Short communication

Development and characterization of mouse monoclonal antibodies reactive with chicken CD83

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A R T I C L E   I N F O
Article history:
Received 9 October 2011
Received in revised form
25 November 2011
Accepted 28 November 2011

Keywords:
Dendritic cell
Hybridoma
Maturation
Chicken CD83
Monoclonal antibodies

A B S T R A C T
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+ cells with morphologic and subcellular localization properties comparable to mammalian DCs. In vitro stimulation of spleen mononuclear cells with concanavalin A (Con A) decreased the percentage of chCD83+ 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+ 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; Prazman et al., 2007; Jin et al., 2010) and, to a lesser extent, on activated T and B lymphocytes (Kretschmer et al., 2007; Prazman 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 (MHCI), 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 (Prazman et al., 2007; Lutjhe 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; Prazman et al., 2007).

In humans and mice, CD83+ 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+ 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 chCD83 (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 rlgG4 portion by enterokinase digestion (EKMak, Invitrogen, Eugene, OR) performed on the protein G column. This procedure allowed the elution of the rCD83 in PBS after digestion, while the rlgG4 portion was still bound by protein G. Afterwards, the rlgG4 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% 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% NaN3 (Sigma), and/or 0.5% saponin for CHO cells stably transfected with chCD83/IgG4. The cells (1.0 × 10⁶/100 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 a FACS Calibur flow cytometer (BD, Franklin Lakes, NJ). Data was obtained from a total of 1.0 × 10⁶ 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...
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 °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 μg/ml streptomycin (Sigma). To determine the effect of concanavalin A (Con A) on chCD83 expression, the cells [5.0 × 10^5/well] were seeded in 6-well plates and incubated at 41 °C in a humidified incubator (Forma, Marietta, OH) with 5% CO₂ for 0–8 days with 5.0 μg/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 μg/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 ± 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
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+ cells in all tissues examined (Fig. 3). Morphologically, chCD83+ cells were round, or elongated with long and thick processes reminiscent of mammalian DCs. In the caecal tonsils, chCD83+ cells were present in the submucosa of diffuse lymphoid tissues and germinal centers (Fig. 3A). In the bursa of Fabricius, chCD83+ 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+ 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+ 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+ 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+ cells similar to mammalian DCs, (d) Con A-stimulated spleen...
mononuclear cells had a decreased percentage of chCD83+ 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 MHCI+ 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.
As would be expected for MHCII expression following in vitro Con A stimulation (Lee et al., 2011a), we observed an increased percentage of MHCII+ cells in Con A-stimulated splenocytes compared with unstimulated cells. Further, addition of chCD83-227 mAb significantly inhibited the Con A-dependent MHCII+ 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+ 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.

Acknowledgements

This project was supported, in part, by the National Research Initiative of the USDA, U.S. Veterinary Immune Reagent Network Grant (NIFA #2010-65121-20649, USDA-CSREES #2005-01812) and the Next-Generation BioGreen 21 Program (No. PJ008084), Rural Development Administration, Republic of Korea. The authors thank Ms. Myeong Seon Park, Dr. Duk Kyoung Kim, Ms. Marjorie Nichols, and Ms. Stacy Torreyson for their contribution to this research.

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