathogen-associated molecular pattern receptors, such as Toll-like receptors, which could recognize residues of *E. coli* and negatively affect cellular proliferation. Since NIH/3T3 proliferation was unaffected by the scFv solution, there is an indication that bacterial contaminants were not an issue. Nevertheless, macrophages are more sensitive to some bacterial products than the fibroblast cell line and could have been more negatively affected by the presence of residues [45, 46].

Confirming that the binding properties of the antibody were greatly changed by mutation of CDR 2, the scFv were shown to specifically bind to unfixed monocytes from canine peripheral blood. This is in contrast with the parental mAb 3.1, which was useful for staining fixed tissues but could not identify cells from fresh blood. This confirms the specificity of the scFv to CSF-1R, since other peripheral blood cells do not express this receptor [47]. The scFv was also effective in reducing the attachment of CSF-1 to canine BMDM or PBMC. Upon ligand interaction, CSF-1R dimerizes, stabilizing the contact with CSF-1. The scFv may have hampered CSF-1 attachment by blocking the dimerization of the receptor.

Overall, these results confirm that it is possible to change and improve antibody specificity and binding through the construction and selection of small libraries of mutant antibodies created with only selected CDR alterations. Therefore, the present study was novel in the selection of the mutated region and in its reduced size [48], while still showing antibody improvements. A shortfall of the method was in not selecting for amino acids that confer higher specificity. Nevertheless, these

findings enable further enhancements of the anti-CSF-1R antibodies through further mutations of the already improved antibodies. Subsequent improvements of the mutated scFv could be achieved by blocking the library with proteins of similar structure to CSF-1R, such as c-Kit and the platelet-derived growth factor receptor (PDGFR) [49]. In this manner, antibodies that undesirably recognized these related proteins would be depleted. Still, the scFv demonstrated here already shows promise for future animal trials.

This article was assessed by reviewers during the unique time of the coronavirus disease (COVID)-19 pandemic. Further experiments were then deemed necessary, but the authors were unable to proceed with them due to the closure of the research facility where the work was carried out. These experiments were for SPR comparison of mAb 3.1 and the scFv and a demonstration of colocalization of CD163 with the staining from mAb 3.1. The authors acknowledge the need for these experiments, and this shall be the subject of our future work.

Conflict of interest statement

None declared.

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REFERENCES

1. Raposo, T, Gregório, H, Pires, I *et al.* Prognostic value of tumour-associated macrophages in canine mammary tumours. *Vet Comp Oncol* 2012. doi: 10.1111/j.1476-5829.2012.00326.x.
2. Chambers, SK, Kacinski, BM, Ivins, CM *et al.* Overexpression of epithelial macrophage colony-stimulating factor (CSF-1) and CSF-1 receptor: a poor prognostic factor in epithelial ovarian cancer, contrasted with a protective effect of stromal CSF-1. *Clin Cancer Res* 1997; **3**: 999–1007.
3. Paulus, P, Stanley, ER, Schäfer, R *et al.* Colony-stimulating factor-1 antibody reverses chemoresistance in human MCF-7 breast cancer xenografts. *Cancer Res* 2006; **66**: 4349–56.
4. Wyckoff, JB, Wang, Y, Lin, EY *et al.* Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer Res* 2007; **67**: 2649–56.
5. Mitchem, JB, Brennan, DJ, Knolhoff, BL *et al.* Targeting tumor-infiltrating macrophages decreases tumor-initiating cells, relieves immunosuppression, and improves chemotherapeutic responses. *Cancer Res* 2013; **73**: 1128–41.
6. Wyckoff, J, Wang, W, Lin, EY *et al.* A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res* 2004; **64**: 7022–9.
7. 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.
8. Haegel, H, Thioudellet, C, Hallet, R *et al.* A unique anti-CD115 monoclonal antibody which inhibits osteolysis and skews human monocyte differentiation from M2-polarized macrophages toward dendritic cells. *MAbs*. 2013; **5**: 736–47.
9. Murayama, T, Yokode, M, Kataoka, H *et al.* Intraperitoneal administration of anti-c-fms monoclonal antibody prevents initial events of atherogenesis but does not reduce the size of advanced lesions in apolipoprotein E-deficient mice. *Circulation* 1999; **99**: 1740–6.
10. Fend, L, Accart, N, Kintz, J *et al.* Therapeutic effects of anti-CD115 monoclonal antibody in mouse cancer models through dual inhibition of tumor-associated macrophages and osteoclasts. *PLoS One* 2013; **8**: e73310.
11. Ries, CH, Cannarile, MA, Hoves, S *et al.* Targeting tumor-associated macrophages with anti-CSF-1R antibody reveals a strategy for cancer therapy. *Cancer Cell* 2014; **25**: 846–59.
12. Cassier, PA, Gomez-Roca, CA, Italiano, A *et al.* Phase 1 study of RG7155, a novel anti-CSF1R antibody, in patients with locally advanced pigmented villonodular synovitis (PVNS). *ASCO Annual Meeting Proceedings* 2014; 10504.
13. Haegel, H, Thioudellet, C, Geist, M *et al.* Antibody against the CSF-1R, 2013.
14. Yamate, J, Yoshida, H, Tsukamoto, Y *et al.* Distribution of cells immunopositive for AM-3K, a novel monoclonal antibody recognizing human macrophages, in normal and diseased tissues of dogs, cats, horses, cattle, pigs, and rabbits. *Vet Pathol Online* 2000; **37**: 168–76.
15. Aguiar, PHP, Borges dos Santos, RR, Larangeira, DF *et al.* A novel monoclonal antibody against canine monocytes/macrophages. *Hybrid Hybridomics* 2004; **23**: 250–7.
16. Liu, H, Leo, C, Chen, X *et al.* The mechanism of shared but distinct CSF-1R signaling by the non-homologous cytokines IL-34 and CSF-1. *Biochimica Et Biophysica Acta-Proteins and Proteomics* 2012; **1824**: 938–45.
17. 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.
18. Ma, X, Lin, WY, Chen, Y *et al.* Structural basis for the dual recognition of helical cytokines IL-34 and CSF-1 by CSF-1R. *Structure* 2012; **20**: 676–87.
19. Wei, S, Nandi, S, Chitu, V *et al.* Functional overlap but differential expression of CSF-1 and IL-34 in their CSF-1 receptor-mediated regulation of myeloid cells. *J Leukoc Biol* 2010; **88**: 495–505.
20. Bradbury, ARM, Sidhu, S, Dübel, S *et al.* Beyond natural antibodies: the power of in vitro display technologies. *Nat Biotechnol* 2011; **29**: 245–54.
21. Li, W, Stanley, ER. Role of dimerization and modification of the CSF-1 receptor in its activation and internalization during the CSF-1 response. *EMBO J* 1991; **10**: 277.
22. Kapetanovic, R, Fairbairn, L, Beraldi, D *et al.* Pig bone marrow-derived macrophages resemble human macrophages in their response to bacterial lipopolysaccharide. *J Immunol* 2012; **188**: 3382–94.
23. Beirão, BCB, Raposo, T, Pang, LY *et al.* Canine mammary cancer cells direct macrophages toward an intermediate activation state between M1/M2. *BMC Vet Res* 2015; **11**: 151.
24. Bradbury, A. Cloning hybridoma cDNA by RACE. In: Kontermann, R, Dubel, S (eds). *Antibody Engineering*. Berlin: Springer, 2010, 15–20.
25. Kontermann, R. Immunotube selections. In: Kontermann, R, Dübel, S (eds). *Antibody Engineering SE - 9*. Berlin Heidelberg: Springer, 2010, 127–37
26. Gow, DJ, Sauter, KA, Pridans, C *et al.* Characterisation of a novel Fc conjugate of macrophage colony-stimulating factor. *Mol Ther* 2014; **22**: 1580–92.
27. Pridans, C, Raper, A, Davis, GM *et al.* Pleiotropic impacts of macrophage and microglial deficiency on development in rats with

targeted mutation of the Csf1r locus. *J Immunol* 2018. doi: 10.4049/jimmunol.1701783.
28. Hawley, CA, Rojo, R, Raper, A *et al.* Csf1r-mApple transgene expression and ligand binding in vivo reveal dynamics of CSF1R expression within the mononuclear phagocyte system. *J Immunol* 2018; **200**: 2209–23.
29. Lacey, DL, Tan, HL, Lu, J *et al.* Osteoprotegerin ligand modulates murine osteoclast survival in vitro and in vivo. *Am J Pathol* 2000; **157**: 435–48.
30. Bonde, A-K, Tischler, V, Kumar, S *et al.* Intratumoral macrophages contribute to epithelial-mesenchymal transition in solid tumors. *BMC Cancer* 2012; **12**: 35.
31. Marabelle, A, Kohrt, H, Caux, C *et al.* Intratumoral immunization: a new paradigm for cancer therapy. *Clin Cancer Res* 2014; **20**: 1747–56.
32. Pyonteck, SM, Akkari, L, Schuhmacher, AJ *et al.* CSF-1R inhibition alters macrophage polarization and blocks glioma progression. *Nat Med* 2013; **19**. doi: 10.1038/nm.3337.
33. Sasmono, RT, Oceandy, D, Pollard, JW *et al.* A macrophage colony-stimulating factor receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse. *Blood* 2003; **101**: 1155–63.
34. Komohara, Y, Hirahara, J, Horikawa, T *et al.* AM-3K, an anti-macrophage antibody, recognizes CD163, a molecule associated with an anti-inflammatory macrophage phenotype. *J Histochem Cytochem* 2006; **54**: 763–71.
35. Bain, CC, Scott, CL, Uronen-Hansson, H *et al.* Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors. *Mucosal Immunol* 2013; **6**: 498–510.
36. Jämsä, J, Huotari, V, Savolainen, E *et al.* Kinetics of leukocyte CD11b and CD64 expression in severe sepsis and non-infectious critical care patients. *Acta Anaesthesiol Scand* 2015.
37. Adams, CW, Allison, DE, Flagella, K *et al.* Humanization of a recombinant monoclonal antibody to produce a therapeutic HER dimerization inhibitor, pertuzumab. *Cancer Immunol Immunother* 2006; **55**: 717–27.
38. Kelley, TW, Graham, MM, Doseff, AI *et al.* Macrophage colony-stimulating factor promotes cell survival through Akt/protein kinase B. *J Biol Chem* 1999; **274**: 26393–8.
39. Mahon, CM, Lambert, MA, Glanville, J *et al.* Comprehensive interrogation of a minimalist synthetic CDR-H3 library and its ability to generate antibodies with therapeutic potential. *J Mol Biol* 2013; **425**: 1712–30.
40. Xu, JL, Davis, MM. Diversity in the CDR3 region of V(H) is sufficient for most antibody specificities. *Immunity* 2000; **13**: 37–45.
41. Julian, MC, Li, L, Garde, S *et al.* Efficient affinity maturation of antibody variable domains requires co-selection of compensatory mutations to maintain thermodynamic stability. *Sci Rep* 2017; **7**: 45259.
42. Chang, H-J, Jian, J-W, Hsu, H-J *et al.* Loop-sequence features and stability determinants in antibody variable domains by high-throughput experiments. *Structure* 2014; **22**: 9–21.
43. Birtalan, S, Zhang, Y, Fellouse, FA *et al.* The intrinsic contributions of tyrosine, serine, glycine and arginine to the affinity and specificity of antibodies. *J Mol Biol* 2008; **377**: 1518–28.
44. Peng, H-P, Lee, KH, Jian, J-W *et al.* Origins of specificity and affinity in antibody–protein interactions. *Proc Natl Acad Sci* 2014; **111**: E2656–65.
45. Lin, Y, Lee, H, Berg, AH *et al.* The lipopolysaccharide-activated toll-like receptor (TLR)-4 induces synthesis of the closely related receptor TLR-2 in adipocytes. *J Biol Chem* 2000; **275**: 24255–63.
46. Jung, DY, Lee, H, Jung, B-Y *et al.* TLR4, but not TLR2, signals autoregulatory apoptosis of cultured microglia: a critical role of IFN-β as a decision maker. *J Immunol* 2005; **174**: 6467–76.
47. Moffat, L, Rothwell, L, Garcia-Morales, C *et al.* Development and characterisation of monoclonal antibodies reactive with porcine CSF1R (CD115). *Dev Comp Immunol* 2014; **47**: 123–8.
48. Chames, P, Coulon, S, Baty, D. Improving the affinity and the fine specificity of an anti-cortisol antibody by parsimonious mutagenesis and phage display. *J Immunol* 1998; **161**: 5421–9.
49. Qiu, FH, Ray, P, Brown, K *et al.* Primary structure of c-kit: relationship with the CSF-1/PDGF receptor kinase family–oncogenic activation of v-kit involves deletion of extracellular domain and C terminus. *EMBO J* 1988; **7**: 1003–11.
50. McLellan, GJ, Cappello, R, Mayhew, IG *et al.* Clinical and pathological observations in English cocker spaniels with primary metabolic vitamin E deficiency and retinal pigment epithelial dystrophy. *Vet Rec* 2003; **153**: 287–92.