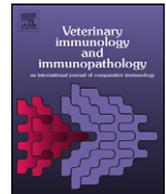




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Short communication

### Development and characterization of mouse monoclonal antibodies reactive with chicken CD83

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#### ABSTRACT

This study was carried out to develop and characterize mouse monoclonal antibodies (mAbs) against chicken CD83 (*chCD83*), a membrane-bound glycoprotein belonging to the immunoglobulin superfamily that is primarily expressed on mature dendritic cells (DCs). A recombinant *chCD83*/IgG4 fusion protein containing the extracellular region of *chCD83* was expressed in Chinese Hamster Ovary (CHO) cells and isolated from the spent cell culture medium by protein G affinity chromatography. The extracellular region of the *chCD83* protein was purified and used to immunize mice. A cell fusion was performed, from which 342 hybridomas were screened for mAbs to *chCD83*. Two mAbs, *chCD83*-159 and *chCD83*-227, stained the greatest percentage of *chCD83*-transfected CHO cells and were selected for further characterization. By flow cytometry, both mAbs reacted with a chicken macrophage cell line, HD11. Both mAbs also recognized a single 53 kDa protein on Western blots of lysates from lipopolysaccharide-stimulated spleen mononuclear cells or unstimulated HD11 cells. Immunostaining of chicken secondary lymphoid organs identified *chCD83*<sup>+</sup> cells with morphologic and subtissue localization properties comparable to mammalian DCs. *In vitro* stimulation of spleen mononuclear cells with concanavalin A (Con A) decreased the percentage of *chCD83*<sup>+</sup> cells compared with cells treated with medium alone. Interestingly, spleen cells treated with Con A in the presence of *chCD83*-227 mAb exhibited decreased percentage of MHCII<sup>+</sup> cells compared with cells treated with an isotype-matched negative control mAb. These *chCD83* mAbs may be useful for future investigations of chicken immune cell maturation and mechanisms of action.

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## 1. Introduction

CD83 is a cell surface glycoprotein predominantly expressed on mature human and mouse dendritic cells (DCs) (Zhou et al., 1992; Prazma et al., 2007; Jin et al., 2010) and, to a lesser extent, on activated T and B lymphocytes (Kretschmer et al., 2007; Prazma et al., 2007; Wolenski et al., 2003), regulatory T cells (Reinwald et al., 2008), and activated macrophages (Cao et al., 2005; Nicod et al., 2005). DCs constitute a fundamental bridge between innate immune recognition of pathogen-associated molecular patterns (PAMPs) and acquired immunity. Activation of DCs by PAMPs up-regulates MHC class II (MHCII), CD80, and CD86 cell surface molecules that are required for DC interaction with naive T cells (Lee et al., 2011a; Caux et al., 1994). On T cells, CD83 acts as a costimulatory molecule (Su et al., 2009; Aerts-Toegaert et al., 2007) that promotes T cell survival in the periphery (Prazma et al., 2007; Luthje et al., 2008). On B cells, CD83 is an early activation marker that it is up-regulated following engagement of Toll-like receptor 4 by lipopolysaccharide (LPS) (Breloer et al., 2007; Prazma et al., 2007).

In humans and mice, CD83<sup>+</sup> DCs are localized in the T cell areas of the secondary lymphoid organs (Cramer et al., 2000; Zhou et al., 1992). Hansell et al. (2007) reported a similar distribution of chicken CD83<sup>+</sup> cells and characterized the structural properties of the chicken CD83 (*chCD83*) protein. Aside from this report, much less is known about the expression and function of *chCD83* compared with its mammalian counterpart. Therefore, the current study was conducted to develop mouse monoclonal antibodies (mAbs) against *chCD83* for the purpose of defining its immunological, cellular, and biochemical characteristics.

## 2. Materials and methods

### 2.1. Expression and purification of recombinant *chCD83*

The extracellular region of the chicken CD83 gene was cloned into the pcDNA/IGHG4 expression vector as described earlier (Wagner et al., 2008). Recombinant soluble chicken CD83/IgG4 fusion protein (*rCD83/IgG4*) was expressed in Chinese Hamster Ovary (CHO) cells, following a procedure described previously in detail (Wagner et al., 2005). In brief, the recombinant CD83 (*rCD83*) protein was purified from serum free supernatant of a stable CD83/IgG4 transfectant using protein G affinity purification. The *rCD83* was separated from the *rIgG4* portion by enterokinase digestion (EKMax, Invitrogen, Eugene, OR) performed on the protein G column. This procedure allowed the elution of the *rCD83* in PBS after digestion, while the *rIgG4* portion was still bound by protein G. Afterwards, the *rIgG4* heavy chain constant region dimer was eluted using 0.1 M glycine pH 2.0. The protein concentration of purified *chCD83* was determined by a BCA assay (Pierce, Rockford, IL) and protein purity was confirmed by Coomassie staining following SDS-PAGE (Wagner et al., 2005).

### 2.2. Hybridoma production

BALB/c mice (Taconic Laboratories, Germantown, NY) were immunized by intraperitoneal injection of 50 µg of

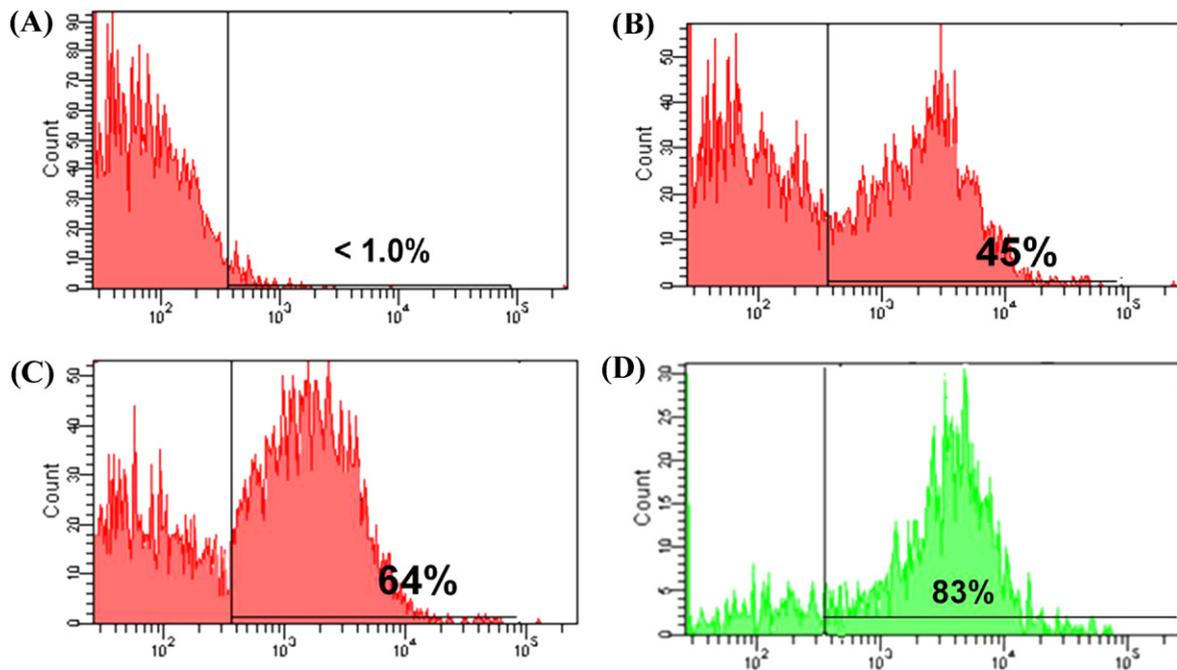
purified *chCD83* protein combined with Gerbu adjuvant (Accurate Chemical, Westbury, NY). The mice were boosted with 25 µg of *chCD83* plus Gerbu adjuvant at days 14 and 21 post-primary immunization, followed by 25 µg of *chCD83* alone on days 28, 29, and 30. Mice were euthanized and spleen lymphocytes 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 *chCD83/IgG4*-transfected CHO cells by flow cytometry as described (Lee et al., 2011a,b). Two mAbs, *chCD83-159* and *chCD83-227*, were selected for further study.

### 2.3. Flow cytometry

Single cell suspensions of CHO cells stably transfected with *chCD83/IgG4* and HD11, an avian myelocytomatosis virus (MC29)-transformed macrophage cell line (Klasing and Peng, 1987), 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 (FBS), 0.02% NaN<sub>3</sub> (Sigma), and/or 0.5% saponin for CHO cells stably transfected with *chCD83/IgG4*. The cells (1.0 × 10<sup>6</sup>/100 µl) were incubated on ice for 45 min with 100 µl of appropriately diluted *chCD83-159* or *chCD83-227* mAbs. HB2, an anti-human T cell mAb, was used as a negative control (Lee et al., 2011a,b). The cells were washed twice with 2.0 ml of PBS 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<sup>4</sup> viable cells.

### 2.4. SDS-PAGE and Western blotting

Spleen mononuclear lymphocytes from 3-week-old broiler chickens (Ross/Ross, Longenecker's Hatchery, Elizabethtown, PA) were stimulated with 5.0 µg/ml of *Escherichia coli* lipopolysaccharide (LPS, Sigma) for 24 h (Lechmann et al., 2002; Lee et al., 2011a). Spleen cells and unstimulated HD11 cells were 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 sec, centrifuged at 4 °C for 10 min at 12,000 rpm, and the supernatants were collected. Cell extracts (15.0 µg) and purified *chCD83* protein (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, and heated at 100 °C for 5 min. The proteins were resolved on 10–20% gradient SDS-acrylamide gels (Bio-Rad, Hercules, CA) and transferred to PVDF membranes (Millipore, Bedford, MA) (Towbin et al., 1979). The membranes were blocked with Superblock T20 (PBS) (Thermo Fisher Scientific, Rockford, IL), incubated overnight at 4 °C with *chCD83-159* or *chCD83-227* mAbs (1:100), and bound Abs were detected with horseradish peroxidase-conjugated



**Fig. 1.** Flow cytometric analysis of *chCD83* mAbs using CHO-*chCD83/IgG4* and HD11 cells. (A–C) CHO cells stably transfected with *chCD83/IgG4* ( $1.0 \times 10^6$ ) were fixed in 2.0% formaldehyde for 20 min at room temperature and washed with PBS. Cell suspensions ( $100 \mu\text{l}$ ) were incubated for 45 min on ice with (A) HB2 mAb (negative control), (B) *chCD83-159* mAb, or (C) *chCD83-227* mAb. (D) HD11 cells ( $100 \mu\text{l}$ ,  $1.0 \times 10^6$  cells) prepared as above were incubated for 45 min on ice with *chCD83-159* mAb. 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  $1.0 \times 10^4$  viable cells. The vertical bars in each panel represent the maximum fluorescence signals in the absence of primary antibody (i.e., background staining by secondary antibody alone). The horizontal bars in each panel represent positively staining cells, and the percentages of positively staining cells above background levels are listed in each panel.

rabbit anti-mouse IgG secondary Ab (1:1000) plus 4-chloro-1-naphthol substrate (Sigma).

## 2.5. Indirect immunofluorescence

Spleen, bursa of Fabricius, and caecal tonsil 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  $4^\circ\text{C}$  with *chCD83-227* mAb (1:200), followed by 30 min incubation with FITC-conjugated rabbit anti-mouse IgG secondary Ab (1:2000) (Lee et al., 2011a,b). 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. Concanavalin A-induced *chCD83* and MHCII expression

Freshly prepared spleen mononuclear cells were resuspended in RPMI 1640 medium containing 5% FBS, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin (Sigma). To determine the effect of concanavalin A (Con A) on *chCD83* expression, the cells ( $5.0 \times 10^6/\text{well}$ ) were seeded in 6-well plates and incubated at  $41^\circ\text{C}$  in a humidified incubator (Forma, Marietta, OH) with 5%  $\text{CO}_2$  for 0–8 days with 5.0  $\mu\text{g}/\text{ml}$  of Con A, or medium alone as a negative control. At the end of incubation, the cells were harvested, washed, and analyzed by flow cytometry with

*chCD83-227* mAb as described above. To assess the ability of *chCD83-227* mAb to inhibit MHCII expression, the cells were seeded and incubated as above with culture medium alone, 5.0  $\mu\text{g}/\text{ml}$  of Con A plus *chCD83-227* mAb, or Con A plus an isotype-matched negative control (NC) mAb. The cells were harvested, washed, and analyzed by flow cytometry with a chicken MHCII mAb (Lee et al., 2011c).

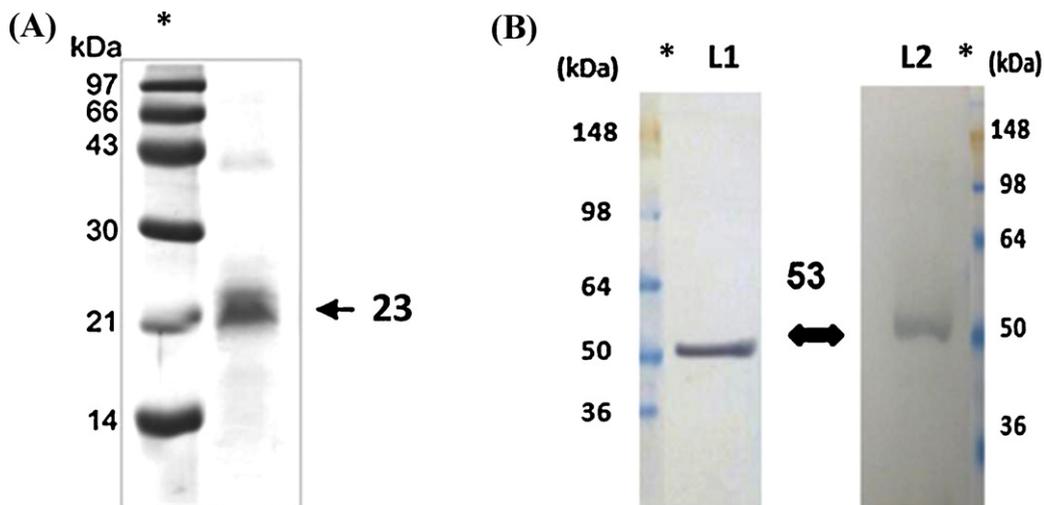
## 2.7. Statistical analysis

Statistical analyses were performed using SPSS 15.0 software for Windows. All data were expressed as mean  $\pm$  S.D. values ( $n=4$ ). Differences between mean values were compared using the Student's *t* test or the Duncan's multiple range test and were considered statistically significant at  $P < 0.05$ .

## 3. Results

### 3.1. Production of *chCD83* mAbs

Three hundred forty-two hybridomas were isolated from spleen cells of mice immunized with purified recombinant *chCD83* protein. Among these hybridomas, *chCD83-159* and *chCD83-227* secreted mAbs that stained the highest percentage (45% and 64%, respectively) of CHO cells stably transfected with *chCD83/IgG4* by flow cytometry (Fig. 1B and C). The *chCD83-159* mAb also stained 83% of HD11 macrophages (Fig. 1D). An irrelevant mAb



**Fig. 2.** Determination of the molecular size of *chCD83*. (A) Purified recombinant *chCD83* protein was resolved by reducing SDS-PAGE and stained with Coomassie blue. (B) Extracts of spleen mononuclear cells (L1) and HD11 cells (L2) were analyzed by Western blotting using *chCD83*-227 mAb. The asterisks (\*) indicate the position of protein molecular weight markers in kDa. The arrows indicate the 23 kDa (A) and 53 kDa (B) proteins.

against human T cells (HB2) stained less than 5% of CHO-*chCD83*/IgG4 cells (Fig. 1A) and of HD11 macrophages (data not shown). Therefore, these 2 hybridomas were cloned by limiting dilution and their mAbs secreted into the cell culture media were used for all subsequent analyses. Characterization of the remaining 340 hybridomas and their mAbs is currently ongoing in our laboratory. The *chCD83*-159 and *chCD83*-227 mAbs retained their binding activities for CHO-*chCD83*/IgG4 cells following re-cloning of their hybridomas, but were non-reactive with CHO cells transfected with an irrelevant gene, *chCD80*/IgG4, encoding the chicken CD80/IgG4 fusion protein (data not shown).

### 3.2. Determination of molecular size of *chCD83*

The recombinant *chCD83*/IgG4 protein in spent CHO cell culture medium was adsorbed to a protein G affinity column and the purified protein was released by enterokinase digestion. Coomassie blue-stained SDS-acrylamide gels under reducing conditions revealed a 23 kDa protein band corresponding to the extracellular region of *chCD83* (Fig. 2A). Western blot analysis of lysates from LPS-stimulated spleen cells and from unstimulated HD11 macrophage cells resolved under identical conditions and using *chCD83*-227 mAb identified a 53 kDa band (Fig. 2B).

### 3.3. Immunostaining in chicken lymphoid tissues with *chCD83* mAbs

Immunofluorescence staining of the caecal tonsils, bursa of Fabricius and spleen identified *chCD83*<sup>+</sup> cells in all tissues examined (Fig. 3). Morphologically, *chCD83*<sup>+</sup> cells were round, or elongated with long and thick processes reminiscent of mammalian DCs. In the caecal tonsils, *chCD83*<sup>+</sup> cells were present in the submucosa of diffuse lymphoid tissues and germinal centers (Fig. 3A). In the bursa of Fabricius, *chCD83*<sup>+</sup> cells were identified in the medulla and cortical area of the follicles, but not in the

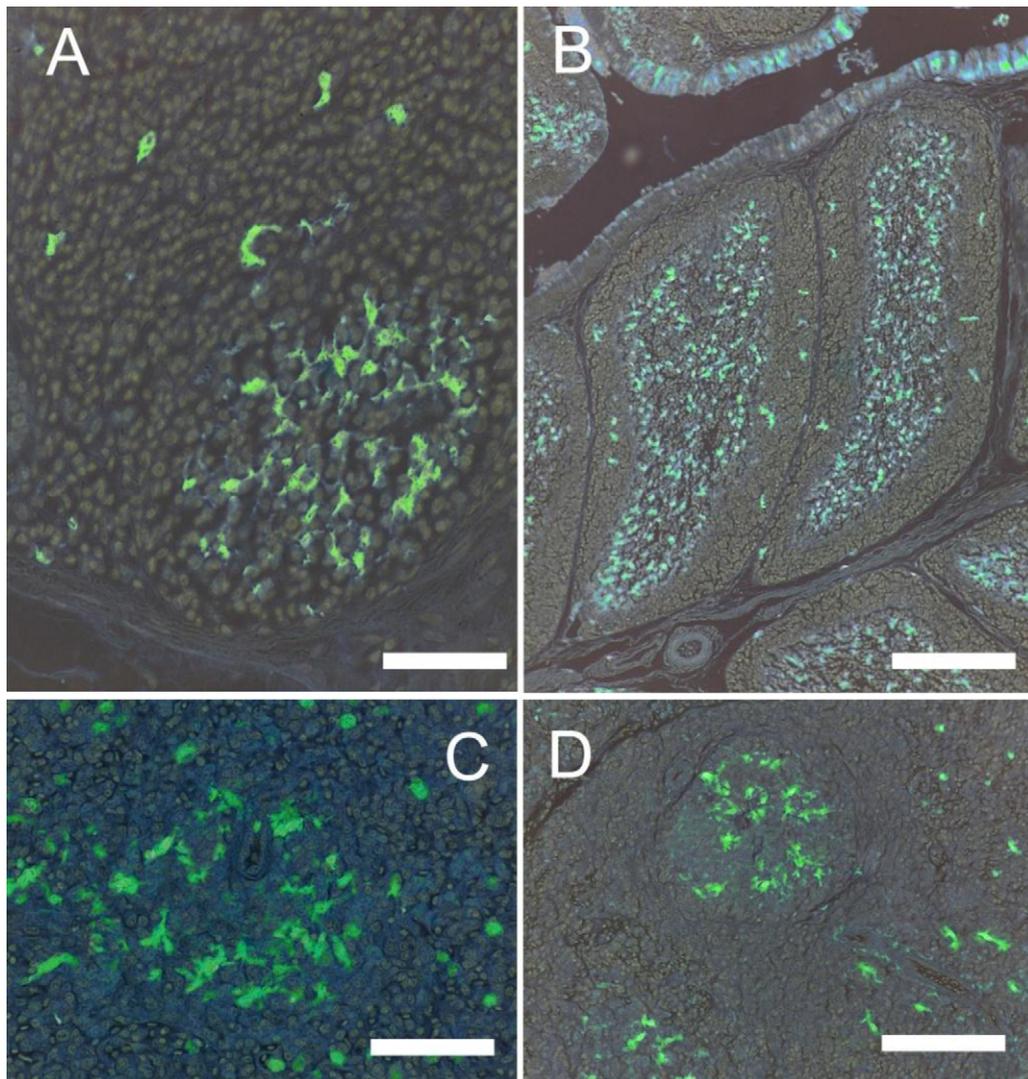
follicle-associated epithelium or the interfollicular areas (Fig. 3B). In the spleen, *chCD83*<sup>+</sup> cells were found around the penicilliform capillary, either on the surface of the ellipsoid or inside the ellipsoid (Fig. 3C). In addition, immunostaining cells were located in the red pulp and inside germinal centers, where positive cells formed a non-continuous network between lymphocytes in the periarteriolar lymphoid sheaths (Fig. 3D).

### 3.4. Con A-stimulated *chCD83* and MHCII expression

A previous report demonstrated that human CD83<sup>+</sup> DCs express cell surface ligands recognized by a variety of lectins, including Con A (El Sherbini et al., 2000). Therefore, we examined *chCD83* surface expression on spleen mononuclear cells following Con A treatment. Con A stimulation increased *chCD83* expression on splenocytes at day 1 post-treatment, but decreased *chCD83* expression at days 2–8 post-treatment (Fig. 4A). Next, we tested the ability of *chCD83* mAb to neutralize Con A-stimulated MHCII antigen expression based on the recent study that CD83 increases MHCII expression on mouse DCs (Tze et al., 2011). Treatment of spleen mononuclear cells with Con A plus *chCD83*-227 mAb for 1–8 days significantly reduced the percentage of MHCII<sup>+</sup> cells compared with cells treated with Con A plus an isotype-matched negative control (NC) mAb (Fig. 4B).

## 4. Discussion

The results of this study demonstrate that: (a) *chCD83*-159 and *chCD83*-227 mAbs reacted with a chicken macrophage cell line, HD11, (b) both mAbs recognized a 53 kDa protein on Western blots of lysates from LPS-stimulated spleen mononuclear cells or unstimulated HD11 cells, (c) fluorescent mAb staining of caecal tonsils, bursa of Fabricius, and spleen identified *chCD83*<sup>+</sup> cells similar to mammalian DCs, (d) Con A-stimulated spleen

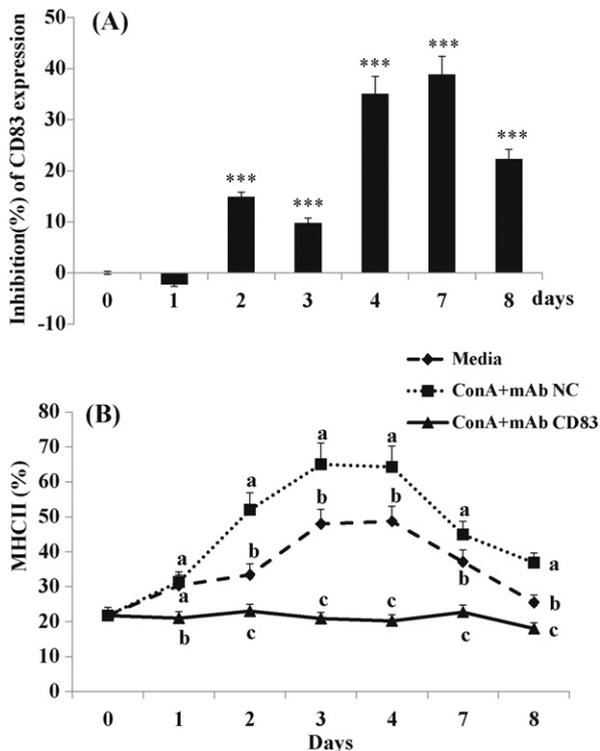


**Fig. 3.** Immunolocalization of *chCD83* in lymphoid organs. (A) Caecal tonsil, (B) bursa of Fabricius, and (C and D) spleen were frozen in liquid nitrogen and tissue sections were stained with *chCD83*-227 mAb for 18 h, followed by FITC-conjugated goat anti-mouse IgG secondary antibody for 30 min. Immunostaining cells were visualized by fluorescence microscopy. Scale bars represent 200  $\mu\text{m}$  (A), 100  $\mu\text{m}$  (B), 150  $\mu\text{m}$  (C), and 100  $\mu\text{m}$  (D).

mononuclear cells had a decreased percentage of *chCD83*<sup>+</sup> cells compared with cells treated with medium alone, and (e) spleen cells treated with Con A in the presence of *chCD83*-227 mAb exhibited a decreased percentage of MHCII<sup>+</sup> cells compared with cells treated with Con A plus an isotype-matched negative control mAb. Chicken CD83-159 mAb showed similar results to *chCD83*-227 mAb (data not shown).

CD83 is a highly sensitive and early activation marker of mammalian DCs (Zhou et al., 1992; Prazma et al., 2007). In these cells, the CD83 gene is expressed as a 205-amino acid polypeptide with a predicted molecular weight of 23 kDa (Prazma and Tedder, 2008). However, the mature CD83 protein expressed by normal cells has an observed molecular weight of 45–50 kDa due to N-linked glycosylation of its ectodomain (Zhou et al., 1992; Cao et al., 2005). Aberrant glycosylation of CD83 in the Hodgkin's disease-derived cell line, KM-H2, further increases its molecular

weight to 53 kDa (Hock et al., 2001). In this study, we identified a 53 kDa protein in lysates of spleen cells and HD11 macrophages that was greater than the observed 23 kDa of *chCD83* recombinant protein purified from CHO cells. Because CHO cells are reportedly deficient in some glycosyltransferases necessary for normal protein glycosylation (Bäckström et al., 2003), this discrepancy in molecular weights may be due to hypoglycosylation of the recombinant protein compared with the native molecule. It is also reported that glycosylation of native mammalian CD83 influences its immunoreactivity on Western blots (Lechmann et al., 2002). Alternatively, we cannot rule out the possibility that over-digestion of recombinant *chCD83* with enterokinase released a smaller molecular weight polypeptide fragment of the full-length *chCD83* from the protein G column, which was subsequently used to generate the *chCD83* mAbs recognizing the larger, intact protein.



**Fig. 4.** Effects of Con A on *chCD83* and MHCII expression. (A) Spleen mononuclear cells ( $5.0 \times 10^6$ ) were incubated with culture medium alone or  $5.0 \mu\text{g/ml}$  of Con A for 0–8 days, and analyzed by flow cytometry using *chCD83-227* mAb as described in Fig. 1. The percent inhibition of *chCD83* expression was calculated as  $(\% \text{ medium} - \% \text{ Con A}) / (\% \text{ medium}) \times 100$ , where % medium represents medium-cultured spleen mononuclear cells immunostained with *chCD83-227* mAb and % Con A represents Con A-cultured spleen mononuclear cells immunostained with *chCD83-227* mAb. Each value represents the mean  $\pm$  S.D. value ( $n=4$ ). \*\*\* $P \leq 0.001$  comparing % inhibition at days 1–8 with day 0 by the Student's *t*-test. (B) Spleen mononuclear cells ( $5.0 \times 10^6$  cells/ml) were incubated with culture medium alone, Con A plus *chCD83-227* mAb, or Con A plus isotype-matched negative control (NC) mAb for 8 days, and analyzed by flow cytometry for MHCII expression. Each value represents the mean  $\pm$  S.D. value ( $n=4$ ). Data points with different letters are significantly different ( $P \leq 0.05$ ) according to the Duncan's multiple range test.

As would be expected for MHCII expression following *in vitro* Con A stimulation (Lee et al., 2011a), we observed an increased percentage of MHCII<sup>+</sup> cells in Con A-stimulated splenocytes compared with unstimulated cells. Further, addition of *chCD83-227* mAb significantly inhibited the Con A-dependent MHCII<sup>+</sup> spleen cell expression. In accordance with the latter observation, Kretschmer et al. (2007) reported that decreased expression of CD83 on murine B cells was correlated with reduced LPS-stimulated MHCII expression. Unexpectedly, however, *in vitro* stimulation of spleen cells with Con A decreased the percentage of *chCD83*<sup>+</sup> cells compared with cells treated with medium alone. Because overexpression of CD83 interfered with calcium signaling, immunoglobulin secretion, and IL-10 production (Kretschmer et al., 2007) and reduced antigen-specific T cell-dependent and -independent antibody production by mouse B cells (Breloer, 2008), we hypothesize that down-regulation of *chCD83* expression following cell activation allows for normal maturation and function

of the relevant cell populations. Future studies are needed to define the immunoregulatory roles of *chCD83* in normal and disease states.

In summary, this paper describes the production and characterization of two mouse mAbs against *chCD83*. It seems that the *chCD83* expression pattern on ConA-activated splenocytes from chicken or mammals differ. The results collectively suggest that *chCD83* may play an important role in the adaptive immune response to avian pathogens and these *chCD83* mAbs are useful for investigations of chicken immune cell maturation and mechanisms of action. These mAbs will serve as valuable immune reagents for basic and applied research in poultry immunology.

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