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Research paper

Development and characterization of mouse monoclonal antibodies reactive with chicken interleukin-2 receptor αlpha chain (CD25)

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ABSTRACT

This study was carried out to develop and characterize mouse monoclonal antibodies (mAbs) against chicken CD25 (*ch*CD25), the alpha chain of the interleukin-2 (IL-2) receptor. A recombinant chimeric *ch*CD25/IgG4 fusion protein was expressed in Chinese hamster ovary (CHO) cells and isolated from spent cell culture medium by protein G affinity chromatography. Purified *ch*CD25 protein was used to immunize mice, from which 54 stable hybridomas secreting *ch*CD25 mAbs were produced. Two mAbs, *ch*CD25-32 and *ch*CD25-54, with high binding affinity for *ch*CD25-expressing CHO cells were selected for further characterization. By flow cytometry, both mAbs detected cells in the spleen, bursa of Fabricius, intestinal duodenum, and immunostained established chicken T cell, B cell, and macrophage cell lines. Both mAbs reacted with a 55 kDa protein on Western blots of lysates from concanavalin A (Con A)-stimulated spleen mononuclear cells. Intraperitoneal injection of chickens with bacterial lipopolysaccharide increased the percentage of *ch*CD25$^+$ spleen cells by approximately 4-fold compared with untreated animals. *In vitro* stimulation of spleen cells with Con A increased the percentage of *ch*CD25$^+$ cells by up to 50-fold compared with cells treated with medium alone. Finally, the *ch*CD25-32 mAb suppressed IL-2-driven spleen cell proliferation and reduced IL-2-induced nitric oxide production. These mAbs may be useful for future investigation of chicken regulatory T cells.

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1. Introduction

Interleukin-2 (IL-2) is a 15.5 kDa polypeptide that is composed of 133 amino acids arranged in a short-chain, four helical bundle topology (Bazan, 1990; Morgan et al., 1976; Smith, 1980). IL-2 is synthesized and secreted mainly by activated T lymphocytes and plays a pivotal role in the growth and differentiation of T and B lymphocytes, monocytes, and natural killer cells (Rubin, 1995). IL-2

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exerts its effects following binding to the high affinity, heterotrimeric cell surface IL-2 receptor (IL-2R), consisting of a non-covalent complex between the IL-2Rα (CD25), IL-2Rβ (CD122), and IL-2Rγ (CD132) polypeptide chains (Sharon et al., 1986; Tsudo et al., 1986; Waldmann, 1989; Smith, 1988). The α and β chains are involved in binding IL-2, while signal transduction through the mitogen activated protein (MAP) kinase, phosphoinositide 3-kinase (PI3K), and JAK-STAT pathways is carried out by the β and γ chains.

IL-2Rα is a transmembrane glycoprotein containing N- and O-linked glycosyl units with an apparent molecular weight of 55 kDa (Leonard et al., 1984). IL-2Rα can bind to IL-2Rβ prior to interaction with IL-2. IL-2R γ alone has a weak binding affinity for IL-2, but after IL-2 is bound to the α/β heterodimer, the γ chain becomes recruited to the α and β chains to form a very stable macromolecular complex (Minami et al., 1993; Kishimoto et al., 1994; Waldmann et al., 1992). IL-2Rβ and IL-2Rγ additionally form complexes for receptors for IL-3, -4, -5, -6, -7, -9, -11, -13, and -15, as well as for granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor, growth hormone, and erythropoietin (Kishimoto et al., 1994). Given the important role of T cell activation in a variety of pathological conditions, such as acute graft rejection, the IL-2R complex is a popular target for preparing neutralizing monoclonal antibodies (mAbs) for therapeutic applications in human clinical medicine (Bingyi et al., 2003).

The characteristic and the function of CD25 protein in chicken have been reported by Teng et al. (2006) and Gu et al. (2010a,b). The identities for nucleic and amino acid sequences were 24.2%, and 15.6% between chicken and human CD25, and 28.9% and 19.3% between chicken and mouse CD25 (Teng et al., 2006). The residues ^{35}proline and ^{41}cysteine of chicken IL-2 are critical for binding to chicken CD25 (Gu et al., 2010a,b). However, compared with mammalian IL-2R subunits, much less is known about the structure, expression, and function of the chicken IL-2R. Therefore, the current study was conducted to prepare and characterize mAbs against chicken CD25 (*ch*CD25).

2. Materials and methods

2.1. Expression and purification of recombinant chCD25

A full-length cDNA encoding *ch*CD25 (GenBank accession No. AF143806) was cloned in the pcDNA/IGHG4 expression vector as described (Wagner et al., 2008). Chinese hamster ovary (CHO) cells were stably transfected with the *ch*CD25/IgG4 recombinant plasmid as described (Wagner et al., 2005) and the chimeric *ch*CD25/IgG4 protein was purified from the serum-free cell culture medium by protein G affinity chromatography. The recombinant *ch*CD25 polypeptide was separated from IgG4 and eluted from the column by *in situ* enterokinase digestion (EnterokinaseMax™, Invitrogen, Carlsbad, CA). Protein concentration of purified *ch*CD25 was determined by the Bradford assay (Lee et al., 2011). Final protein purity was confirmed by SDS-PAGE.

2.2. Hybridoma production

BALB/c mice (Taconic Laboratories, Germantown, NY) were immunized by intraperitoneal injection of 50 μg of purified *ch*CD25 protein combined with Gerbu adjuvant (Acurrate Chemical, Westbury, NY) and boosted with 25 μg of *ch*CD25/adjuvant at days 14 and 21 post-primary immunization, followed by 25 μg of *ch*CD25 alone on days 28, 29, and 30. Mice were euthanized and spleen lymphocytes were fused with non-secreting mouse myeloma X63-Ag8.653 cells at day 3 post-immunization. Hybridomas were selected in RPMI 1640 medium supplemented with hypoxanthine–aminopterin–thymidine (Sigma, St. Louis, MO) and cell culture supernatants were screened for mAbs binding to *ch*CD25/IgG4-transfected CHO cells by flow cytometry as described (Lee et al., 2011).

2.3. Flow cytometry

One-day-old broiler chickens (Ross/Ross, Longenecker's Hatchery, Elizabethtown, PA) were housed in wire cages and provided with feed and water *ad libitum*. At 3 weeks of age, spleens and bursa of Fabricus were obtained and single cell suspensions were prepared as described (Lee et al., 2011). All animal protocols were approved by the Beltsville Area Institutional Animal Care and Use Committee. In addition, the following cell lines were analyzed: CHO cells stably transfected with *ch*CD25/IgG4; CHO cells stably transfected with a chicken CD80/IgG4 fusion protein (*ch*CD80/IgG4); HD11, an avian myelocytomatosis virus (MC29)-transformed macrophage cell line (Klasing and Peng, 1987); HTC, an avian leukosis virus-transformed macrophage cell line (Hong et al., 2006); UD35, a RB1B strain Mareck's disease virus (MDV)-transformed T cell line (Parcells et al., 1999); UA53, a TK strain MDV-transformed T cell line (Santin et al., 2006); and CU60, a reticuloendothelial virus-transformed B cell line (Keller, 1992). Single cell suspensions were fixed for 20 min at room temperature in 2.0% formaldehyde, washed twice with PBS, pH 7.2, and resuspended in 1.0 ml of PBS supplemented with 0.5% bovine serum albumin, 0.5% saponin, and 0.02% NaN_3 (Sigma). The cells (1.0×10^6 in 100 μl) were incubated on ice for 45 min with 100 μl of appropriately diluted *ch*CD25-32 or *ch*CD25-54 mAb. HB2, an anti-human T cell mAb was used as a negative control (Lee et al., 2011). The cells were washed twice with 2.0 ml of saponin buffer, incubated on ice for 30 min with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG secondary Ab (Sigma), washed twice, resuspended in 1.0 ml, and analyzed with an FACSCalibur flow cytometer (BD, Franklin Lakes, NJ). Data was obtained from a total of 1.0×10^4 viable cells.

2.4. Western blot

Freshly prepared spleen mononuclear cells (1.0×10^7 cells/ml) from 3-week-old chickens were incubated for 24 h at 41 °C with 1.0 ml of 5.0 μg/ml of concanavalin A (Con A) (Sigma), the cells were washed, and disrupted using glass beads as described (Lee et al., 2011). Cellular protein (5.0 μg) was mixed with an equal volume of 0.125 M Tris–HCl, pH 6.8, 4.0% SDS, 20% glycerol, 10%

2-mercaptoethanol, and 0.004% bromophenol blue, heated for 5 min at 100 °C, and resolved by SDS-polyacrylamide gel electrophoresis. Separated proteins were transferred to PVDF membrane (Millipore, Bedford, MA) (Towbin et al., 1979) and the membrane was blocked with Superblock T20 (PBS) blocking buffer (Thermo Fisher Scientific, Rockford, IL), incubated overnight at 4 °C with *ch*CD25-32 or *ch*CD25-54 mAb, and bound Ab was detected with horseradish peroxidase-conjugated rabbit anti-mouse IgG secondary Ab (1:1000) plus 4-chloro-1-naphthol substrate (Sigma).

2.5. Indirect immunofluorescence

Spleen, bursa of Fabricius, and intestinal duodenum tissues from 3-week-old broiler chickens were snap frozen in liquid nitrogen. Cryostat sections on glass slides were blocked for 10 min with 10% normal horse serum and incubated for 18 h at 4 °C with *ch*CD25-32 mAb (1:200), followed by 30 min with FITC-conjugated rabbit anti-mouse IgG secondary Ab (Lee et al., 2011). Tissue sections were observed with an Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan). As negative controls, the procedure was repeated in the absence of primary mAb.

2.6. LPS-induced chCD25 expression in vivo

Four-week-old chickens ($n = 5$) were untreated or were intraperitoneally injected with 1.0 mg/kg of LPS (*Escherichia coli* O111:B4, Sigma). At day 4 post-treatment, spleens were removed and cell suspensions were prepared by gently passing through a cell strainer followed by centrifugation at 2000 rpm for 20 min at room temperature in Histopaque-1077 (Sigma) as described (Lee et al., 2009). Spleen mononuclear cells (1.0×10^7 cells/ml) were analyzed by flow cytometry for *ch*CD25$^+$ cells as described above.

2.7. IL-2-induced spleen cell proliferation and nitric oxide production

Freshly prepared spleen mononuclear cell suspension (2.0×10^6 cells/ml) in RPMI 1640 medium containing 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma) were incubated for 48 h at 41 °C with 2.0 μg/ml of Con A. The cells were treated for 15 min at 41 °C with 0.05 M α-methyl mannoside (Sigma) to remove Con A, washed, centrifuged on Histopaque-1077, and 100 μl/well (2.0×10^5 cells) dispensed into 96-well plates containing 50 μl/well of *ch*CD25-32 or *ch*CD25-54

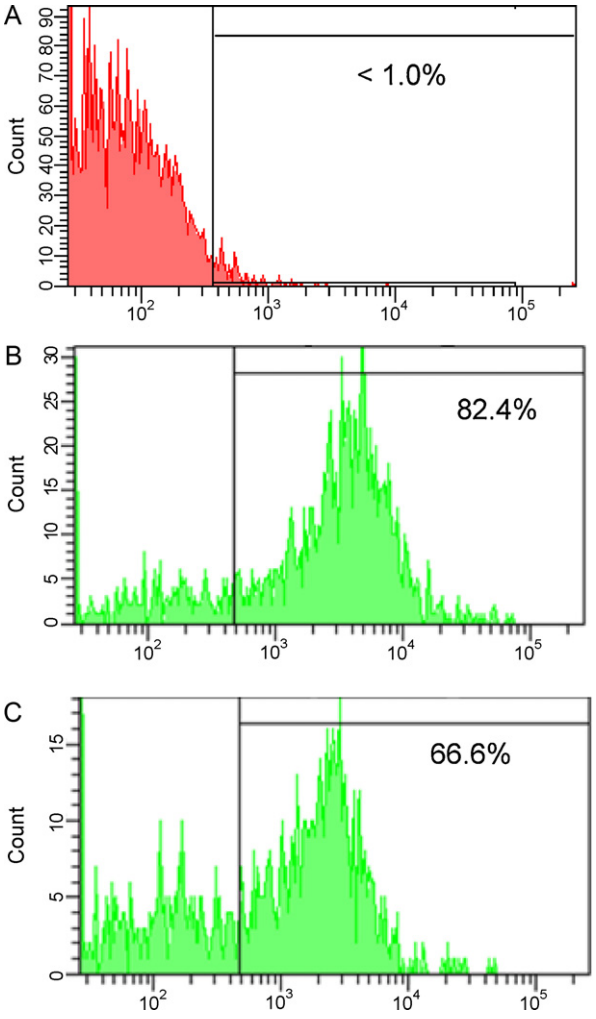

Fig. 1. Flow cytometric analysis of *ch*CD25 mAbs using CHO-*ch*CD25 cells. CHO cells stably transfected with *ch*CD25/IgG4 (1.0×10^6) were fixed in 2.0% formaldehyde for 20 min at room temperature and washed with PBS. Cell suspensions (100 μl, 1.0×10^6 cells) were incubated for 45 min on ice with HB2 mAb (negative control, A) or with *ch*CD25-32 (B) or *ch*CD25-54 (C) mAbs. The cells were washed, incubated with FITC-conjugated rabbit anti-mouse IgG secondary Ab for 30 min on ice, and analyzed by flow cytometry. Data was obtained from a total of 1.0×10^4 viable cells. The percentage of positively staining cells above background levels indicated by the vertical lines are listed in each panel.

Table 1
Flow cytometric analysis of *ch*CD25 mAbs.

mAb	CHO-*ch*CD25/IgG4[a]	CHO-*ch*CD80/IgG4	Spleen[b]	Bursa	HD11
*ch*CD25-32	82.8 ± 2.0[c,d]	2.5 ± 1.8	5.9 ± 0.3	2.7 ± 0.4	66.3 ± 4.1
*ch*CD25-54	63.6 ± 4.2	2.2 ± 1.4	3.5 ± 0.2	1.5 ± 0.4	35.2 ± 3.2

[a] CHO cells were stably transfected with *ch*CD25/IgG4 or *ch*CD80/IgG4.
[b] Single cell suspensions of spleen or bursa of Fabricius, or the HD11 macrophage cell line, were prepared.
[c] All cells (1.0×10^6) were fixed in 2.0% formaldehyde for 20 min at room temperature and washed with PBS. Cell suspensions (100 μl, 1.0×10^6 cells) were incubated for 45 min on ice with *ch*CD25-32 or *ch*CD25-54 mAbs, washed, incubated for 30 min on ice with FITC-conjugated rabbit anti-mouse IgG secondary Ab, and analyzed by flow cytometry.
[d] Data was obtained from a total of 1.0×10^4 viable cells. Each value represents the mean ± S.D. value ($n = 3$).

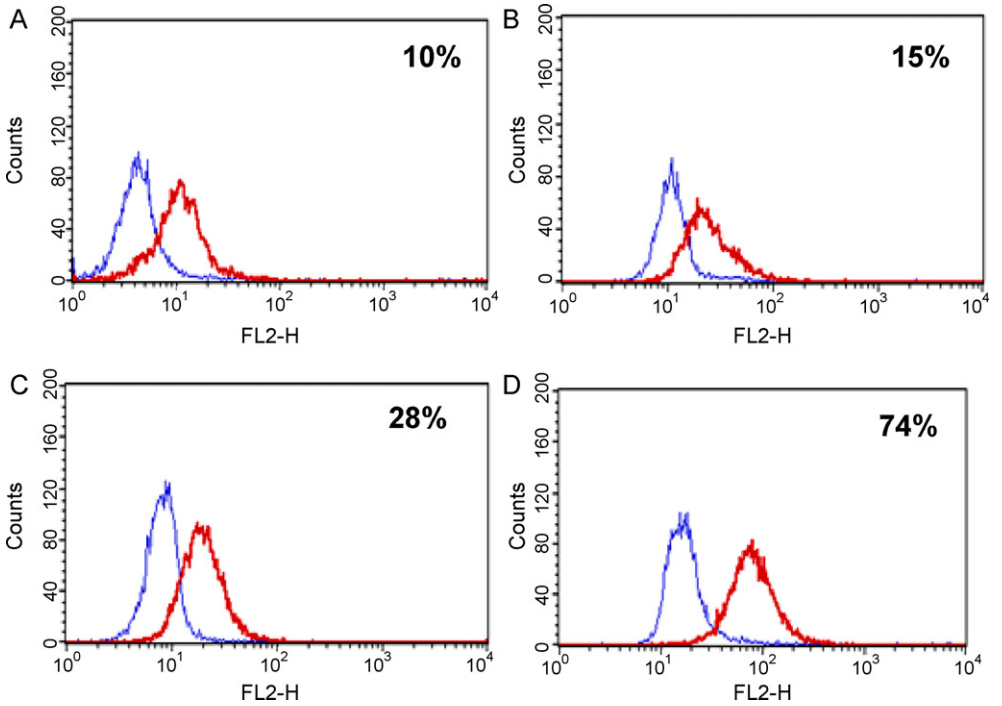

Fig. 2. Flow cytometric analysis of *ch*CD25 mAbs using established lymphoid cell lines. Single cell suspensions of UD35 (A), UA53 (B), CU60 (C), and HTC (D) cells were fixed in 2.0% formaldehyde for 20 min at room temperature and washed with PBS. Cell suspensions (100 μl, 1.0×10^6 cells) were incubated for 45 min on ice with *ch*CD25-32 mAb. The cells were washed, incubated for 30 min on ice with FITC-conjugated rabbit anti-mouse IgG secondary Ab, and analyzed by flow cytometry. Data was obtained from a total of 1.0×10^4 viable cells. The percentage of positively staining cells above background levels are listed in each panel.

mAb serially diluted from 1:10 to 1:320. As a negative control, the cells were incubated with HB2 mAb. The cells were incubated for 2 h at 41 °C in a humidified incubator, 50 μl/well of recombinant chicken IL-2 (Miyamoto et al., 2001) were added, the cells were incubated for 48 or 72 h, and proliferation was measured using WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, Cell Counting Kit-8, Dojindo Molecular Technologies, Gaithersburg, MD) by measuring optical density (OD) at 450 nm using a microplate spectrophotometer (BioRad, Hercules, CA) as described (Lee et al., 2011). In other experiments, nitric oxide (NO) levels in the mAb-treated, IL-2-stimulated cell culture media were determined using the Griess reagent (Sigma) as described (Lee et al., 2009).

2.8. Statistical analysis

Statistical analyses were performed using SPSS 12.0 software for Windows. All data were expressed as means ± S.D. values. Differences between mean values were compared using the Student's *t* test and were considered statistically significant at $P < 0.05$.

3. Results

3.1. Production of chCD25 mAbs

Fifty-four hybridomas were isolated using mice immunized with purified recombinant *ch*CD25 protein. Among these hybridoma clones, the clones designated *ch*CD25-32 and *ch*CD25-54 secreted mAbs detecting 82.4% and 66.6%, respectively, of CHO cells stably transfected with *ch*CD25/IgG4 by flow cytometry (Fig. 1). An irrelevant mAb against human T cells (HB2) stained less than 1% of these cells. Therefore, these 2 hybridomas were cloned by limiting dilution and their mAbs secreted into the

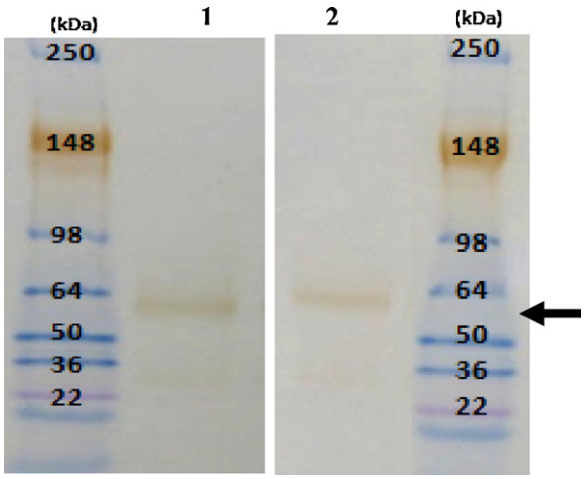

Fig. 3. Western blot analysis of *ch*CD25. Lysates from Con A-stimulated spleen cells were resolved by SDS-PAGE and analyzed by Western blotting using *ch*CD25-32 (lane 1) and *ch*CD25-54 (lane 2) mAbs. Protein molecular weight markers are shown on the left or the right. The arrow indicates the 55 kDa *ch*CD25 protein.

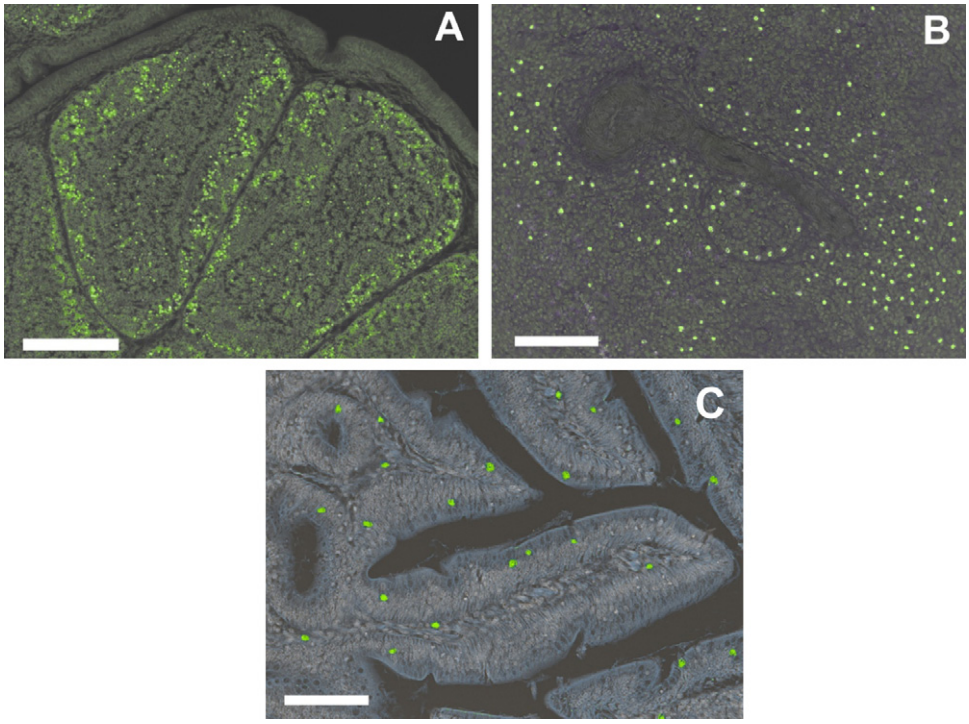

Fig. 4. Immunolocalization of *ch*CD25 in tissues. Bursa of Fabricius (A), spleen (B), and intestinal duodenum (C) were frozen in liquid nitrogen and tissue sections were stained with *ch*CD25-32 mAb plus FITC-labeled rabbit anti-mouse IgG secondary Ab. Scale bars represent 200 μm (A), 150 μm (B) or 100 μm (C).

cell culture media were used for all subsequent analyses. Characterization of the remaining 52 hybridomas is currently ongoing in our laboratory. As shown in Table 1, the *ch*CD25-32 and *ch*CD25-54 mAbs retained their binding activity for CHO-*ch*CD25/IgG4 cells following re-cloning of their hybridomas, but were non-reactive with CHO cells transfected with an irrelevant gene, *ch*CD80/IgG4, encoding the chicken CD80 cell surface protein/IgG4 fusion protein. Further, these mAbs stained 66.3% and 35.2% of HD11 chicken macrophages, 5.9% and 3.5% of spleen mononuclear cells, and 2.7% and 1.5% of bursa-derived lymphocytes, respectively. *Ch*CD25-32 mAb, that detected the highest percentage of CHO-*ch*CD25/IgG4 cells, stained 10% and 15% of MDV-transformed T cell lines, UD35 and US53 respectively, 28% of a reticuloendothelial virus-transformed B cell line, CU60, and 74% of the avian leukosis virus-transformed macrophage cell line, HTC (Fig. 2).

3.2. Western blot analysis of chCD25

Western blot analysis of lysates from Con A-stimulated spleen cells using *ch*CD25-32 and *ch*CD25-54 mAbs identified a 55 kDa band (Fig. 3).

3.3. ChCD25 immunostaining in chicken lymphoid tissues

Immunofluorescence staining of the bursa of Fabricius, spleen, and intestinal duodenum identified *ch*CD25$^+$ cells in all tissues examined (Fig. 4). Morphologically, *ch*CD25$^+$ cells appeared round in shape and lacked notable cellular processes. In the bursa, these cells were located in the follicles at the outer portion of the cortex, but were not observed in the follicle-associated epithelium or in the interfollicular areas (Fig. 4A). A few immunostained cells, however, were observed in the medulla of the follicles. In the spleen, *ch*CD25$^+$ cells were found throughout the red and white pulp and frequently in the periarteriolar lymphoid sheath, with a minority of cells

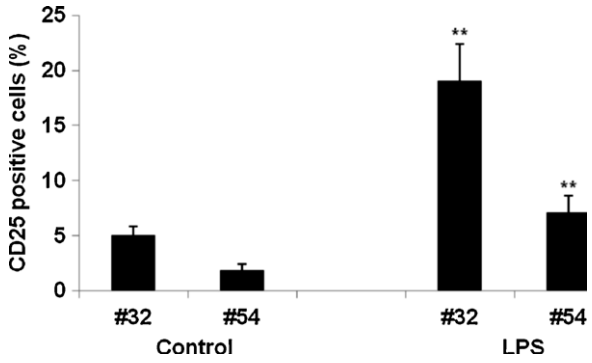

Fig. 5. *In vivo* LPS-stimulated *ch*CD25 expression. Four-week-old broiler chickens were injected intraperitoneally with PBS control or 1.0 mg LPS/kg body weight and the percentages of *ch*CD25$^+$ spleen cells were determined by flow cytometry using *ch*CD25-32 and *ch*CD25-54 mAbs. Each bar represents the mean ± S.D. ($n = 3$) value. **, $P < 0.01$ comparing LPS-treated with control-treated animals for each mAb using the Student's *t* test.

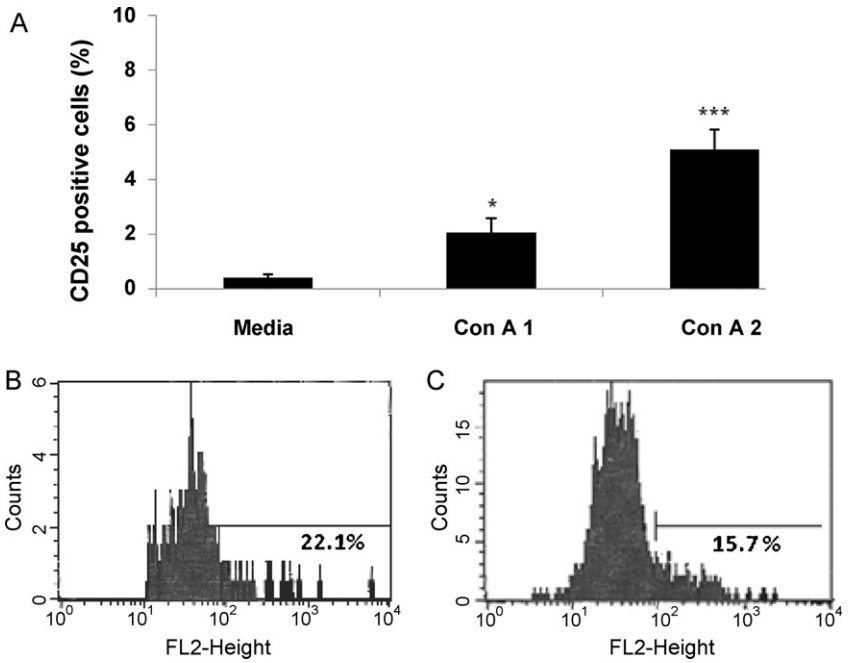

Fig. 6. *In vitro* Con A-stimulated *ch*CD25 expression. (A) Spleen cells were incubated for 72 h at 41 °C with medium alone, 1.0 μg/ml Con A (Con A 1), or 2.0 μg/ml Con A (Con A 2). The cells were harvested and the percentages of *ch*CD25$^+$ cells were determined by flow cytometry using the *ch*CD25-54 mAb. Each bar represents the mean ± S.D. value ($n = 3$). *, $P < 0.05$ and ***, $P < 0.001$ comparing Con A-treated with medium-treated cells using the Student's *t* test. Spleen cells were treated with 5.0 μg/ml of Con A as above and *ch*CD25$^+$ cells were determined by flow cytometry using the chCD25-32 (B) and *ch*CD25-54 (C) mAbs. The percentage of positively staining cells above background levels are listed in each panel.

inside germinal centers (Fig. 4B). *Ch*CD25$^+$ cells were not observed in the periellipsoidal white pulp of the spleen. In the duodenum, *ch*CD25$^+$ cells were present within the epithelium covering the villi and lining the crypt lumen (Fig. 4C). Immunostained cells were observed at all levels of each villus and crypt. Also of significance was the presence of *ch*CD25$^+$ cells beneath the epithelium within the loose connective tissue located in both the core of the villus and the lamina propria surrounding the crypts.

3.4. In vivo LPS- and in vitro Con A-stimulated chCD25 expression

The percentages of *ch*CD25$^+$ spleen cells obtained 4 days following intraperitoneal injection of LPS were measured by flow cytometry using *ch*CD25-32 and *ch*CD25-54 mAbs, and compared with cells from animals given the PBS control. *Ch*CD25$^+$ cells significantly increased from 4.9 ± 0.9% using PBS to 19.3 ± 3.0% using LPS by staining with *ch*CD25-32, and from 2.2 ± 0.7% using PBS to 6.5 ± 1.1% using LPS by staining with *ch*CD25-54 (Fig. 5). Next, the percentages of *ch*CD25$^+$ spleen cells were measured by flow cytometry following *in vitro* stimulation with 1.0, 2.0, or 5.0 μg/ml of Con A, and compared with cells treated with the medium control. *Ch*CD25-54-staining cells significantly increased from 0.3 ± 0.1% using medium to 2.0 ± 0.3% using 1.0 μg/ml of Con A, and to 5.2 ± 0.4% using 2.0 μg/ml of Con A (Fig. 6A). At 5.0 μg/ml of Con A, 22.1% and 15.7% of spleen cells were stained by *ch*CD25-32 and *ch*CD25-54 mAbs, respectively (Fig. 6B and C).

3.5. Inhibition of IL-2-dependent spleen cell proliferation and nitric oxide production

Finally, we tested that ability of *ch*CD25 mAbs to block IL-2-dependent spleen cell proliferation and nitric oxide (NO) production. IL-2-stimulated proliferation in the presence of an irrelevant mAb (HB2) was approximately 1.5- to 2.0-fold greater compared with unstimulated cells (Fig. 7). However, dose-dependent inhibition of cell proliferation was observed in the presence of the *ch*CD25-32 and *ch*CD25-54 mAbs diluted from 1:10 to 1:160 ($P < 0.01$ or $P < 0.001$). Similarly, while IL-2-driven NO production was 2.0-fold greater in the presence of the HB2 mAb compared with unstimulated cells, NO levels were dose-dependently decreased in the presence of the *ch*CD25-32 mAb diluted from 1:10 to 1:160 (Fig. 8).

4. Discussion

The results of this study indicate that the mAbs named *ch*CD25-32 and *ch*CD25-54 mAbs are likely to detect *ch*CD25 based on their ability to react with CHO cells transfected with the expressed gene sequence of chicken CD25, but not with other CHO cells. Using primary chicken cells we found the two mAbs recognized cells in the spleen, bursa of Fabricius, intestinal duodenum; in addi-

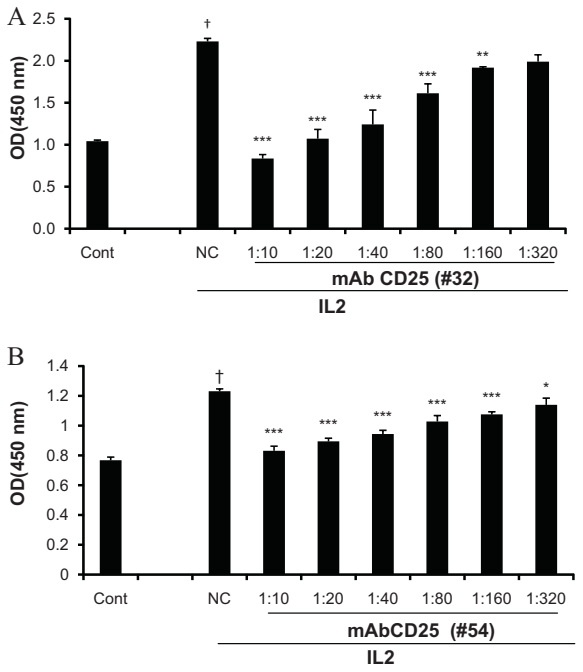

Fig. 7. Inhibition of IL-2-dependent spleen cell proliferation. Spleen cells were incubated for 48 h at 41 °C with 2.0 μg/ml of Con A and were treated for 15 min at 41 °C with 0.05 M α-methyl mannoside. The cells were added to 96-well plates (2.0×10^5 cells/well) and incubated for 2 h with a negative control (NC) mAb (HB2) or with 1:10, 1:20, 1:40, 1:80, 1:160, or 1:320 dilutions of *ch*CD25-32 mAb (A) or *ch*CD25-54 mAb (B). Following incubation, 50 μl/well of recombinant chicken IL-2 was added, the cells were incubated for 72 h (A) or 48 h (B), and cell proliferation was determined at OD (450 nm) using WST-8. Each bar represents the mean ± S.D. value ($n = 4$). †, $P < 0.001$ comparing IL-2-treated (NC) with medium-treated (Cont) cells using the Student's *t* test. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$ comparing IL-2-treated, chCD25-32 or chCD25-54 mAb-treated cells with IL-2-treated HB2 mAb-treated (NC) cells using the Student's *t* test.

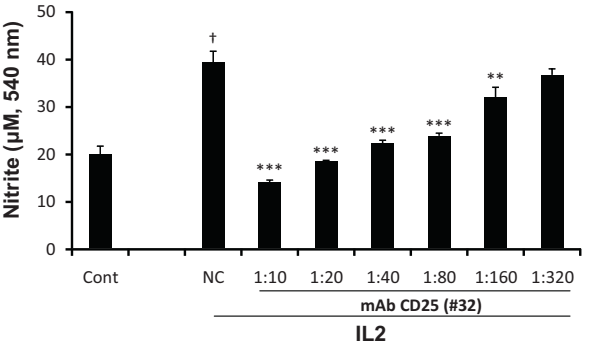

Fig. 8. Inhibition of IL-2-dependent spleen cell NO production. Spleen cells were incubated for 48 h at 41 °C with 2.0 μg/ml of Con A and were treated for 15 min at 41 °C with 0.05 M α-methyl mannoside. The cells were added to 96-well plates (2.0×10^5 cells/well) and incubated for 2 h with a negative control (NC) mAb (HB2) or with 1:10, 1:20, 1:40, 1:80, 1:160, or 1:320 dilutions of *ch*CD25-32 mAb. Following incubation, 50 μl/well of recombinant chicken IL-2 was added, the cells were incubated for 72 h, and NO levels in cell culture media were determined using the Griess reagent. Each bar represents the mean ± S.D. value ($n = 4$). †, $P < 0.001$ comparing IL-2-treated (NC) with medium-treated (Cont) cells using the Student's *t* test. **, $P < 0.01$, and ***, $P < 0.001$ comparing IL-2-treated, chCD25-32 mAb-treated cells with IL-2-treated HB2 mAb-treated (NC) cells using the Student's *t* test.

tion, they immunostained established chicken T cell, B cell, and macrophage cell lines. As would be expected for CD25 expression (Shirai et al., 2004), following intraperitoneal injection of chickens with LPS, there was an increased percentage of *ch*CD25$^+$ spleen cells compared with animals given the PBS control, and *in vitro* stimulation of spleen cells with Con A increased the percentage of *ch*CD25$^+$ cells compared with cells treated with medium alone. Finally, the *ch*CD25 mAbs inhibited IL-2-dependent spleen cell proliferation and decreased IL-2-stimulated NO production as has been documented in other systems for antibodies to the high affinity IL-2 receptor, CD25 (Teng et al., 2006; Miyamoto et al., 2001). While the recognition of a 55 kDa protein on Western blots of lysates from Con A-stimulated spleen cells was greater than the predicted molecular weight of the 211-amino acid *ch*CD25 protein is 23,420 Da (http://www.ncbi.nlm.nih.gov/protein/NP_989927.1.), the larger value observed in this study agrees with those reported for the duck, sheep, mouse, and human proteins (Leonard et al., 1984; Wang et al., 2007; Bujdoso et al., 1992). Discrepancy with the predicted number likely is the result of glycosylation at one or more of the potential N- and/or O-linked glycosylation sites in the *ch*CD25 protein. Our results on the expression patterns agree with that of others. It has been recognized for some time that human CD25 is an early marker of antigen- or mitogen-activated T cells (Smith, 1988; Waldmann, 1986), it is now known that mammalian CD25 also is expressed on activated B cells and antigen presenting cells (Mnasria et al., 2008; Suri and Austyn, 1998). Similarly, we identified a small percentage of *ch*CD25$^+$ cells in the bursa, a predominantly B cell lymphoid organ, and much higher levels of expression on established B cell and macrophage cell lines, thus confirming its presence on cells other than T lymphocytes. The low percentages of *ch*CD25$^+$ cells in normal spleen and bursa is due to the fact that CD25 expression is normally low, but up-regulated following antigen or mitogen stimulation (Smith, 1980, 1988; Waldmann, 1989). Third, comparable with the data presented here, the percentages of *ch*CD25$^+$ (Gu et al., 2010a,b) and duck CD25$^+$ spleen cells (Wang et al., 2007) were shown to be increased following Con A stimulation.

The IL-2R was the first cytokine receptor to be cloned (Smith, 1980) and subsequently the 3 distinct receptor chains, α, β, and γ, were isolated, with α (also known as CD25) being the first to be discovered (Leonard et al., 1984). X-ray crystallographic studies have identified the molecular sites on the IL-2 molecule that interact with these 3 receptor chains (Wang et al., 2005). IL-2 is held between the β and γ chains within the pocket of a "Y-shaped" complex, while the opposite face of the cytokine molecule simultaneously interacts with the α chain. Following binding of IL-2, the β chain undergoes a conformational change that increases its binding affinity for the γ chain, thereby forming the high affinity IL-2R complex, but the high binding affinity of receptor for its ligand ($K_d \approx 10^{-11}$ M) is the net result of a rapid association rate ($k = 10^7$/M/s) contributed by the α chain, and a relatively slow dissociation rate ($k' = 10^{-4}$/s) contributed by the β and γ chains. Thus blocking the α chain would be expected to block IL-2 binding most efficiently. As reported by Teng et al. (2006) and now

confirmed here, mAbs against *ch*CD25 do indeed inhibit IL-2-dependent proliferation of chicken spleen cells. Similar results have been shown in other species as well including human (Nagler et al., 1990; Quéméneur et al., 2002).

Following engagement of the IL-2R with IL-2, the intracellular domains of the β and γ chains recruit and interact with the Janus kinase 1 (JAK1) and JAK3 cytosolic protein tyrosine kinases (Smith, 1988). As a consequence, the JAK-STAT, PI3K, and MAP kinase intracellular signaling pathways are activated. Activation of the JAK-STAT pathway promotes the transcriptional activation of cyclin D and related molecules, while activation of the PI3K pathway leads to the degradation of p27, an inhibitor of cyclin D/cyclin-dependent kinase. The overall effect of these signaling pathways is the progression of the cell through the G1 phase of the cell cycle and the initiation of DNA synthesis and mitosis. Given the important role for IL-2/IL-2R interaction in cell proliferation, mAb-based drugs that inhibit the human IL-2R, such as basiliximab and daclizumab, are currently used to prevent immune rejection of tissue and organ transplants. Hopefully, the chicken IL-2R mAbs described herein, as well as those previously reported (Gu et al., 2010a,b; Teng et al., 2006), will also serve as valuable immune reagents for basic and applied research in poultry immunology.

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