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Development and characterization of mouse monoclonal antibodies reactive with chicken CD80

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ABSTRACT

This study was carried out to develop and characterize mouse monoclonal antibodies (mAbs) against chicken CD80 (*ch*CD80). A recombinant plasmid containing a *ch*CD80/horse IgG4 fusion gene was constructed and expressed in CHO cells to produce recombinant *ch*CD80/IgG4 protein. Chicken CD80 was purified from the *ch*CD80/IgG4 fusion protein following enterokinase digestion, and used to immunize BALB/c mice, resulting in 158 hybridomas that produced mAbs against *ch*CD80. Three mAbs with high binding specificity for recombinant *ch*CD80/IgG4-transfected CHO cells were identified by flow cytometry, and one of these (#112) was selected for further characterization. Immunoprecipitation of CD80/IgG4-CHO cell extract, or lipopolysaccharide (LPS)-treated monocytes identified 35.0 kDa proteins. Immunohistochemical analysis revealed *ch*CD80-expressing cells exclusively in the bursal follicles at the outer portion of the cortex, and throughout the red pulp and the outer boundary of the white pulp in the spleen. By immunofluorescence microscopy, *ch*CD80 was observed on intestinal dendritic cells. LPS treatment of bursa or spleen monocytes for 24 or 48 h increased *ch*CD80 expression. Finally, addition of *ch*CD80 mAb to Con A-stimulated spleen cells inhibited the expression of major histocompatibility complex class II antigens and IL-2-driven proliferation of lymphoblast cells. In summary, these *ch*CD80 mAbs will serve as valuable immunological reagents for basic and applied poultry immunology research.

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1. Introduction

CD80 (Cluster of Differentiation 80, B7.1) is a type I glycoprotein that consists of two extracellular immunoglobulin-like domains, a transmembrane region, and a cytoplasmic tail. Functionally, CD80 on antigen presenting cells (APCs) binds to CD28 on T cells, thereby providing a costimulatory signal necessary for T cell activation. Specifically, engagement of CD80 with CD28 induces the proliferation and differentiation of naïve

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T cells into cytokine-producing effector cells [1–5]. By contrast, engagement of CD80 with CTLA4 (CD152) sends a T cell inhibitory signal [4,6]. Mature dendritic cells that bridge the innate and adaptive immune systems typically express high levels of CD80 [7,8] that can be further up-regulated by treatment with concanavalin A (Con A) or lipopolysaccharide (LPS) [9,10]. Thus, expression of CD80 by these and other APCs plays a critical role in the development of immunity against infectious pathogens, vaccine antigens, and diverse injurious stimuli [11].

In addition to humans and mice, CD80 has been characterized in several veterinary animal species, including pig [12], sheep [13], cat [14–16], and dog [14]. A chicken homologue of CD80 which binds to mammalian CTLA4 has been reported [17]. However, monoclonal antibodies (mAbs) against chicken CD80 (*ch*CD80) that would be useful for further characterization of the expression and immunological function of the avian CD80 molecule have not been described. Therefore, in this report a recombinant *ch*CD80/IgG4 fusion protein was engineered to develop murine mAbs against *ch*CD80 for expression and functional studies.

2. Materials and methods

2.1. Mammalian cell expression and purification of recombinant chCD80

A cDNA encoding the extracellular region of *ch*CD80 was cloned into the pcDNA/IGHG4 expression vector as described [18]. Recombinant *ch*CD80/IgG4 fusion protein was expressed in Chinese hamster ovary (CHO) cells using the previously described method [19]. Cells with stable expression of *ch*CD80/IgG4 were used for *ch*CD80 protein purification. Recombinant protein was purified from the serum-free supernatant of transfected cells using protein G affinity purification. Purified *ch*CD80 was separated from the IgG4 linker by enterokinase digestion (EKMax, Invitrogen, Eugene, OR) on the protein G column, followed by post-digestion elution in PBS.

2.2. Hybridoma production

BALB/c mice (6-week-old, Taconic Laboratories, Germantown, NY) were immunized intraperitoneally with 50 μg of purified *ch*CD80 combined with Gerbu adjuvant (Accurate Chemical, Westbury, NY). Animals were boosted intraperitoneally with 25 μg of the *ch*CD80/adjuvant emulsion at days 14 and 21, followed by injection of 25 μg of *ch*CD80 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 3 days after the final boost. Hybridomas were selected in medium supplemented with HAT (Sigma, St. Louis, MO) and supernatants were screened for binding to CHO cells expressing *ch*CD80/IgG4 using a FACSCalibur flow cytometer (BD, Franklin Lakes, NJ). Hybridomas which showed high binding activity for *ch*CD80/IgG4-CHO cells were cloned by limiting dilution and used for Western blotting, immunohistochemistry, and biological function analyses. The supernatant of a hybridoma which showed no binding activity for *ch*CD80/IgG4-CHO cells was used as a negative control (NC).

2.3. Flow cytometry

Flow cytometric analysis of *ch*CD80/IgG4-CHO cells were performed as previously described [20,21]. Single cell suspensions were fixed in 2% formaldehyde for 20 min at room temperature, washed twice with PBS, and resuspended in 1.0 ml of PBS supplemented with 0.5% bovine serum albumin, 0.5% saponin, and 0.02% NaN_3. Cell suspensions (1.0×10^6 cells in 100 μl) were incubated on ice for 45 min with 100 μl of appropriately diluted *ch*CD80 mAb or with HB2 (American Type Culture Collection, Manassas, VA), a mAb against human T cells, as a negative control. After washing twice with 2.0 ml of PBS/saponin buffer, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG secondary antibody (Sigma) on ice for 30 min, washed twice, resuspended in 1.0 ml, and analyzed with a FACSCalibur flow cytometer. Data was obtained from a total of 1.0×10^4 viable cells. To determine the effect of lipopolysaccharide (LPS) on *ch*CD80 expression, single cell suspensions of mononuclear lymphocytes were prepared from bursa of Fabricius and spleen as described [22,23] by gently flushing through a cell strainer and density gradient centrifugation on Histopaque-1077 (Sigma). Mononuclear lymphocytes (1.0×10^7 cells/ml) were incubated at 41 °C in a humidified incubator (Forma, Marietta, OH) with 5% CO_2 for 72 h with 1.0 ml of culture medium alone or with 5.0 μg/ml of *Escherichia coli* LPS in 6-well tissue culture plates (Costar, Corning, NY). The cells were harvested and used at a concentration of 1.0×10^7 cells/ml for *ch*CD80 mAb staining by flow cytometery.

2.4. SDS–PAGE

*Ch*CD80/IgG4-CHO cells and freshly prepared mononuclear lymphocytes from the bursa of Fabricius which was obtained from 3-week-old broiler chickens (Ross/Ross, Longenecker Hatchery, Elizabethtown, PA) were used to identify the target molecules of *ch*CD80 mAbs. Monocytes were stimulated with 5.0 μg/ml of LPS for 48 h [22,23] and resuspended in 20 mM Tris–HCl, pH 8.3, containing 137 mM NaCl, 10% glycerol, 1.0% Triton X-100, and 2.0 mM EDTA. The cells were disrupted by sonication for 25 s, centrifuged at 4 °C for 10 min at 12,000 rpm, and the supernatants were collected. Cell extracts were incubated overnight with *ch*CD80 mAb at 4 °C with constant agitation and immune complexes were precipitated with protein A agarose (Pierce, Rockford, IL) at room temperature for 2 h. Agarose beads were collected by centrifugation at 4 °C for 5 min at 2,000 rpm, washed with 25 mM Tris–HCl, pH 7.2 containing 150 mM NaCl, and bound proteins were eluted with 0.1 M glycine, pH 2.3 and neutralized with 1.0 M Tris–HCl, pH 7.5 [24]. Eluted proteins (2.0 μg) were mixed with an equal volume of 0.125 M Tris–HCl, pH 6.8, containing 4.0% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.004% bromophenol blue, heated at 100 °C for 5 min, separated by SDS-polyacrylamide gel electrophoresis (Bio-

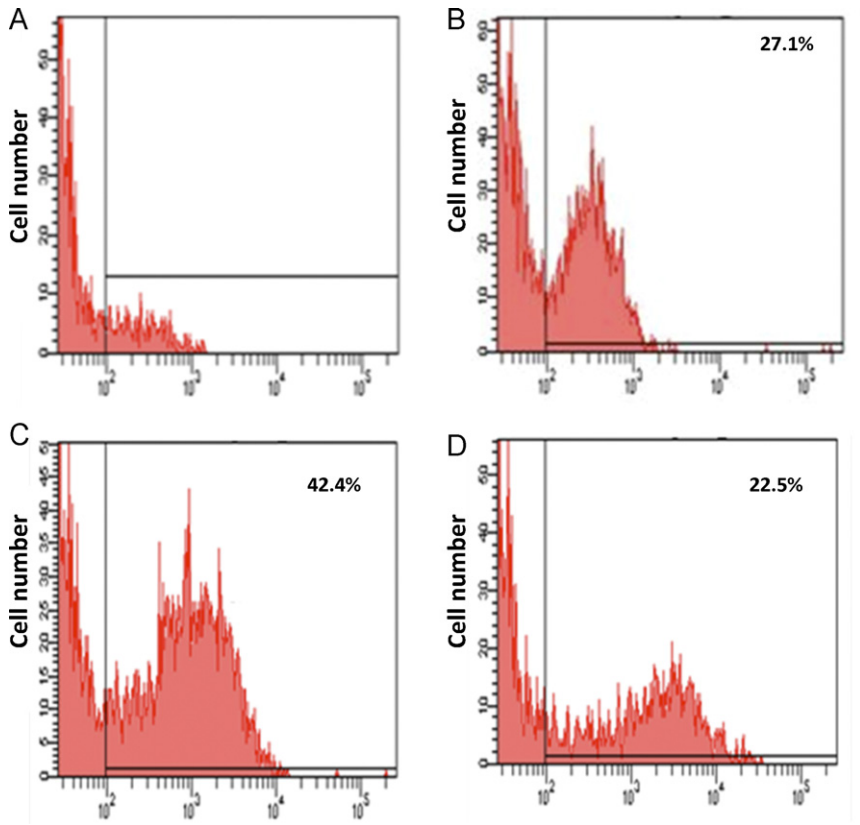

Fig. 1. Flow cytometric analysis of *ch*CD80-expressing cells. CD80/IgG4-CHO cells (1.0×10^6) were stained with: (A) the HB2, an anti-human T cell mAb as a negative control, (B) *ch*CD80 mAb #26, (C) *ch*CD80 mAb #112, and (D) *ch*CD80 mAb #116. Cells were incubated with hybridoma supernatants for 45 min, followed by FITC-conjugated goat anti-mouse IgG secondary antibody for 30 min and cellular fluorescence was analyzed by flow cytometry. The percentage of positively staining cells above background levels indicated by the vertical lines are listed in each panel.

Rad, Hercules, CA), and visualized by staining with 0.025% Coomassie blue.

2.5. Immunohistochemistry

Bursa of Fabricius and spleen from 3-week-old White Leghorn chickens (Doux Iberica, Zaragoza, Spain) were fixed in Bouin's fixative for 2 h, immersed in 70% ethanol for 48 h, embedded in paraffin, and 5 μm sections were prepared. Deparaffinized sections were rinsed in PBS, pH 7.2, and endogenous peroxidase was inactivated with 1.7% H_2O_2 in ethanol for 30 min. The slides were blocked with undiluted normal pig serum at room temperature for 30 min and incubated with *ch*CD80 mAb (1:10 dilution) at 4 °C for 18 h. After washing with PBS, pH 7.2, the slides were incubated with biotinylated goat anti-mouse IgG antibody (Vector, Burlingame, CA) for 30 min. The avidin–biotin-peroxidase complex (Vector) was applied for 45 min and the peroxidase reaction was developed with 0.02% of 3.3′-diaminobenzidine substrate in 0.05 M Tris–HCl, pH 7.6, containing 0.005% H_2O_2 for 5 min.

2.6. Indirect Immunofluorescence staining

Dendritic cells were isolated from the cecal tonsils of chickens infected with *Eimeria tenella* as described [25]. The cells (1.0×10^4 cells/ml) were incubated for 48 h with 1.0 ml of medium alone or with 5.0 μg/ml of LPS as above and the cells were centrifuged at 800 rpm for 2 min (Cytospin 3, Shandon, Tokyo, Japan) onto glass slides precoated with 0.1% poly-L-lysine (Sigma). Immediately after centrifugation, the cells were incubated at −20 °C for 20 min in acetone, washed 3 times with PBS, blocked for 10 min with normal horse serum (Vector), and incubated for 90 min with *ch*CD80 mAb or isotype-matched negative control (NC) mAb. After washing with PBS, the slides were incubated for 30 min with Alexa Fluor 568-labeled goat anti-mouse IgG secondary antibody (1:250 dilution, Invitrogen, Carlsbad, CA), washed with PBS, covered with Vectashield (Vector), and the cells were observed with an Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan).

2.7. Con A-induced spleen lymphocyte differentiation

Freshly prepared spleen lymphocytes (1.0×10^7 cells/ml) from 3-week-old broiler chickens were incubated at 41 °C in a humidified incubator with 5% CO_2 for 24 or 48 h with 1.0 ml of medium alone or with 5.0 μg/ml of Con A plus or minus 1.0 ml of *ch*CD80 mAb (1:5 dilution) in 6-well tissue culture plates. The cells were harvested and 1.0×10^7 cells/ml were analyzed by flow cytometry for

major histocompatibility complex class II (MHCII) antigen expression.

2.8. IL-2-induced spleen lymphocyte proliferation

Freshly prepared splenocytes from 6-week-old broiler chickens were adjusted to 2.0×10^6 cells/ml in RPMI-1640 medium (Sigma) supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin [26]. The cells were incubated at 41 °C for 48 h with 2.0 μg/ml of Con A, treated for 15 min at 41 °C with 0.05 M α-methyl mannoside (Sigma), washed, and purified by density gradient centrifugation over Histopaque-1077. The lymphoblast cells were resuspended in RPMI-1640 medium containing 5% FBS at 2.0×10^6 cells/ml and 100 μl/well added to 96-well plates containing 50 μl of medium alone or chicken recombinant IL-2 [27] in the absence or presence of *ch*CD80 mAbs or isotype-matched negative control mAb [26]. The cells were incubated at 41 °C for 48 h and cell proliferation was measured using WST-8 according to the manufacturer's instruction (Cell Counting Kit-8, Dojindo Molecular Technologies, Gaithersburg, MD).

2.9. Statistical analysis

Statistical analyses were performed using SPSS 12.0 software for Windows. All data was expressed as mean ± SD values ($n = 4$). ANOVA, the Student's *t*-test, and the Duncan's multiple range test were used to evaluate the differences between treatment groups. Differences between mean values were considered statistically significant at $P \leq 0.05$.

3. Results

3.1. Production of chCD80 mouse monoclonal antibodies

One hundred fifty eight murine hybidomas were isolated by growth in HAT selective medium using spleen cells from mice immunized with the purified recombinant *ch*CD80 molecule. Three of these mAbs (#26, #112, and #116) showed 22.5–42.4% staining of CHO cells transiently expressing the *ch*CD80/IgG4 fusion protein (Fig. 1). Monoclonal Ab #112 with the greatest binding activity for *ch*CD80/IgG4-CHO cells was selected for further analysis (Fig. 1C).

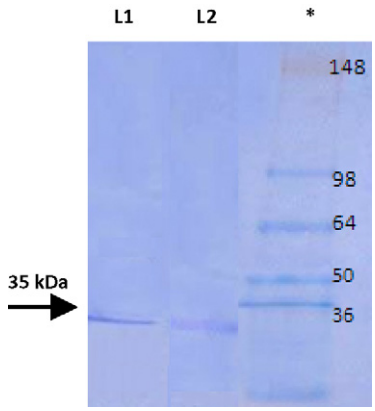

Fig. 2. Determination of the molecular size of *ch*CD80. Extracts of chCD80/IgG4-CHO cells (L1) and LPS-stimulated bursa monocytes (L2) were immunoprecipitated with *ch*CD80 mAb #112, precipitated proteins were resolved by reducing SDS–PAGE, and stained with Coomassie blue. The 35.0 kDa *ch*CD80 proteins are indicated by the arrow on the left. The asterisk (*) indicates the position of prestained molecular weight markers with kDa on the right.

3.2. Determination of the molecular size of chCD80

Immunoprecipitation of *ch*CD80 from transfected CHO cells, or from LPS-stimulated mononuclear lymphocytes, using mAb #112 identified 35.0 kDa proteins on Coomassie blue-stained SDS-polyacrylamide gels (Fig. 2).

3.3. Immunolocalization of chCD80

Immunohistochemical staining of the bursa of Fabricius and spleen by *ch*CD80 mAb #112 identified *ch*CD80$^+$ cells (Fig. 3). In the bursa, *ch*CD80$^+$ cells appeared round in shape, lacked notable cellular processes, and were located in the follicles at the outer portion of the cortex (Fig. 3A). *Ch*CD80$^+$ cells were not observed in the follicle-associated epithelium (FAE), in the medulla of the follicles, or in the interfollicular areas. In the spleen, *ch*CD80$^+$ cells were found throughout the red pulp, both within the cords of Billroth and in the venous sinuses (Fig. 3B). The major-

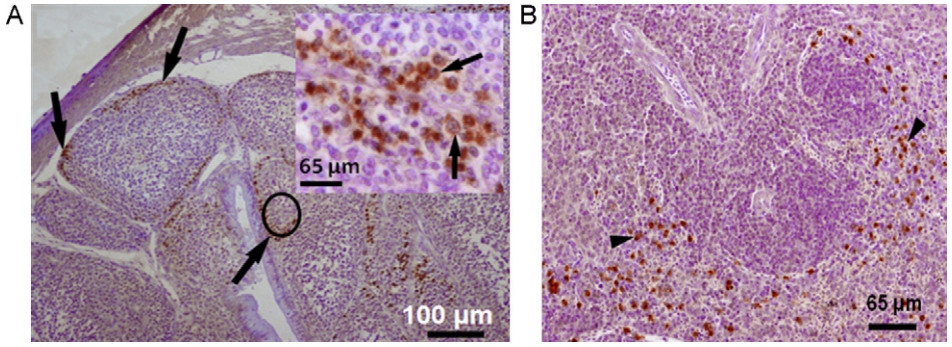

Fig. 3. Immunohistochemical localization of chCD80 in chicken immune tissues. Bursa of Fabricius (A) and spleen (B) were fixed in Bouin's fixative, embedded in paraffin, and sections were stained with *ch*CD80 mAb #112 as described in Section 2. Positive cells are located in the cortical area of the bursa (arrows) and in the red pulp of the spleen (arrowheads).

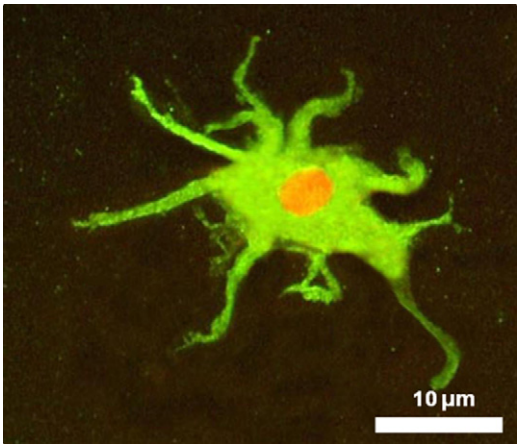

Fig. 4. Expression of *ch*CD80 on LPS-stimulated dendritic cells. Cells (1.0×10^4 cells/ml) were incubated for 48 h with 5.0 μg/ml of LPS, cytospun onto glass slides, incubated with *ch*CD80 mAb #112 for 90 min, followed by Alexa Fluor 568-conjugated goat anti-mouse IgG secondary antibody for 30 min and cells were visualized by fluorescence microscopy.

ity of splenic *ch*CD80$^+$ cells had polygonal or elongated cell body shapes, and were frequently observed near the outer boundary of the white pulp. *Ch*CD80$^+$ cells were not observed in the periarteriolar lymphoid sheath (PALS), inside of the germinal centers, in the ellipsoid, or in the periellipsoidal white pulp.

3.4. Expression of chCD80 on dendritic cells

Immunofluorescence examination of LPS-stimulated intestinal dendritic cells stained with *ch*CD80 mAb #112 revealed staining on the plasma membrane of the cell body, processes, and filiform dendrites (Fig. 4). No detectable immunofluorescence staining was observed on unstimulated dendritic cells, or LPS-stimulated cells reacted with the isotype-matched negative control mAb (data not shown).

3.5. Effect of LPS on chCD80$^+$ monocytes in the bursa of Fabricius and spleen

By flow cytometry, chCD80 mAb #112 detected 2.5 ± 0.2% of monocytes in the bursa and 5.6 ± 2.8% of spleen monocytes (Fig. 5). LPS treatment of bursa monocytes for 48 h increased chCD80-expressing cells to 8.3 ± 0.6%. LPS treatment of spleen monocytes for 24 or 48 h increased chCD80-expressing cells to 8.1 ± 2.4% and 7.3 ± 2.5%, respectively.

3.6. Functional activity of chCD80

Two experimental approaches were used to demonstrate the functional activity of *ch*CD80 mAb #112. In the first, treatment of spleen mononuclear lymphocytes with ConA increased the percentages of MHCII-expressing cells from 24.0% to 50.4% after 24 h incubation, which remained relatively constant at 52.0% after 48 h incubation (Fig. 6). However, addition of *ch*CD80 mAb #112 to the ConA-treated cells reduced the percentage of MHCII$^+$ cells to

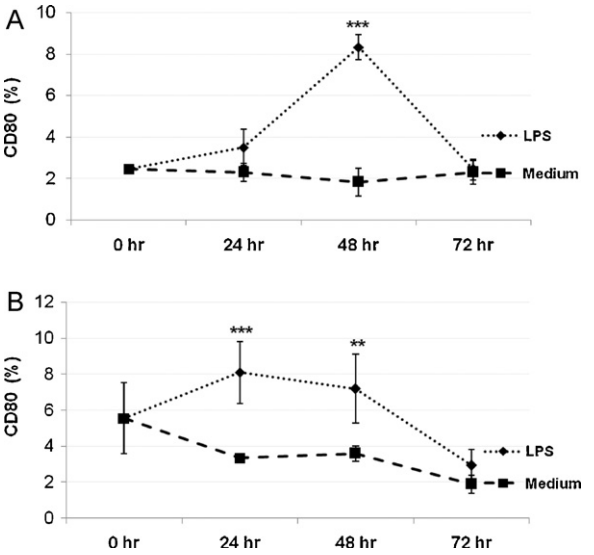

Fig. 5. Expression of *ch*CD80 on LPS-stimulated bursa (A) and spleen mononuclear lymphocytes (B). Cells (1.0×10^7 cells/ml) were untreated (0 h), or were incubated for 72 h with culture medium alone or 5.0 μg/ml of LPS, and analyzed by flow cytometry with *ch*CD80 mAb #112. Each value represents the mean ± SD ($n = 4$). $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ when comparing LPS stimulation with medium control at the respective time points by the Student's *t*-test.

the levels seen by treatment with culture medium alone. Secondly, *ch*CD80 mAb #112 dose-dependently inhibited IL-2-induced spleen lymphocyte proliferation, compared with the isotype-matched negative control mAb (Fig. 7).

4. Discussion

In this report, we describe the production and characterization of mouse mAbs specific for *ch*CD80. The major findings are: (1) 3 mAbs prepared by immunization with a recombinant *ch*CD80 ectodomain protein reacted with CHO cells expressing the *ch*CD80 polypeptide, (2) mAb #112 immunoprecipitated 35.0 kDa proteins from lysates of *ch*CD80/IgG4-CHO cells and from bursa monocytes

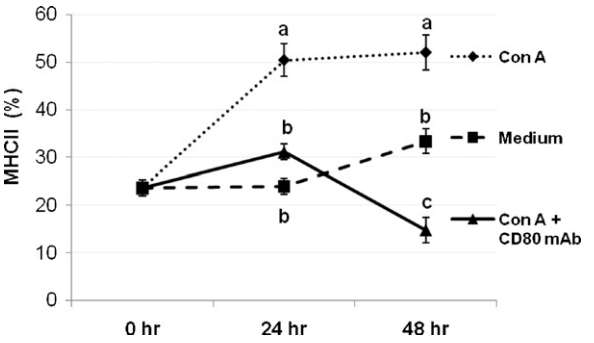

Fig. 6. Effect of *ch*CD80 mAb on ConA-stimulated MHCII antigen expression. Spleen mononuclear lymphocytes (1.0×10^7 cells/ml) were untreated (0 h) or were incubated for 24 or 48 h with culture medium alone, 5.0 μg/ml of ConA, or ConA plus *ch*CD80 mAb #112, and analyzed by flow cytometry for MHCII expression. Each value represents the mean ± SD ($n = 4$). Points with different letters are significantly different ($P \leq 0.05$) according to the Duncan's multiple range test.

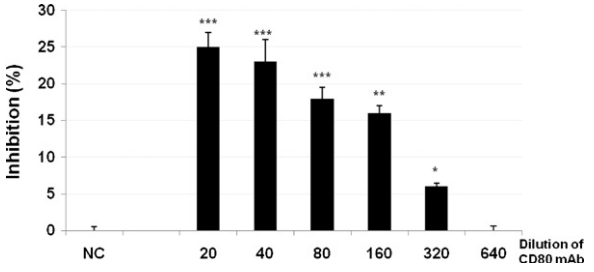

Fig. 7. Effect of *ch*CD80 mAb on IL-2 driven lymphoblast cell proliferation. Spleen lymphoblast cells (1.0×10^6 cells/ml) were cultured for 48 h with medium alone or chicken IL-2 plus the indicated dilutions of *ch*CD80 mAb #112 or an undiluted isotype-matched negative control (NC) mAb. The percent inhibition of cell proliferation was calculated as described in Section 2. Each bar represents the mean ± SD value ($n = 4$). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ when comparing *ch*CD80 mAb with NC by the Student's *t*-test.

expressing the full-length native protein, (3) mAb #112 detected *ch*CD80$^+$ cells in the bursa and spleen, and on the surface of intestinal dendritic cells, (4) LPS increased the percentages of *ch*CD80$^+$ bursa and spleen cells, and (5) mAb #112 inhibited ConA-stimulated spleen cell differentiation into MHCII$^+$ cells and blocked IL-2 induced spleen blast cell proliferation.

None of *ch*CD80 mAbs described in this report reacted with untransfected CHO cells or with CHO cells transfected with an irrelevant gene, *ch*CD83/IgG4 (data not shown). Thus, these *ch*CD80 mAbs showed specificity for *ch*CD80. Further, by SDS–PAGE, the observed molecular weight of 35.0 kDa for the native *ch*CD80 protein expressed by LPS-stimulated monocytes corresponds closely with the theoretical size of 33.0 kDa based upon its amino acid sequence, and is similar to that reported for the human protein on activated B cells, macrophages and dendritic cells (37.0 kDa) [28,29]. It is important to note, however, that glycosylation of the human and chicken proteins may drastically influence their observed mobilities on denaturing polyacrylamide gels [30]. Interestingly, the size of the *ch*CD80/IgG4 fusion protein expressed in CHO cells which only contains the *ch*CD80 ectodomain, matches with that observed for the full-length native molecule expressed by bursa-derived monocytes. Apparently, the contribution to molecular weight of the IgG4 segment in the former fortuitously compensates for the transmembrane and intracellular domains of the latter.

In mammals, CD80 is expressed by professional APCs where it functions as a costimulatory molecule for antigen-driven T cell differentiation and proliferation following ligation with CD28. These APCs include dendritic cells, macrophages/monocytes, and B cells. Our immunohistochemical and immunofluorescence results on *ch*CD80 expression within the bursa and spleen are consistent with these studies. Further, human or mouse B cells with reduced CD80 expression by lentiviral transduction of endoplasmic reticulum-directed CTLA4, or by treatment with CD80 blocking antibody, exhibited suppressed antigen-specific lymphocyte proliferation *in vitro* [31,32]. In addition, LPS, a potent stimulator of dendrtic cell maturation, up-regulated the expression of multiple costimulatory molecules, including CD80 [33,34], and CD80 reportedly influenced T cell autoreactivity to MHCII antigens [35]. All of these observations in mammalian systems are consistent with the results of the current study. CD80 costimulation also regulates T cell differentiation, including through the production of IL-2 and IFN-γ [3,4,36]. In chickens, IL-2 plays an important role as an immunoregulatory molecule, and is functionally identical to mammalian IL-2, as they both stimulate the differentiation of T lymphoblasts, enhance NK cell activity, and promote adaptive immunity against various infectious microorganisms [26,37]. Our results showing the inhibition of IL-2-induced spleen cell differentiation to MHCII$^+$, CD4$^+$, and CD8$^+$ cells by *ch*CD80 mAb further confirms the function of CD80 in chickens [38,39].

In summary, this is the first report describing the production and characterization of mouse mAbs against *ch*CD80. Collectively, these results suggest that the biological function of *ch*CD80 is similar to that of the mammalian proteins by playing important role in APC-lymphocyte interactions that lead to the initiation of adaptive immunological responses to antigens and infectious pathogens. Future studies utilizing these mAbs will contribute to furthering basic and applied poultry immunology research.

Acknowledgments

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