Development and characterization of monoclonal antibodies specific for chicken interleukin-13 and their neutralizing effects in chicken primary monocytes

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ABSTRACT Compared with mammals, the functionality of chicken cytokines is not well understood because of the unavailability of immune reagents. Mammalian interleukin (IL)-13 is an important Th2 type cytokine with well-known biological functions through its 2 receptors, IL-13 receptor (IL-13R)-α1 and IL-13Rα2. In the present study, we developed mouse monoclonal antibodies (**mAb**) against chIL-13 and further investigated their specificity in detecting endogenously produced chIL-13. Upon characterization of mAb using indirect ELISA and Western blot, the capture ELISA was developed for detecting chIL-13. Neutralizing effects were tested by measuring nitric oxide (**NO**) production and inducible nitric oxide synthase (**iNOS**) expression in primary chicken monocytes stimulated with chIL-13, lipopolysaccharide (**LPS**), chIL-13+LPS, or chIL-13+LPS+mAb. In addition, gene expression of *chIL-13Rα1*, *chIL-13Rα2*, and *TGF-β1* was tested in chicken monocytes treated with chIL-13 or chIL-13+mAb. Based on indirect ELISA, 5 mAb that detected recombinant chIL-13 were identified, and all of them specifically detected recombinant chIL-13 protein by Western blotting. An optimal signal was obtained with 2 mAb (#9B11 and #10A2) in a pairing assay, and these 2 mAb were used in a capture assay. A neutralization assay further revealed that chIL-13 reduced LPS-stimulated NO production and iNOS expression in monocytes and macrophage cells, and the 2 mAb (#9B11 and #10A2) abrogated these effects. In addition, chIL-13–induced expressions of *chIL-13Rα2* and *TGF-β1* were neutralized by the 2 mAb. In summary, the present study showed that chIL-13 may be involved in the alternative activation of primary monocytes in chickens and that chIL-13 signaling may be regulated through chIL-13Rα2 binding and TGF-β1 secretion. Importantly, the newly developed anti–chIL-13 mAb will serve as valuable immune reagents for future studies on the biological activity of chIL-13 and its receptors.

Key words: interleukin-13, antigen capture assay, monoclonal antibodies, chicken, alternative activation

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INTRODUCTION

Compared with their mammalian counterparts, the functionality of chicken cytokines is not well understood because of unavailability of immune reagents. More recently, efforts are being directed toward developing monoclonal antibodies (**mAb**) against chicken interleukin (**IL**)-4, IL-8, IL-10, IL-12, and IL-15 to better understand their functionality (Min et al., 2002; Balu et al., 2011; Lee et al., 2014, 2018; Wu et al., 2016; Kim et al., 2017a; Chaudhari et al., 2018). Such reagents can be used effectively to detect these cytokines specifically in several parasitic or bacterial infections in chickens to better understand host–pathogen interactions. They can also be used to investigate the specific functions of the cytokines. For example, newly developed anti–chicken IL-4 antibodies were used to show the regulatory role of chicken IL-4 (**chIL-4**) in the alternative activation of macrophages (Chaudhari et al., 2018). In mammals, IL-13 is a Th2 cytokine and shares many of its biological activities with IL-4 such as B-cell proliferation, immunoglobulin class switching, and anticytotoxic and anti-inflammatory properties in monocytes (Punnonen et al., 1993; Zurawski and de Vries, 1994; Zurawski et al., 2018). Although similar, IL-4 and IL-13 use discrete pathways in either alternative activation of macrophages or allergic disease conditions (LaPorte et al., 2008; Munitz et al., 2008; Bhattacharjee et al., 2013; Bao and Reinhardt, 2015).

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Interleukin-13 produced from monocytes or macrophages exhibits an anti-inflammatory effect by suppressing the production of proinflammatory mediators such as prostaglandins, reactive oxygen and nitrogen species (Doherty et al., 1993; Sozzani et al., 1995), and cytokines including IL-1, IL-6, IL-8, tumor necrosis factor-α, and IL-12 (De Vries, 1998). The anti-inflammatory role of IL-13 is mediated via suppression of the nuclear factor κB pathway (Lentsch et al., 2008). In general, IL-13 signaling has been reported in gastroenteric and autoimmune diseases, asthma, systemic sclerosis, and parasitic infections (Finkelman et al., 1997, 2004; Bao and Reinhardt, 2015; Seyfizadeh et al., 2015; Giuffrida et al., 2019). Of relevance, the role of IL-13 in antihelminth immunity is more prominent than IL-4 in clearing nematode infection in mice (Lawrence et al., 1996; McKenzie et al., 1998; Urban et al., 1998; Bao and Reinhardt, 2015). Interestingly, chIL-13 levels have been reported to be significantly elevated in chickens infected with Marek's disease virus (Heidari et al., 2008). In response to the infection caused by extracellular pathogens, cIL-13 is induced in higher amounts than chIL-4 (Degen et al., 2005; Powell et al., 2009, 2012; Schwarz et al., 2011). More recently, an elevated chIL-13 expression has been reported in chickens after primary and secondary *Eimeria* infections, thus suggesting the role of chIL-13 in Th2-mediated immunity in response to the coccidiosis infection (Hong et al., 2006a,b).

In mammals, IL-13 signaling is mediated through the complex network of IL-13 receptors, IL-13Rα1 and IL-13Rα2. Similar to IL-4 signaling through type II receptor complex (i.e., IL-4 Rα and IL-13Rα1), IL-13 signaling is mediated via its binding specifically to IL-13Rα1 (Seyfizadeh et al., 2015). However, IL-13 has a moderate affinity with IL-13Rα1 and increasing evidence in the recent years suggests that IL-13 signaling could also be mediated through IL-13Rα2, which is otherwise considered a "decoy receptor" (Sato et al., 1993; Silvestri et al., 2006; Lupardus et al., 2010; Andrews et al., 2014; McCormick and Heller, 2015). This signaling through IL-13Rα2 has been reported to induce TGF-β production in IL-13–mediated fibrosis in a colitis model in mice (Mentink-Kane and Wynn, 2004; Fichtner-Feigl et al., 2006, 2008; Fichtner-feigl et al., 2007; Munitz et al., 2008). Despite having a greater than 35% homology, the expression patterns of IL-13Rα1 and IL-13Rα2 are different depending on the cell types in mammals (Seyfizadeh et al., 2015). Both the IL-13 receptors have been widely expressed on macrophages, B cells, eosinophils, endothelial cells, monocytes, and respiratory epithelial cells. However, it was believed that IL-13Rα2 expression is restricted to the spleen and brain and in its soluble form in mouse serum (J. Hilton D., 1997), until it was reported that IL-13Rα2 is more widespread and exists intracellularly in cultured monocytes, respiratory epithelial cells, primary respiratory epithelium, and primary human monocytes (Daines and Hershey, 2002). Miyoshi et al. (2007) showed that in chickens, high levels of chIL-13Rα2 mRNA were expressed in liver, testis, ovary, brain, and monocyte cell lines. Thus, the involvement of both IL-13 receptors in IL-13 signaling is well understood in mammalian species, although some contrasting reports debate on the exact mechanisms (Fichtner-Feigl et al., 2008; Lupardus et al., 2010; Andrews et al., 2014; McCormick and Heller, 2015). Although the expression of chIL-13 receptors has been reported in chickens, the effect of chIL-13 on their expression has not been reported (Miyoshi et al., 2007).

We have recently shown that chIL-4 regulates alternative activation of chicken macrophages, and our findings suggested the existence of M1 or M2 paradigm in chickens (Chaudhari et al., 2018). Owing to the similarities between IL-4 and IL-13 in terms of their biological functions, it is important to investigate whether chIL-13 also mediates the alternative activation of macrophages in chickens. Therefore, in the present study, we developed anti–chIL-13 mouse mAb and further investigated their specificity in detecting endogenously produced chIL-13. We then investigated if chIL-13 can induce expression of *chIL-13Rα1* and *chIL-13Rα2* receptors and the alternative activation of chicken monocytes and whether anti–chIL-13 mAb could have a neutralizing effect on chIL-13 functionality.

MATERIALS AND METHODS

Recombinant chIL-13 Production

Recombinant chIL-13 protein was expressed in *Escherichia coli*. The cDNA encoding chIL-13 (GenBank accession: NM_001195791.1) was cloned into pUC57 and subcloned into the pET-30a(+) bacterial expression vector (Novagen, Madison, WI) incorporating an NH2-terminal polyhistidine tag. Recombinant chIL-13/pET30a was transformed into *E. coli* BL21(DE3) cells (Life Technologies, Grand Island, NY), and expression was induced using 3 mmol isopropyl β-D-1- thiogalactopyranoside in 2x tryptone yeast liquid culture medium containing 16 g/L BD Bacto Tryptone (BD Biosciences, San Jose, CA), 10 g/L yeast extract, and 6 g/L NaCl during the midlog phase (optical density at 600 nm = 0.4) at 37°C for 4 h. The expressed recombinant chIL-13 was purified using $Ni2^{+}$-NTA His•bind Resin (Merck Millipore, Billerica, MA) column chromatography, and lipopolysaccharide (**LPS**) removal was performed before use. The concentration of purified *E. coli* chIL-13 protein was determined using the Bradford assay, and purity was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. To test the cross-reactivity of mAb, another cytokine chicken IL-7 (chIL-7) was cloned into the same vector and the recombinant chIL-7 was expressed and purified as described earlier and was used as a negative antigen. The recombinant yeast–expressed chIL-13 was purchased from Kingfisher Biotech (Catalog No. RP0110C-025, St. Paul, MN) and was used for all the assays.

Production and Characterization of mAb Against chIL-13

The recombinant chIL-13 from *E. coli* was used as an immunogen to generate anti-chIL13 antibodies from mice. All animal protocols were performed as per the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at the Beltsville Agricultural Research Center. BALB/c mice (National Cancer Institute, Frederick, MD) were immunized biweekly (2 boosters) by intraperitoneal and subcutaneous injections with 50 μg of recombinant chIL-13 in Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO), as described elsewhere (Lee et al., 2011). Third booster immunization by intravenous injection with 25 μg of chIL-13 without adjuvant was performed 3 D before cell fusion. Hybridomas were produced by fusion of splenic lymphocytes of the immunized mice with mouse SP2/0 cells (ATCC, Manassas, VA), and hybridomas were selected in Roswell Park Memorial Institute (RPMI)-1640 medium containing hypoxanthine, aminopterin, and thymidine (all from Sigma-Aldrich). Hybridomas secreting anti–chIL-13 mAb were selected by indirect ELISA, as described previously (Min et al., 2002). Briefly, 1.0 μg/well yeast- or *E. coli*–expressed chIL-13 antigen was coated on 96-well microtiter plates overnight at 4°C, followed by blocking with phosphate-buffered saline (**PBS**) containing 1.0% bovine serum albumin for 1 h and washed with PBS (pH 7.2) containing 0.05% Tween 20 (PBS-T). After blocking, the plates were incubated at room temperature for 1 h with 100 μL/well undiluted hybridoma culture supernatant and then washed 5 times with PBS-T. A recombinant chIL-7 was used as a negative control. Detection using horseradish peroxidase (**HRP**)–conjugated rabbit anti–mouse IgG secondary Ab (1/10,000 dilution), 3,3′,5,5′-tetramethylbenzidine (**TMB**) substrate, and H_2O_2 (all from Sigma-Aldrich) was carried out at room temperature. Optical density was measured at 450 nm (OD450) using a microplate reader (BioTek, VT). Five reacting mAb were selected for limiting dilution and further characterization, and the mAb from hybridoma supernatants were purified by protein G agarose chromatography, followed by immunoglobulin isotyping using a mouse mAb isotyping kit (Sigma-Aldrich, St Louis, CA).

Western Blot Analysis

Equal volumes of sample and sample buffer [0.125 mol Tris-HCl (pH 6.8), 4.0% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.004% bromophenol blue] were mixed and heated at 95°C for 5 min. For Western blot detection of chIL-13 using the anti–chIL-13 mAb, yeast- or *E. coli*–expressed recombinant chIL-13 (1 μg/lane) was resolved on a 15% SDS-PAGE gel followed by electroblotting onto a nitrocellulose membrane (Immobilon-P, Millipore, Bedford, MA). Similarly, the cross-reactivity of these mAb was determined using chIL-10 and chIFN-γ at a concentration of 1 μg/lane (all from Kingfisher Biotech, St. Paul, MN). Blocking of the membranes was performed using SuperBlock Blocking Buffer in PBS (Thermo Fisher Scientific, Waltham, MA) followed by washing with 1x PBS-T. The membranes were independently incubated with 5 anti–chIL-13 mAb (1.0 μg/mL). HRP-conjugated rabbit anti–mouse IgG secondary Ab (1/1,000 dilution) was used to determine the immunoreactivity, and the bands were detected using a Clarity Western ECL Substrate (Bio-Rad, Hercules, CA); the membranes were visualized using the ChemiDoc imaging system (Bio-Rad, Hercules, CA).

Pairing Assay

Antibody pairing assay was performed using the 5 clones indicated above as #1D6, #2A12, #9B11, #10A2, and #15F. HRP conjugation of all these clones was performed using HRP conjugation kit (Cat. No. ab102890, Abcam, Cambridge, UK) as per manufacturer's instructions. All non–HRP-conjugated clones were coated on a 96-well microtiter plate as a capture antibody (2 μg/mL) and incubated for 2 h at 37°C, followed by blocking with 1.0% bovine serum albumin in PBS for 1 h and washed with PBS-T. After blocking, yeast-expressed chIL-13 was added to each well (500 ng/mL), and the plates were incubated at 37°C for 2 h, followed by washing 5 times with PBS-T. Furthermore, HRP-conjugated mAb were added to the plate as a detection antibody (2 μg/mL) for 1 h at 37°C, in a way that each capture antibody–chIL-13 complex will be detected by 5 independent HRP-conjugated antibodies in the respective wells. Detection was carried out using TMB substrate and H_2O_2 (all from Sigma-Aldrich) at room temperature. Optical density was measured at 450 nm (OD450) using a microplate reader (BioTek, VT). Based on the pairing assay, the best capture assay pair, #9B11 as the capture antibody and #10A2-HRP as the detection antibody, was further optimized by titration assay using 4 concentrations at 0.25, 0.5, 1, and 2 μg/mL for capture and detection antibodies.

A Standard Curve Generation Using Yeast-Expressed chIL-13 Assay

Capture ELISA using anti–chIL-13 mAb, #9B11 as the capture antibody and #10A2 (HRP-conjugated) as the detection antibody, was developed in the present study. Based on the titration experiment, 96-well flat-bottomed microtiter plates were coated with 1 μg/mL of #9B11 antibody and sealed plates were incubated at 37°C for 2 h followed by blocking and washing as described earlier. Yeast-expressed chIL-13 was added in different concentrations (range, 0.125–64 ng/mL) directly to the respective wells and incubated for 2 h at 37°C, washed with PBS-T, and detection antibody #10A2 (2 mg/mL) was added for 1 h at 37°C. The plates were washed using PBS-T and developed with TMB substrate solution. Optical densities at 450 nm were measured, and a standard curve was generated.

Table 1. Oligonucleotides used in the study for quantitative real-time PCR.

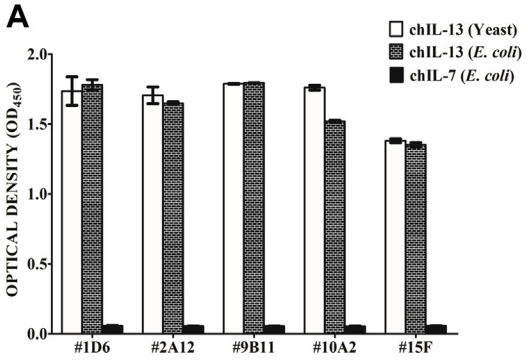
Gene	Forward (5′–3′)	Reverse (5′–3′)	Reference	Accession No.
β-actin	CACAGATCATGTTTGAGACCTT	CATCACAATACCAGTGGTACG	(Kim et al., 2017b)	NM_205518
chIL-13Rα1	GAAAAGGCCTGAGGACCATT	CACCCATCTCAAAGGATCCA	(Li et al., 2007)	XM_420218
chIL-13Rα2	AGCGGCCGCCTTTCGGGTCCCACTGC	CCCTCGAGGGCCAGGTTGTGAAGACG	(Miyoshi et al., 2007)	NM_001048078
TGF-β1	CGGGACGGATGAGAAGAAC	CGGCCCACGTAGTAAATGAT	This study	NM_001318456

Neutralization Assay

The neutralizing effect of newly developed anti–chIL-13 mAb was investigated using primary monocytes. This assay was based on nitric oxide (**NO**) assay as described previously (Chaudhari et al., 2018). Chicken primary monocytes were isolated as described previously (Arsenault et al., 2013). Briefly, peripheral blood mononuclear cells were isolated from healthy chickens by a Histopaque (Sigma-Aldrich) density gradient method and maintained in RPMI-1640 medium containing 10% fetal bovine serum and antibiotics (100 U penicillin/mL and 100 μg streptomycin/mL, 5 μg of gentamycin/mL). Peripheral blood mononuclear cells (3×10^7/mL) were seeded into 24-well plates and incubated at room temperature for 3 h followed by 3 times washing with media to remove nonadherent cells. The adhered monocytes were cultured for 18 h in the same media. The cells were washed twice with media and stimulated with 500 ng/mL yeast chIL-13, 1 μg/mL of LPS, chIL-13+LPS, chIL-13+LPS+#9B11, or chIL-13+LPS+#10A2 in RPMI media at 41°C for 24 h. The concentration of mAb was 10 μg/mL for neutralization assay as described earlier (Chaudhari et al., 2018). After the treatment, media were collected and the NO assay was performed using Griess reagent (Sigma-Aldrich) as described earlier (Chaudhari et al., 2018). A standard curve was generated using known concentrations of sodium nitrite (Sigma-Aldrich) in cell-free medium, and the amount of NO produced was measured. Expression of inducible nitric oxide synthase (**iNOS**) protein was investigated by Western blot analysis as previously described (Kim et al., 2017b). Briefly, the cells were lysed after treatment in lysis buffer [(25 mmol HEPES (pH 7.4), 100 mmol NaCl, 1 mmol EDTA, 5 mmol MgCl2, 0.1 mmol dithiothreitol] and protease inhibitor cocktail (Sigma, #P8340). Total protein (40 μg/mL) of each sample was run on 8–15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Rabbit anti-iNOS (Abcam, Cambridge, UK) and rabbit anti-β-actin antibodies (Cell Signaling Technology, Danvers, MA) were used for immunodetection. The band intensities were measured by laser scanning densitometry (Bio-Rad, Hercules, CA) and expressed as relative expression of iNOS compared with β-actin.

Neutralizing Effect on Expression of chIL-13Rα1, chIL-13rα2, and TGF-β1

In mammals, IL-13 signaling occurs via 2 receptors, IL-13 receptor alpha 1(IL-13Rα1) and IL-13Rα2 and IL-13 stimulation induce TGF-B1 production (Seyfizadeh et al., 2015). In chickens, the functional role of IL-13 is yet to be explored. Therefore, the present study investigated mRNA levels of c*chIL-13Rα1, chIL-13Rα2,* and *TGF*-β1using quantitative real-time PCR. The oligonucleotides used in the present study are described in Table 1. Primary monocytes were treated with 500 ng/mL of yeast chIL-13, chIL-13+#9B11, and chIL-13+#10A2 at 41°C for 24 h. The mAb concentration used was 10 μg/mL based on a previous study (Chaudhari et al., 2018). Nonstimulated cells were used as control cells. Total RNA was extracted by QIAcube using RNeasy Mini kit (Qiagen, Germany) followed



B

Clone No	Isotype	ELISA reactivity against IL-13 antigen	
		Yeast IL-13	*Ecoli* IL-13
1D6	IgG2b, k	✓	✓
2A12	IgG1, k	✓	✓
9B11	IgG1, k	✓	✓
10A2	IgG1, k	✓	✓
15F	IgG1, k	✓	✓

Figure 1. (A) Indirect ELSIA against recombinant yeast–expressed and *E. coli*–expressed chIL-13 protein. Specificity of mouse anti–chIL-13 mAb was determined against yeast- and *E coli*–expressed chIL-13 proteins by indirect ELISA. *E. coli*–expressed chIL-7 protein was used as a negative control. All 5 antibodies specifically recognized recombinant chIL-13 expressed in yeast and *E. coli* as compared to negative control chIL-7. (B) Isotype analysis revealed that 4 of 5 mAb (#2A12, #9B11, #10A2, and #15F) were of IgG1, κ chain, whereas #1D6 mAb was IgG2, κ.

by DNAse digestion and RNA quantification using a NanoDrop spectrophotometer at 260 nm/280 nm (GE Healthcare Life Sciences, Pittsburg, PA). cDNA synthesis was performed in a 20-µl reaction volume using an Applied Biosystems High Capacity cDNA Reverse Transcriptase Kit (Life Technologies, Grand Island, NY). The expression of the *chIL-13Rα1, chIL-13Rα2*, and *TGF*-β1 genes was quantified using RT^2 SYBR Green ROX qPCR Mastermix (Qiagen, Germany) per manufacturer's instructions. DNA was amplified in a Stratagene M x 3000P system (Agilent Technologies Inc., Santa Clara, CA) with PCR conditions as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. Melt curve analysis for each sample was performed by using MxPro software provided in the Stratagene M x 3000P system and was considered valid when a single peak was observed. The data obtained from 3 independent experiments were used to analyze the relative gene expression compared with nontreated samples by the $2^{-\Delta\Delta Ct}$ method.

Statistical Analyses

All data are expressed as the mean ± standard deviation (SD) unless otherwise specified. Statistical analyses were performed using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test using GraphPad Prism, version 5, software (GraphPad Software, Inc., La Jolla, CA). Statistical differences were considered when the *P*-values were ≤0.05 or ≤0.01.

RESULTS

Production of anti–chIL-13 mAb

Five clones, #1D6, #2A12, #9B11, #10A2, and #15F, producing anti–chIL-13 mAb were identified through their strong ELISA reactivity to recombinant yeast-expressed chIL-13 and *E. coli*-expressed chIL-13 (Figure 1). All mAb specifically detected chIL-13 and showed no cross-reactivity in the ELISA against the negative control chIL-7 antigen (Figure 1A). Isotype analysis showed that 4 mAb (#2A12, #9B11, #10A2, and #15F) were IgG_1, κ, whereas #1D6 was identified as IgG_{2a}, κ (Figure 1B). Recombinant *E. coli*-expressed chIL-13 showed an expected band of ∼13.9 kD on SDS-PAGE (Figure 2A). The recombinant *E. coli* chIL-13 was used for immunization of mice, and all 5 mAb recognized a specific band around ∼13.9 kD upon Western blot analysis (Figure 2B). The SDS-PAGE analysis of yeast chIL-13 showed that the antigen has post-translational modifications with 2 characteristic bands between 20 and 15 kD (Figure 2C), and all 5 mAb detected these 2 bands by Western blotting (Figure 2D). None of the mAb reacted with other cytokines from chickens such as chIL-10 or chIFN-γ (Figure 2D), suggesting the specificity of the antibodies to detect chIL-13.

Paring Assay

To develop an antigen-specific capture ELISA for chIL-13, a pairing assay was performed to determine which 2 mAb can be paired as capture and detection antibody for better detection of chIL-13. As shown in Figure 3A, when captured with #9B11 and detected with HRP-conjugated #10A2 antibodies, a maximum signal was obtained compared with all other combinations (indicated by a black solid arrow). In addition, better signals were obtained with the pairing of #10A2 and HRP-conjugated #9B11 compared with other combinations (Figure 3A). Thus, a vice versa pairing with 2 mAb (#9B11 and #10A2) showed maximum signal and the combination of #9B11 as a capturing antibody and #10A2-HRP as a detecting antibody showed the best sensitivity (Figure 3A). Furthermore, we performed a titration experiment to determine the optimum concentrations of these 2 mAb to develop a capture assay. Titration with different concentrations of capture (#9B11) and detection (#10A2-HRP) antibodies showed that both the antibodies indicated a high signal when used at a concentration as 2 µg/mL (Figure 3B). However, capture with #9B11 at 1 µg/mL and detection by #10A2-HRP at 2 µg/mL yielded the optimum signal (as indicated by a dotted black arrow in Figure 3B). Lower concentrations of both the antibodies (0.5 and 0.25 µg/mL) yielded a low signal (Figure 3B).

Detection of Sensitivity of Capture ELISA by Standard Curve Generation Using Yeast chIL-13

A capture assay using #9B11 as a capture antibody and #10A2-HRP as a detection antibody was performed to detect yeast-expressed chIL-13. The results are presented in Figure 4. A standard curve was generated using different known concentrations of chIL-13 (0.125-64 ng/mL), and the result indicated that the lowest chIL-13 concentration detected by capture assay was 125 pg/mL (Figure 4A and B). The linear standard curve was generated for determining the chIL-13 concentration (Figure 4B). Our results indicate that the capture ELISA developed in the present study could detect endogenous yeast chIL-13 production with a sensitivity of 125 pg/mL.

Neutralizing Effect of Anti–chIL-13 mAb

The neutralization assay was based on a previously published work in which a Th2 cytokine (chIL-4) inhibited LPS-induced NO production (He et al., 2011; Chaudhari et al., 2018). We hypothesized that chIL-13 may have a similar effect on LPS-induced NO production, and the 2 mAbs (#9B11 and #10A2) may reverse this effect. The results are presented in Figure 5. As expected, LPS induced significantly higher levels of NO in primary monocytes, and treatment with chIL-13+LPS showed a significant decrease in NO levels

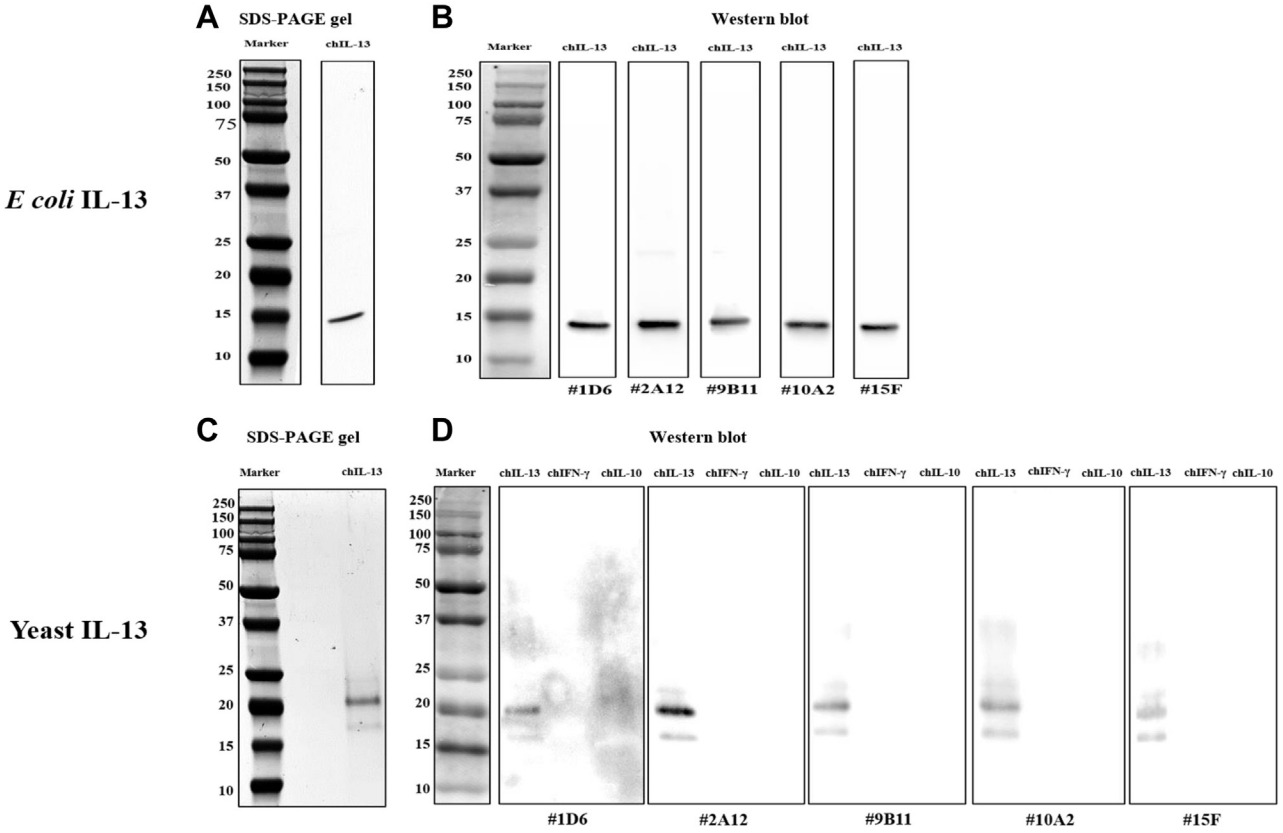


Figure 2. SDS-PAGE and Western blot analysis of recombinant yeast and *E. coli* chIL-13. (A, B) SDS-PAGE of recombinant *E. coli*–expressed chIL-13 showed a specific band at ∼13.9 kD, and all 5 anti–chIL-13 monoclonal antibodies (#1D6, #2A12, #9B11, #10A2 and #15F) specifically detected the band. (C, D) Two specific bands between ∼20 and 15 kD were observed for yeast-expressed chIL-13 protein upon SDS-PAGE analysis, and these bands were specifically detected by all 5 anti–chIL-13 mAb. The cross-reactivity of the mAb with chIFN-γ and chIL-10 was also performed, and all 5 mAb did not show any cross-reactivity. Molecular weight (kD) marker is presented on the left.

(P value = 0.001, Figure 5A). On the contrary, both the mAb neutralized the chIL-13 effect on the inhibition of LPS-induced NO production and showed significantly higher NO levels (P value = 0.001, Figure 5A). Similarly, iNOS protein was highly induced in LPS-stimulated primary monocytes and the iNOS expression in chIL-13+LPS-stimulated cells was observed to be significantly lower (Figure 5B). Both mAb reversed this effect, and iNOS expression was significantly higher in primary monocytes (Figure 5B).

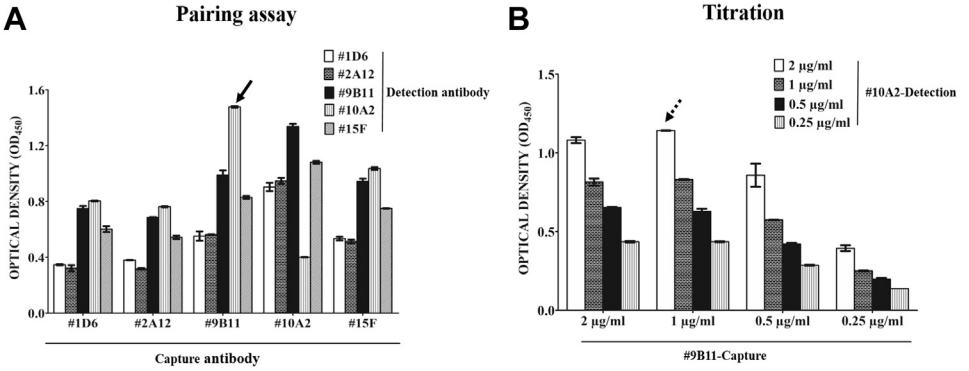


Figure 3. Antibody pairing capture assay and titration assay. (A) All 5 anti–chIL-13 monoclonal antibody (mAb) clones were horseradish peroxidase (HRP)-conjugated and used to determine the best antibody pairing combination with the highest signal in a capture ELISA assay. All the clones were coated as the capture antibody on the plate, followed by addition of 500 ng/mL of chIL-13. Then, all 5 HRP-conjugated mAb were added as a detection antibody. Pairing between same clones (i.e., #1D6 as the capture antibody and #1D6-HRP as the detection antibody) was considered as negative controls. The strongest signal was observed for antibody pairing, #9B11 as the capture and #10A2-HRP as the detection antibody, indicated by black solid arrow. (B) Titration assay for the capture assay pair, #9B11 as the capture and #10A2-HRP as the detection antibody, was performed using 4 different concentrations (0.25, 0.5, 1, and 2 µg/mL) of capture as well as detection antibodies. The dotted black arrow indicates the optimal concentrations. Data represent 2 independent experiments.

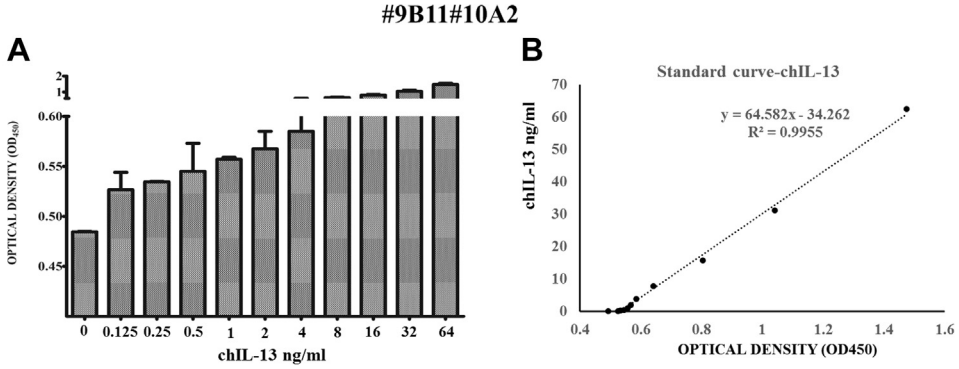


Figure 4. A standard curve using yeast-expressed chIL-13 using capture ELISA. (A) A capture assay with #9B11 as the capture antibody and #10A2-horseradish peroxidase (HRP) as the detection antibody was developed to generate the standard curve using different concentrations of recombinant yeast–expressed chIL-13 (range, 0.125–64 ng/mL), and the optical density (OD) values are presented for each concentration of yeast-expressed chIL-13. (B) The standard curve was generated with the known chIL-13 concentrations. Data represent 3 independent experiments and mean ± SD and $^{**}P < 0.01$ as compared to respective control sample.

Neutralizing Effect of mAb on chIL-13–induced Gene Expression

We investigated the gene expression levels of 2 chIL-13 receptors (*chIL-13Rα1* and *chIL-13Rα2*) and *TGF-β1* in chIL-13 stimulated primary monocytes and further investigated whether mAb could neutralize the chIL-13 effect. There was no change in the gene expression of the *chIL-13Rα1* in chIL-13–stimulated cells, whereas the expression of the *chIL-13Rα2* and *TGF-β1* genes was significantly upregulated in chIL-13–stimulated monocytes compared with nonstimulated cells (Figure 6A–C). This stimulatory effect on expression levels of *chIL-13Rα2* and *TGF-β1* was significantly reversed when cells were incubated with chIL-13+#9B11 and chIL-13+#10A2 (Figure 6B and C).

DISCUSSION

In the present study, anti–chIL-13 mAb were developed to specifically detect recombinant chIL-13. These antibodies were specific to chIL-13 and did not show any cross-reactivity with other cytokines in chickens. To our knowledge, the present study is the first to report the applicability of anti–chIL-13 mAb, and our results indicate that 5 mAb specifically detected recombinant chIL-13 expressed by *E. coli*, as well as from yeast, by indirect ELISA and Western blotting. Although *E. coli*-expressed chIL-13 was used for immunization to generate the mAb, all the 5 mAb also detected yeast-expressed chIL-13 specifically. Our results demonstrated that yeast-expressed chIL-13 may have post-translational modifications indicated by the presence of

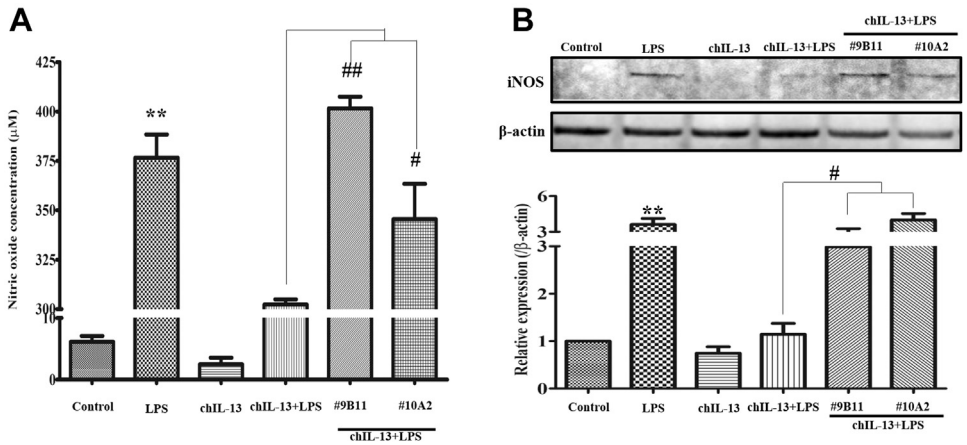


Figure 5. Neutralization of chIL-13 by #9B11 and #10A2 mAb in primary chicken monocytes. (A) Neutralization of chIL-13 by #9B11 and #10A2 was based on the hypothesis that the 2 mAb will reverse the chIL-13 inhibition of lipopolysaccharide (LPS)-induced nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression in primary chicken monocytes. The cells were treated for 24 h with respective treatments. The 2 mAb neutralized the chIL-13 inhibition of LPS-induced NO production and showed elevated NO production. (B) Western blot analysis of iNOS protein expression showed that LPS induced robust iNOS expression which was inhibited by coincubation with chIL-13. Both the mAb reversed this effect which showed detection of iNOS bands. The densitometric analysis for each band was performed. Data represent 3 independent experiments and the mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared to nontreated sample, whereas $\#P < 0.05$ and $\#\#P < 0.01$ as compared to chIL-13+LPS samples.

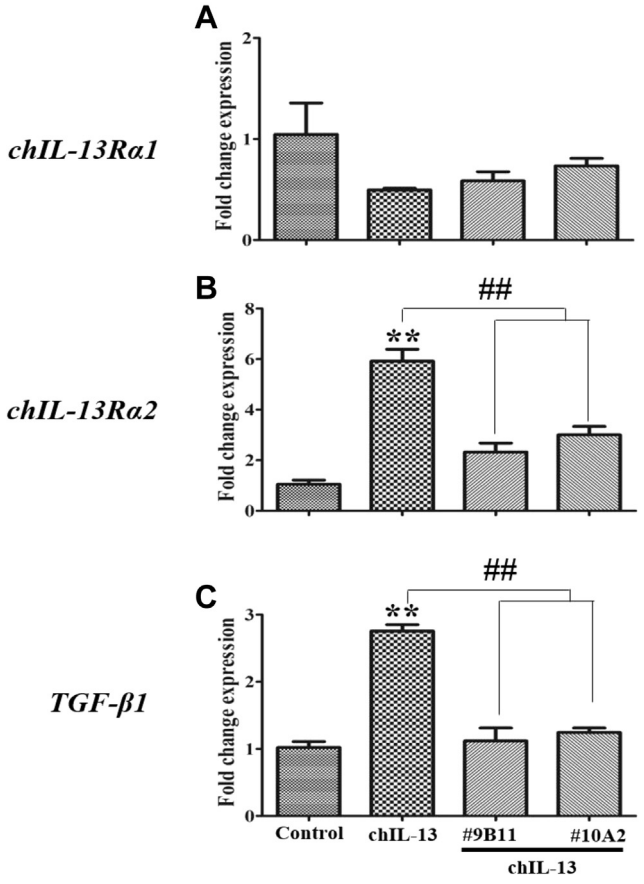


Figure 6. Neutralizing effect on expression of *chIL-13Rα1, chIL-13Rα2, and TGF-β1* in primary monocytes. The expression *chIL-13Rα1, chIL-13Rα2, and TGF-β1* in chIL-13–stimulated primary monocytes was investigated, and the neutralizing effect of 2 mAb, #9B11 and #10A2, was examined. The cells were stimulated with yeast-expressed chIL-13 (500 ng/mL), chIL-13+#9B11, and chIL-13+#10A2 at 41°C for 24 h. Nonstimulated cells were indicated as control cells. Gene expressions of (A) *chIL-13Rα1*, (B) *chIL-13Rα2*, and (C) *TGF-β1* were investigated. Values are expressed as fold change expression compared with nontreated cells. All data are presented as mean ± SD of the results determined using at least 3 biological replicates $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared to nontreated sample, whereas $\#P < 0.05$ and $\#\#P < 0.01$ as compared to chIL-13–stimulated samples.

2 bands between ∼15-20 kD and that all 5 mAb specifically detected these bands upon blot analysis. These results suggest that the mAb developed in the present study could be used to detect endogenously produced chIL-13. Pairing assay analysis showed that #9B11 as a capture antibody and #10A2-HRP as a detection antibody detected the highest signal compared with any other combination. Therefore, the capture ELISA was developed using this pair to determine chIL-13 specifically and the standard curve analysis showed that the sensitivity of the capture assay was as low as 125 pg/mL.

It was previously reported that infection with *Eimeria tenella* parasites induces significantly high levels of mRNA transcripts of chIL-13 in intestinal lymphocytes until 10 D after primary infection and the expression remains elevated after the secondary infection when compared with the noninfected control chickens (Hong et al., 2006a). These findings suggest the role of chIL-13 in Th2-mediated immune responses in chickens in response to the coccidian infection. As an integral part of the Th2-mediated response, IL-13 plays an important role in mammalian helminthic parasitic infections possibly by increasing the expulsion of the parasite (Bao and Reinhardt, 2015; McCormick and Heller, 2015). Therefore, it will be interesting to design future experiments to detect circulating chIL-13 in *Eimeria*-infected chicken serum using the capture ELISA developed in the present study. In addition, IL-13 stimulation has been shown to induce secretion of TGF-β in pancreatic cells or in colitis models in mammals (Fichtner-Feigl et al., 2006, 2008; Fichtner-feigl et al., 2007; Shimamura et al., 2010; Giuffrida et al., 2019). It also warrants further investigation that similar to mammals, chIL-13 regulates the expression of TGF-β1 in chickens. At present, our results clearly indicated that the sensitivity of the capture assay in the present study could be increased and may require further optimization. Nevertheless, our results report that the capture assay using anti–chIL-13 mAb can specifically detect endogenously produced yeast chIL-13.

Furthermore, anti–chIL13 mAb were investigated for their neutralizing effect. The neutralization assay was based on the hypothesis that IL-4, a Th2 cytokine with homology to IL-13, inhibits LPS-induced NO production in chicken monocytes (Chaudhari et al., 2018). It was reported that chIL-4 regulates alternative activation of macrophages in chickens by inhibiting LPS-induced NO production and thus overriding the LPS functionality (Chaudhari 2018). In mammals, IL-4 and IL-13 share similar biological functions. However, the literature also suggests that they have discreet mechanisms and there are conflicting reports on IL-13 inhibition of the LPS-induced NO pathway (El Gayar et al., 2003; Sinha et al., 2005; Authier et al., 2008). In chickens, recombinant chIL-4 and chIL-13 have been shown to induce proliferation of chicken B cells, indicating a possible functional redundancy between these 2 molecules (Avery et al., 2004). Our results showed that chIL-13 inhibited the NO production by LPS, when the primary chicken monocytes were coincubated with chIL-13+LPS with abrogation of iNOS protein expression and that the mAb reversed this effect. Monocytes/macrophages play an indispensable role in innate immunity and are critical in terms of responding to various stimuli such as microbial proteins, necrotic cells, and so forth and thus play an important role in immunomodulation to maintain tissue integrity, organ development, and tissue regeneration (Pollard, 2009; Luzina et al., 2012; Osborn and Olefsky, 2012; Martinez and Gordon, 2014; Roszer, 2015). Activation of macrophages is categorized as M1 and M2 polarization (Sica and Mantovani, 2012; Novak and Koh, 2013; Martinez and Gordon, 2014). Lipopolysaccharide-stimulated macrophages are the M1 phenotype, whereas M2 or alternatively activated macrophages are produced in response to Th2 cytokines such as IL-4 and IL-13 in mammals (Ma et al., 2003; Gordon and Martinez, 2010; Luzina et al., 2012; Roszer, 2015). As opposed to M1 macrophages, M2 macrophages serve regulatory

functions because of their anti-inflammatory properties (Pollard, 2009; Murray et al., 2014). That is why LPS-induced NO production is inhibited by IL-4 and IL-13, as these cytokines cause M2 polarization in macrophages in mammals (Pollard, 2009; Murray et al., 2014). More recently, M2 polarization of chicken macrophages by chIL-4 has been reported (He et al., 2011; Chaudhari et al., 2018) and, owing to the functional redundancy, chIL-13 may have similar effects on chicken monocytes/macrophages. The results of the present study partly support this notion by demonstrating inhibition of LPS-induced NO production by chIL-13 and neutralizing this effect by anti–chIL-13 mAb. These results indicate the possibility of the alternative activation of macrophages by another Th2 cytokine in chickens, although this requires further investigation.

To further investigate the neutralization effect of anti–chIL-13 mAb, we investigated the expression of chIL-13 receptors (*chIL-13Rα1* and *chIL-13Rα2*) and *TGF-β1* in chIL-13–stimulated primary monocytes. Our results demonstrated that chIL-13 significantly induced the expression of *chIL-13Rα2* and *TGF-β1* in monocytes, whereas *chIL-13Rα1* expression remained unchanged. The upregulation of *chIL-13Rα2* and *TGF-β1* was reversed when the mAb were incubated together with chIL-13. In mammals, IL-13 signaling is mediated through 2 receptors, the type II IL-13Rα1/IL-4Rα complex and IL-13Rα2 receptors. Mammalian IL-13 has a stronger affinity for IL-13Rα2 than IL-13Rα1. The IL-13Rα2 is a so-called decoy receptor which has been shown to be important for IL-13–mediated signaling in intestinal diseases (Giuffrida et al., 2019). Research findings now suggest that IL-13Rα2 has more than a decoy activity and leads to fibrosis in a colitis model by producing higher secretions of TGF-β1 (Fichtner-feigl et al., 2007; Giuffrida et al., 2019). However, all these findings are reported in mammals and there is no such information on chIL-13 signaling through either of its receptors in chickens. In addition, it is unknown whether chIL-13 has a regulatory role in the expression of IL-13Rα1 or IL-13Rα2 in chickens. The results of the present study shed some light on the receptor expressions upon chIL-13 stimulation. As in mammals, our results too showed that chIL-13 stimulation increases *chIL-13Rα2* and *TGF-β1* production, which is suggestive of a possible mechanism involving chIL-13 signaling through the chIL-13Rα2 that eventually induces TGF-β1 expression. Interestingly, the expression of *chIL-13Rα1* was not observed upon chIL-13 stimulation of monocytes. This phenomenon is interesting and requires further investigation. Without any more information, it could be an overstatement that chIL-13 signaling is mainly mediated through chIL-13Rα2 and TGF-β1 signaling. The results of this study provide new information on chIL-13 and its receptors and suggest that *TGF-β1* overexpression could be attributed to upregulated *chIL-13rα2* expression in chicken monocytes stimulated with chIL-13. Most importantly, our results showed the neutralizing effect on chIL-13 functionality.

To summarize, the present study describes new anti–chIL-13 mAb and a capture assay for the detection of chIL-13. Our results also suggest that similar to chIL-4, chIL-13 may also be involved in alternative activation of primary monocytes in chickens. More importantly, the results presented here suggest that chIL-13 signaling may be regulated through chIL-13Rα2 and TGF-β1 secretions, based on the findings that anti–chIL-13 mAb neutralized the chIL-13–induced expression of both. These newly developed mAb will serve as valuable immune reagents for future studies on the biological activity of chIL-13 and its receptors in chickens.

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