


# Development of a sandwich ELISA for the detection of chicken colony-stimulating factor 1

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**ABSTRACT** Macrophage colony-stimulating factor-1 (**M-CSF-1** or **CSF-1**) is a hematopoietic growth factor that stimulates the survival, proliferation, and differentiation of the mononuclear phagocyte lineage and is involved in bone metabolism, fertility, pregnancy, inflammatory processes, and homeostasis. CSF-1-activated macrophages display unique features, such as distinguishable cell surface antigens, enhanced Fc $\gamma$ -receptor-mediated phagocytosis, intensified reactive oxygen species activity, enhanced proliferation, and enhanced chemotaxis. Five mouse monoclonal antibodies (**mAbs**) for the detection of chicken CSF-1 were developed and characterized using western blot, indirect ELISA, and in vitro functional assays. One of the anti-chCSF-1 mAbs, 8A12, showed neutralization of chicken macrophage cell line (**HD11**) proliferation and CSF-1-induced nitric oxide release, whereas mAb 1G4 inhibited the phagocytosis of fluorescent-labeled *E. coli* by HD11

cells in vitro. For the quantitative assessment of native chCSF-1 in biological samples from chickens, a sensitive sandwich ELISA was developed using the best capture and detection pair of mAbs that were selected from newly developed anti-chCSF-1 mAbs. Chickens that were challenged with *Eimeria acervulina*, *E. maxima*, and *E. tenella* showed a steady increase in the circulating levels of serum CSF-1, starting from day 1 to 7 post-challenge reaching their peak levels at day 10 postchallenge infection. The CSF-1 synthesis induced by 3 different species of *Eimeria* was quite similar, even though these they are reported to be phenotypically and immunologically different. Therefore, this mAb-based sandwich ELISA will be a valuable tool for the detection of CSF-1 production during various poultry infections, and these new anti-chCSF-1 mAbs will facilitate the fundamental and applied research related to CSF-1 function in normal and disease states in chickens.

**Key words:** colony-stimulating factor-1, chicken, macrophages, sandwich ELISA, coccidiosis

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## INTRODUCTION

Macrophage colony-stimulating factor-1 (**M-CSF-1** or **CSF-1**) is a homodimeric growth factor involved in the development, proliferation, chemotaxis, phagocytosis, and survival of the mononuclear phagocyte lineage (Pixley and Staley, 2004). CSF-1 is a disulfide-linked dimer consisting of 2 bundles of 4  $\alpha$ -helices with an inter-chain disulfide bond and an antiparallel  $\beta$ -sheet (Pandit et al., 1992).

The shorter CSF-1 precursor is expressed as a membrane-spanning glycoprotein of 256 amino acids in humans (Kawasaki et al., 1985) and murine CSF-1 cDNA was identified via immunological screening of a mouse expression library (Rajavashisth et al., 1987).

Chicken CSF-1 was isolated from chicken stage 20 HH (Hamburger Hamilton) embryo RNA samples using RT-PCR (Garceau et al., 2010).

Using labeled <sup>125</sup>I-CSF-1, the cell surface CSF-1 receptor (**CSF-1R**) was identified in mouse peritoneal exudate- or bone marrow-derived macrophages and their progenitors (Guilbert and Stanley, 1986).

To understand the role of CSF-1 in the immune homeostasis in chickens, Garcia-Morales et al. (2014) produced a mAb (ROS-AV170) against chCSF-1R that did not block chCSF-1 activity, even though it could detect chCSF-1R on the cell surface of monocytes and its predecessors on the cell surface, but not on lymphocytes in blood and tissues. This characteristic contrasts with 2 well-known anti-CSF-1R rat mAbs, AFS98, and M279, which have been used mostly in mouse to remove resident macrophage populations in vivo (Hume and MacDonald, 2012). Additionally, McDonald et al. (2010) has reported a reduction in peritoneal, liver, epidermis, dermis, small intestine, stomach, colon, bladder, pancreas, testis, and kidney using M279 (anti-CSF-1R)

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mAb in McGreen mice. Moreover, [Sehgal et al. \(2018\)](#) has reported a prolonged CSF1R blockage in gut, Peyer patches and bone marrow macrophages by M279 mAb in Csf1r-EGFP mice. Finally, [Wu et al. \(2020\)](#), has reported the use of mouse anti-CSF-1 mAb (ROS-AV183) in peripheral mononuclear cells, liver, and bone osteoclast on CSF1R-eGFP transgenic chicken.

To better understand and study the immunobiology of chCSF-1, we developed and characterized new sets of mouse mAbs that specifically detect native CSF-1 in chicken sera. Using these mAbs, a sensitive sandwich ELISA was developed to monitor the circulating levels of chCSF-1 during *E. acervulina*, *E. maxima*, and *E. tenella* infections and to study the various effector functions (proliferation, nitric oxide production, and phagocytosis) of CSF-1 in inflammation and immune homeostasis using an established chicken macrophage cell line, HD11.

## MATERIALS AND METHODS

### Recombinant chCSF-1

The recombinant chCSF-1 protein (rchCSF-1) was obtained from Kingfisher Biotech, Inc. (Saint Paul, MN). The protein concentration of rchCSF-1 was determined using a Bicinchoninic Acid (BCA) protein assay kit (Thermo-Scientific-Pierce, Waltham, MA), and its purity was assessed using 12% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE).

### Production and Purification of chCSF-1 mAbs

All procedures using mice, including immunization and cell fusion, were conducted by GenScript Inc. (Piscataway, NJ). Briefly, rchCSF-1 (1.5–2 mg) was used for Balb/c mice ( $N = 5$ ) prime-boost immunization. Mice with high anti-chCSF-1 antibody titers as determined using indirect ELISA were selected for fusion. Hybridomas secreting chCSF-1 mAb were grown, screened, and isotyped using indirect ELISA. Briefly, 96-well high-binding microtiter plates (Corning, Bedford, MA) were coated with rchCSF-1 (1  $\mu\text{g}/\text{well}$ ) overnight at 4°C, followed by blocking of the nonspecific sites with PBS/1.0% BSA for 1 h. After washing with PBS/0.05% Tween 20 (PBS/T), the plates were incubated at room temperature for 1 h with 100  $\mu\text{L}/\text{well}$  of undiluted hybridoma culture supernatants and then washed 5 times with PBS/T. CHO-derived recombinant chicken IL7 (Panebra et al., 2021) was used as a negative control. The antigen-antibody reaction was detected using horseradish peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (1:10,000 dilution), followed by a color reaction by adding 3,3',5,5'-tetramethylbenzidine (TMB) substrate and  $\text{H}_2\text{O}_2$  (all from Sigma-Aldrich, St. Louis, MO) at room temperature for 20 min. The reaction was stopped by adding 0.05 mL/well of 2 N  $\text{H}_2\text{SO}_4$  and the optical density was

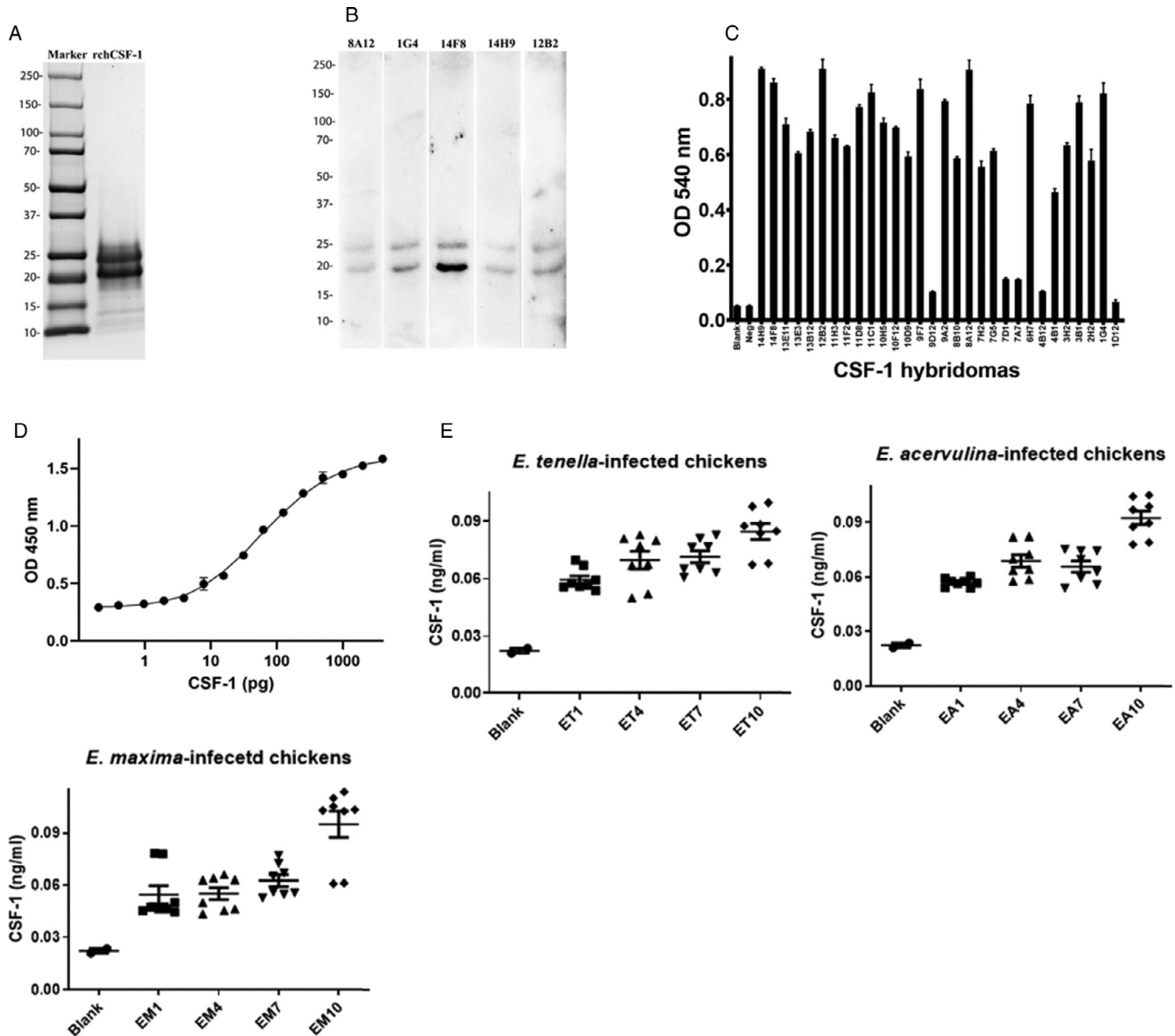
measured at 450 nm ( $\text{OD}_{450}$ ) using a microplate reader ELx800 (BioTek, Winooski, VT). Hybridomas secreting anti-chCSF-1 mAbs were single-cell cloned via limiting dilution and the cloned mAbs were isotyped using an Iso Quick kit for mouse monoclonal isotyping (Sigma-Aldrich). Monoclonal antibodies were purified from the hybridoma cell culture supernatants using affinity chromatography on protein-G agarose columns according to the manufacturer's instructions (Pierce, Rockford, IL). Purified mAbs were biotinylated using an EZ-Link NHS-Biotin kit (Pierce) according to the manufacturer's instructions.

### Western Blot Analysis

Recombinant chCSF-1 (1  $\mu\text{g}/\text{well}$ ) were resolved using 12% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. Blots were treated with Superblock Blocking Buffer (Thermo Fisher Scientific, Waltham, MA), followed by washing with 1X Tris-Borate-Saline buffer (TBS)/0.05% Tween 20 (TBS/T). Membranes were incubated with 1  $\mu\text{g}/\text{mL}$  anti-chCSF-1 mAbs at 4°C overnight, washed, and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich) (1:10,000) in blocking buffer at room temperature for 1 h with gentle shaking. After washing, immunoreactivity was visualized using Clarity Western ECL Substrate and recorded using a ChemDoc Imaging System (both from Bio-Rad, Hercules, CA).

### Establishment of the Sandwich ELISA

All five chCSF-1 mAbs selected for their high binding activity with rchCSF-1 were tested for their capture or detection abilities to identify the compatible mAb pairs for the antigen capture ELISA. To establish a sandwich ELISA, flat-bottomed 96-well high-binding ELISA plates were coated with each capture chCSF-1 mAb candidates in PBS (10  $\mu\text{g}/\text{mL}$ ) and incubated at 4°C overnight. Plates were washed with PBST and then blocked with 1% BSA/PBS at room temperature for 1 h, followed by a final incubation with 0.1 mL of CSF-1 (0.1  $\mu\text{g}/\text{mL}$  in 0.1% BSA/PBS) or chicken sera (diluted to 1:5 with 0.1% BSA/PBS) at 37°C for 2 h. After washing, 0.1 mL of biotin-labeled detecting chCSF-1 mAb candidates (1  $\mu\text{g}/\text{mL}$  in 0.1% BSA/PBS) were added and incubated at 37°C for 1 h. Plates were then washed with PBST and incubated with 0.1 mL avidin HRP in PBS/0.1% BSA (Sigma-Aldrich) (1:10,000) at 37°C for 1 h and developed using an Ultra TMB peroxidase substrate solution (eBioscience, San Diego, CA) at room temperature for 20 min. The reaction was stopped by adding 0.05 mL of 2 N  $\text{H}_2\text{SO}_4$  and the OD 450 nm was measured using a microplate reader ELx-800 (BioTek). A standard curve was created in parallel, and the sensitivity of the ELISA was determined to be 1 pg/mL (Figure 1D).



**Figure 1.** Molecular weight determination, reactivity of chicken CSF-1 mAbs to rhCSF-1, and sandwich ELISA. (A) A total of 1  $\mu\text{g}$  per lane of yeast-derived CSF-1 (lane 1) was resolved using 12% SDS-PAGE (Figure 1A). (B) Blotted onto a PDVF membrane and probed using the following CSF-1 mAbs: 8A12 (lane 2), 1G4 (lane 3), 14F8 (lane 4), 14H9 (lane 5), and 12B2 (lane 6) (Figure 1B). (C) Screening of the hybridomas secreting CSF-1 mAbs against rhCSF-1 using indirect ELISA (Figure 1C). (D) ChCSF-1 sandwich ELISA standard curve using unlabeled 14H9 mAb (capture) and biotinylated-1G4 mAb (detecting) (Figure 1D). (E) Monitoring of the circulating levels of chCSF-1 in chickens infected with *E. acervulina*, *E. maxima*, and *E. tenella* at different time points (0, 1-, 4-, 7-, and 10-d postchallenge) (Figure 1E). Abbreviations: mAb, monoclonal antibodies; SDS-PAGE, sodium dodecyl polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.

### Inhibition of Macrophage Proliferation by mAbs

To evaluate whether 8A12 mAb could neutralize the rhCSF-1-mediated HD11 proliferation, freshly passaged HD11 cells were prepared in complete RPMI 1640 medium containing 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 4 mM glutamine, and 100  $\mu\text{g}/\text{mL}$  Pen-Strep. A flat-bottomed 96-well plate (ThermoFisher Scientific) was seeded with HD11 cells ( $2 \times 10^7$  cells/mL) in complete RPMI medium and incubated at 41°C overnight. A volume of 0.05 mL of rhCSF-1 (0.1  $\mu\text{g}/\text{mL}$ ) was preincubated (in triplicate) with 0.05 mL of 8A12 mAb at different concentrations, ranging from 0.15 to 5  $\mu\text{g}/\text{mL}$ , at 37°C for 2 h. Then, the mixture was added to the HD11 cells after removing the extra

medium, followed by incubation at 41°C for 24 h. Finally, the CCK8 reagent (Dojindo, Rockville, MD) was added (10  $\mu\text{L}/\text{well}$ ), and the plates were incubated at 41°C for 2 h before the optical density was measured at 450 nm.

### Inhibition of Nitric Oxide Release by Macrophages by mAbs

HD11 cells ( $2 \times 10^7$  cells/mL) were seeded on flat-bottomed 96-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) and incubated overnight at 41°C with high glucose DMEM supplemented with 10% FBS and 100  $\mu\text{g}/\text{mL}$  penicillin and streptomycin. Recombinant chCSF-1 (0.1  $\mu\text{g}/\text{mL}$ ) was preincubated with

increasing concentrations of 8A12 mAb (0.15–5  $\mu\text{g}/\text{mL}$ ) at 37°C for 2 h. Then, the mixture was added to HD11 cells, followed by incubation at 41°C for 4 h. Afterward, aliquots of 50  $\mu\text{L}$  of conditioned culture media were transferred to a new flat-bottomed 96-well tissue culture plate, and an equal volume of Griess reagent (Sigma-Aldrich) was added for incubation at room temperature for 10 min in the dark. Finally, the absorbance was measured at 540 nm, and the results were expressed as micromolar amounts of nitrite ( $\mu\text{M}$ ) per  $10^6$  cells. A standard curve was created in parallel using different concentrations of nitrite, ranging from 1.56 to 100  $\mu\text{M}$  (data not shown).

### **Inhibition of Phagocytosis by mAbs**

HD11 cells grown in RPMI-1640 complete medium supplemented with 10% FBS, 1 mM pyruvate, and 100  $\mu\text{g}/\text{mL}$  Penn-Strep were harvested by gentle scraping using a rubber policeman, centrifuged, and resuspended in complete media. Viability, as determined using trypan blue exclusion dye, was  $\geq 90\%$  and the cell concentration was adjusted to  $1 \times 10^6$  cells/mL for seeding on a 96-well black clear bottom plate (Thermo Scientific, Grand Island, NY), which was incubated overnight at 41°C. The next day, 0.05 mL of rchCSF-1 (0.1  $\mu\text{g}/\text{mL}$ ) was preincubated (in quintuplicate) with 0.05 mL of 1G4 mAb at different concentrations ranging from 0.15 to 5  $\mu\text{g}/\text{mL}$  at 41°C for 2 h. Then, they were added to the HD11 cells and incubated at 41°C for 4 h. An *E. coli* BioParticle suspension (0.1 mL) from the Vybrant Phagocytosis assay kit (Thermo Scientific) was then added to each well and incubated at 41°C for 2 h. Afterward, a trypan blue suspension (0.1 mL) was added to each well, incubated for 1 min, aspirated, and the relative fluorescence units (RFU) were measured using a Synergy HTX Multimode reader (BioTek) at excitation and emission wavelengths of 480 and 520 nm, respectively.

### **Eimeria Infection**

Two-wk-old chickens ( $N = 36$  total) were orally inoculated with  $1 \times 10^4$  sporulated oocysts/mL of *E. acervulina* (ARS strain 12) ( $N = 12$ ), *E. maxima* (41A strain) ( $N = 12$ ), and *E. tenella* (WR1 strain) ( $N = 12$ ), respectively, via oral gavage. Chickens were bled at pre-infection and 1, 4, 7, and 10 d after infection, and the serum samples ( $N = 36$  total,  $N = 12$  for each *Eimeria* spp. strain used) were processed via centrifugation at  $1,000 \times g$  for 10 min and stored at  $-20^\circ\text{C}$ . The serum CSF-1 levels induced by coccidiosis, were monitored using sandwich ELISA, as described above. The animal trial procedures and experimental details were approved by the Beltsville Institutional Animal Care and Use Committee, Agriculture Research Services, U.S. Department of Agriculture (Animal Use Protocol approval #20-015).

### **Statistical Analysis**

All data are expressed as the mean  $\pm$  SD unless otherwise specified. Analyses were performed using GraphPad Prism version 9 software (GraphPad Software Inc., La Jolla, CA). Statistical differences were evaluated using a one-way analysis of variance, followed by Tukey's test. The differences were considered statistically significant when the  $P$  values were  $< 0.05$ .

## **RESULTS AND DISCUSSION**

### **Production of Chicken CSF-1 mAb**

The recombinant chCSF-1 protein consisted of 240 amino acids with a predicted molecular weight of 27.2 kDa. In 12% SDS-PAGE gel stained with Coomassie brilliant blue R-250 and western blot, rchCSF-1 protein run as a doublet (at approximately 22 and 24 kDa) (Figure 1A and B, respectively).

After the initial screening of hybridomas for their binding activity to rchCSF-1, 30 hybridoma clones were chosen based on their higher binding activities, compared to negative control which was a mAb detecting chIL-7 and 5 hybridoma clones (8A12, 1G4, 14F8, 14H9, and 12B2) were selected based on their high binding activities (around 15xOD compared to negative control) (Figure 1C). These were then used to select the best capture and detecting mAb pairs to develop a sandwich ELISA for the chCSF-1 protein.

### **Pairing Assay and Sandwich ELISA Development**

ChCSF-1 mAb pairings were assessed using sandwich ELISA to identify the best pairs for the quantification of chCSF-1 using unlabeled (capture) and biotinylated (detecting) chCSF-1 mAbs. Serially diluted rchCSF-1 (from 4 ng to 1 pg) was used to generate a standard curve for chCSF-1 detection (Figure 1D). Among the 5 mAbs that were tested, 14H9 mAb was the best capture mAb at 10  $\mu\text{g}/\text{mL}$  and the biotinylated-1G4 mAb was selected as the best detecting mAb at 1  $\mu\text{g}/\text{mL}$  to detect native chCSF-1 in the serum samples obtained from chickens infected with *E. acervulina*, *E. maxima*, and *E. tenella*. The serum samples were collected at different time points after coccidia infection, and all of these showed significantly higher circulating CSF-1 levels compared to the serum from unchallenged control chickens. Briefly, significantly higher CSF-1 levels were detected in the serum samples from *E. acervulina*- and *E. tenella*-infected chickens (50–80  $\text{pg}/\text{mL}$ ,  $P < 0.001$ ) at 1-, 4-, and 7-d postchallenge with *Eimeria* infection, reaching their peak at 10 d postchallenge (80–100  $\text{pg}/\text{mL}$ ,  $P < 0.001$ ) compared to the unchallenged control group (20  $\text{pg}/\text{mL}$ ). In *E. maxima*-infected chickens, in which parasites primarily undergo intracellular development in the midgut, significantly higher CSF-1 levels (50–60  $\text{pg}/\text{mL}$ ,  $P < 0.05$ ) were detected in the serum at 1-, 4-, and 7-d postchallenge, reaching a peak

at 10 days postchallenge (90–110 pg/mL,  $P < 0.001$ ) (Figure 1E). Interestingly, although *E. acervulina*, *E. maxima*, and *E. tenella* infect the different niches in the chicken intestine (duodenal loop, midgut, and ceca, respectively) and they elicit phenotypically and immunologically different responses, all of them induced quite similar chCSF-1 synthesis pattern and this finding agrees with a recent report (Liu et al., 2021) that showed similar biological responses at the system biology level in chickens infected with 3 above-mentioned *Eimeria* species.

Most studies on circulating levels of CSF-1 have been done in human (Roth and Stanley, 1995) and mice (Roth and Stanley, 1996). In both cases, the circulating CSF-1 levels were monitored until 5 d postnatal age and were around 30 to 40 ng/mL. Regarding CSF-1 circulating levels in chickens, Wu et al. (2020), detected up to 14 d posthatch and their levels were around 5 to 20 ng/mL but these chickens were not infected (broilers and layers). Although, Cheers et al. (1988) has reported elevated levels of CSF-1 in serum (14 pmol/mL) postchallenge with *Listeria monocytogenes* in Balb/cJ mice and approximately 1 pmol/mL in C57BL/10 at 24 h by RIA, however having large standard deviation, reflecting high mouse to mouse variation. Our data are in the range of 60 to 100 pg/mL that is not far away from those reported by them. However, we cannot rule out the possibility that beyond 10 d postchallenge, the levels of chCSF-1 could be higher. Also, we believe that increasing the challenging dose could lead to higher chCSF-1 circulating levels. In our coccidiosis model, we use  $1 \times 10^4$  sporulated oocyst as the optimal dose used to measure body weight gain, lesion scores and oocyst shedding.

### **Inhibition of Macrophage Proliferation by Anti-CFS-1 mAbs**

There was a numerical, although not statistically significant, inhibition of the proliferation of HD11 macrophages by 8A12 mAb at concentration ranges between 0.3 and 0.6  $\mu\text{g/mL}$  compared to the positive control (0.1  $\mu\text{g/mL}$  CSF-1). Moreover, at 1.25  $\mu\text{g/mL}$ , there was a clear and significant ( $P \leq 0.05$ ) inhibition of HD11 cell proliferation, which was more pronounced at 2.5 and 5  $\mu\text{g/mL}$  ( $P \leq 0.01$ ) (Figure 2A). Other CSF-1 mAbs did not show any inhibitory activities on HD11 cell proliferation at the doses tested (data not shown).

Early studies have reported the proliferation of stably transfected Ba/F3 [pro-B murine cell line dependent on interleukin-3 (IL-3) for growth]-chCSF-1R or bone marrow-derived cells induced by chCSF-1, as determined using MTT assays (Garceau et al., 2010). Furthermore, Wu et al. (2020) demonstrated that anti-CSF-1 mAb ROS-AV183 neutralized the chCSF-1-induced proliferation of Ba/F3-chCSF-1R stable clones either ex vivo embryo- or bone marrow-derived activated macrophages. In our study, 8A12 mAb showed a dose-dependent neutralization of the CSF-1-induced proliferation

of HD11 cells like the results as previously reported (Wu et al., 2020), although two different systems were used.

### **Inhibition of Nitric Oxide Production by Anti-CSF mAbs**

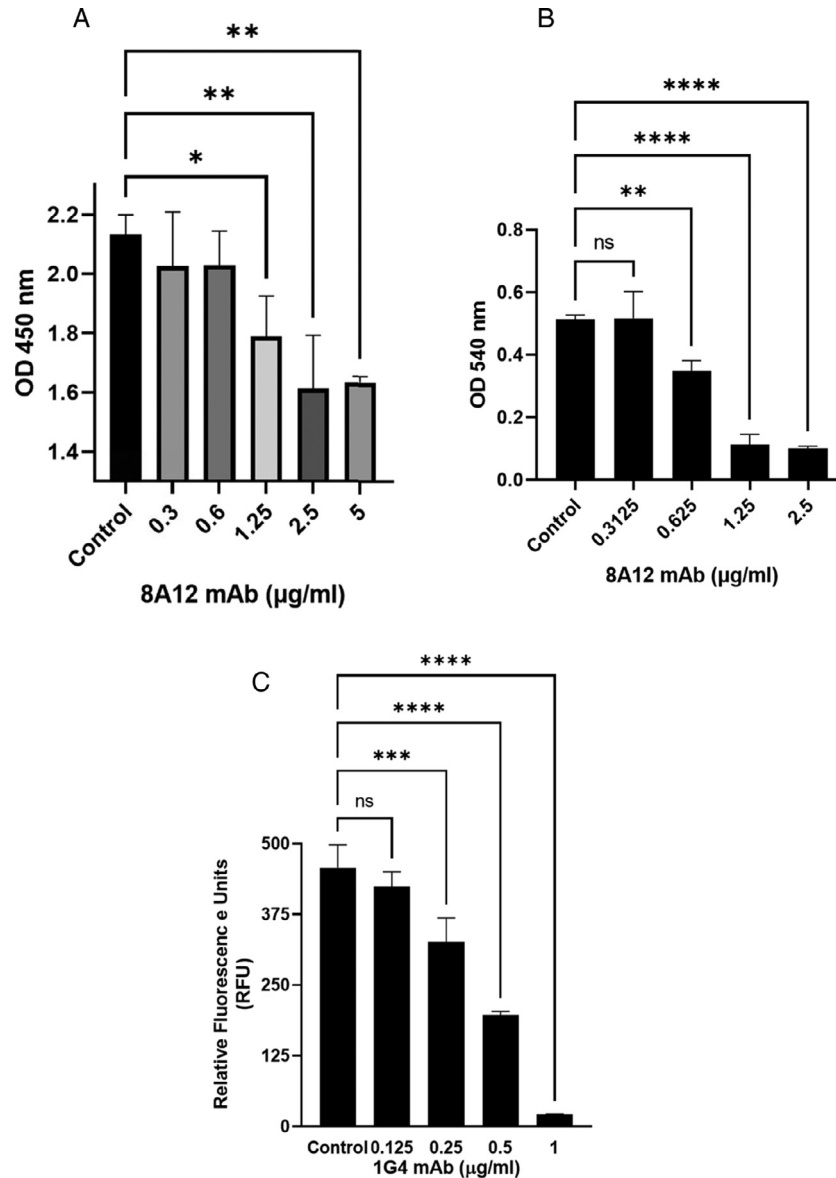
Of the 5 anti-CSF-1 mAbs that we tested, only 8A12 mAb inhibited the nitrite release by CSF-1-activated HD11 cells. Inhibition was dose-dependent and at a lower 8A12 mAb concentration (0.3  $\mu\text{g/mL}$ ), no significant nitric oxide release inhibition was seen compared to that of the positive control (CSF-1-treated cells). In contrast, inhibition of nitric oxide release by macrophages was highly significant ( $P \leq 0.0001$ ) at higher 8A12 mAb concentrations (0.6, 1.25, 2.5, and 5  $\mu\text{g/mL}$ ) as shown in Figure 2B. This is the first report showing that 8A12 mAb neutralizes the nitric oxide release from CSF-1-activated HD11 cells in a dose-dependent manner.

### **Inhibition of Phagocytosis Mediated by Anti-CSF mAb**

There was no inhibition of phagocytosis at a low 1G4 mAb concentration (0.125  $\mu\text{g/mL}$ ) compared with the chCSF-1 positive control (CSF-1, 0.1  $\mu\text{g/mL}$ ). In general, phagocytosis inhibition was dose-dependent, being more pronounced and highly significant ( $P \leq 0.001$ ) at a higher 1G4 mAb concentration (0.25  $\mu\text{g/mL}$ ) with the highest inhibition at 0.5 and 1  $\mu\text{g/mL}$ , compared with the corresponding control (Figure 2C). All the other CSF-1 mAbs tested did not affect phagocytosis (data not shown). Like the neutralization of the nitrite release by HD11 cells, this is the first report on the neutralization of phagocytosis mediated by 1G4 mAb in HD11 cells.

In summary, new sets of CSF-1 mAbs and a sandwich ELISA to monitor the native CSF-1 levels in the serum of coccidiosis-infected chickens were developed. Furthermore, we evaluated the effects of these mAbs on various immune functions of CSF-activated macrophages including the proliferation, nitric oxide release, and phagocytosis inhibition.

There is some report on the role of anti-CSF-1 mAbs in blocking CSF-1 biological activity in vivo in different animal models. For example, Lokeshwar and Lin (1988) have reported anti-CSF-1 rat 5A1 mAb inhibit colony formation of tissue mononuclear phagocyte colony-forming cells in mice. CSF-1 complexed with 5A1 mAb did not bind either to its cell surface receptor of peritoneal exudate macrophages, or complex with cell-bound CSF-1, and inhibit proliferation of bone marrow cell-derived macrophages. Moreover, Cenci et al. (2000) has reported that neutralizing CSF-1 with 5A1 mAb in vivo, prevent the rise of; osteoclast number, increase in bone resorption and bone loss due to ovariectomy in mice. Furthermore, Campbell et al. (2000) has reported the involvement of CSF-1 in collagen-induced arthritis



**Figure 2.** Inhibition of the proliferation, nitric oxide release, and phagocytosis of HD11 cells mediated by CSF-1 mAbs. (A) CSF-1-treated HD11 cell proliferation inhibition mediated by different concentrations of 8A12 mAb (0.3, 0.6, 1.25, 2.5, and 5  $\mu\text{g}/\text{mL}$ ). Data represent the means  $\pm$  SD of triplicate samples from two independent experiments.  $*P < 0.05$  or  $**P < 0.01$ , compared with the CSF-1 (0.1  $\mu\text{g}/\text{mL}$ )-treated control. (B) The inhibition of nitric oxide release by CSF-1-treated HD11 cells was carried out using different concentrations of 8A12 mAb (0.3, 0.6, 1.25, and 2.5  $\mu\text{g}/\text{mL}$ ). Data represent the means  $\pm$  SD of triplicate samples from 2 different experiments. ns, not significant;  $**P < 0.01$  or  $****P < 0.0001$ , compared with the CSF-1 (0.1  $\mu\text{g}/\text{mL}$ )-treated control. (C) The inhibition of phagocytosis by CSF-1-treated HD11 cells was carried out using different concentration of 1G4 mAb (0.125, 0.25, 0.5, and 1  $\mu\text{g}/\text{mL}$ ). Data represent the means  $\pm$  SD of triplicate samples from two different experiments. ns, not significant;  $***P < 0.001$  or  $****P < 0.0001$ , compared with the CSF-1 (0.1  $\mu\text{g}/\text{mL}$ )-treated control.

(CIA), a murine model of rheumatoid arthritis by 5A1 mAb in mice. Besides, Radi et al. (2011) has reported a human PD-0360324 mAb that neutralize CSF-1 and reduced CD14<sup>+</sup>CD16<sup>+</sup> monocyte population, depleted liver Kupfer cells (KC) and increased aspartate amino transferase and creatine kinase enzyme levels in serum in cynomolgus macaques, in osteopetrotic (Csf1<sup>OP</sup>/Csf1<sup>OP</sup>) mice that have reduced levels, of KC and higher serum enzyme than wild-type mice littermates. Finally, Wu et al. (2020) has reported a chicken anti-CSF-1 mAb (ROS-AV183) impacted on some tissue macrophage populations but not on monocytes, as well as CSF1R-transgene expressing cells were reduced in Bursa

de Fabricius, ceca tonsil, TIM4<sup>+</sup> Kupfer cells in liver were abated, reduced bone density, trabecular volume, and TRAP<sup>+</sup> osteoclast in chicken.

Furthermore, we cannot exclude the possibility that the inhibition of nitric oxide release and phagocytosis of HD11 cell are linked to the inhibition of macrophage proliferation due to chCSF-1 mAbs.

These new CSF-1 mAbs are valuable immune tools for studying the role of CSF-1 in macrophage biology and immune homeostasis.

Because the epitopes which are recognized by anti-CSF-1R mAbs are likely to be different, we expect different mAb will affect differently macrophage functions.

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## DISCLOSURES

The authors declare no conflict of interest.

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