Generation and validation of novel anti-bovine CD163 monoclonal antibodies ABM-1A9 and ABM-2D6

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Abstract

The scavenger receptor CD163 is widely used as a cell signature for alternatively active "M2" macrophages in mammals. In this study, we generated two monoclonal antibodies, ABM-1A9 and ABM-2D6, against the extracellular region of bovine CD163. Conventional Western blotting using the antibodies yielded immunoreactive bands of bovine CD163 at 130 and 150 kDa in non-reduced and reduced spleen lysates, respectively. The minimum limit of detectable concentration of both antibodies was relatively lower (5.0 ng/mL) than that of the anti-human CD163 monoclonal antibody AM-3K (> 1.0 μg/mL), which has been used previously for the detection of bovine CD163. An immunohistochemical study using formalin-fixed paraffin-embedded sections revealed that ABM-1A9 and ABM-2D6 clearly stained some $Iba1^{+}$ macrophages in the lymph nodes of cattle with mastitis. Moreover, the CD163-stained macrophages were frequently observed engulfing leukocytes. ELISA using ABM-2D6 distinguished levels of circulating soluble CD163 in healthy cattle (less than 16.9 pmol/mL) and cattle with mastitis (more than 33.7 pmol/mL). These new monoclonal antibodies can be used in the diagnosis and evaluation of inflammatory disease

prognosis in cattle with immunohistological analyses and blood test applications.

Keywords

CD163; M2 macrophage; monoclonal antibody; inflammation; immunohistochemistry; ELISA

1. Introduction

Growing evidence indicates that inflammation is the basis for canonical inflammatory disease, as well as for many metabolic disorders (Bessueille and Magne, 2015; Freitas Lima *et al*., 2015; Saito *et al*., 2017). Accurate assessment of the inflammatory state will lead to further understanding of the pathology of a wide variety of diseases. In humans and mice, it has been proposed that the progression of inflammation is controlled by two distinct macrophage subpopulations: canonical inflammatory macrophages (also called "M1" or "M1-like") and alternatively active anti-inflammatory macrophages (also called "M2" or "M2-like") (Olefsky and Glass, 2010; Sica *et al*., 2015). M1 macrophages secrete pro-inflammatory cytokines such as IL-1β and TNF-α, while M2 macrophages secrete anti-inflammatory cytokines such as IL-10 and TGF-β (Sica *et al*., 2015). In addition, M2 macrophages phagocytose microbes and cellular debris, which can inhibit lesion expansion (A-Gonzalez *et al*., 2017). Although some studies suggest that there is impairment of M2 macrophages in inflammatory disease and metabolic disorders in cattle (Contreras *et al*., 2016; Thirunavukkarasu *et al*., 2015), the roles of the macrophage subpopulations in cattle

remain elusive. The lack of highly sensitive antibodies against bovine macrophage populations is a potential reason for this limitation.

CD163 belongs to the scavenger receptor cysteine-rich superfamily class B, and is widely used as a canonical anti-inflammatory macrophage marker in humans, mice, and dogs (Almeida *et al*., 2017; Kurahara *et al*., 2011; Martínez-Fernández *et al*., 2017). CD163 contributes to the endocytosis of the hemoglobin-haptoglobin complex, as well as circulating neutrophils to prevent local inflammation (A-Gonzalez *et al*., 2017; Kristiansen *et al*., 2001). Moreover, CD163-dependent phagocytosis activates the gene expression of anti-inflammatory molecules in macrophages (Alvarado-Vazquez *et al*., 2017). There are two distinct forms of CD163, membrane-associated and secreted, both of which attenuate the inflammatory response (Moestrup and Møller, 2004). Patients suffering from inflammatory disease exhibit higher circulating soluble CD163; serum CD163 levels have thus been used as an inflammation marker in human blood tests (Etzerodt and Moestrup, 2013; Møller, 2012; Møller *et al*., 2002).

To date, bovine CD163 has been detected using commercially available anti-human CD163 antibodies. Yamate *et al*. (2000) demonstrated that the human CD163

monoclonal antibody AM-3K stained cells in Zamboni's solution-fixed paraffin sections of lymph nodes. More recently, the monoclonal antibody MCA1853 stained some Iba-1^{+} macrophages in formalin-fixed and paraffin-embedded bovine lung sections (Fry *et al*., 2016). Despite these pioneering studies, it is still unclear whether these antibodies are widely applicable to bovine CD163 detection using different techniques, such as Western blot analysis and enzyme-linked immunosorbent assay (ELISA). In this study, we generated two monoclonal antibodies against the extracellular region of bovine CD163 and validated them using several different analytical methods.

2. Materials and Methods

Animals

Bovine spleen and lymph node samples were collected from cattle afflicted with bacterial mastitis at the time of pathology dissection. Serum samples were obtained from blood that was collected from the jugular vein of clinically healthy cattle as well as individuals with mastitis, centrifuged at 1,200 × g for 10 min, and stored at –80°C. Detailed information on the cattle used in this study is shown in Table 1.

The mouse spleen sample was obtained from a male C57BL/6 strain mouse (Japan SLC, Hamamatsu, Japan) at 20 weeks of age after cervical dislocation under anesthetization with IsoFlo (Abbott Laboratories, IL, USA).

BALB/c mice (Japan SLC) were subcutaneously inoculated at seven weeks of age with a suspension mixture of complete Freund's adjuvant (Difco Laboratories, Franklin Lakes, NJ, USA) and N-terminal keyhole limpet hemocyanin (KLH)-conjugated synthetic peptide (sequence CGLALEAGKEA) corresponding to amino acids 975–985 of bovine CD163 (NCBI accession: NP001156885.1) under anesthesia with IsoFlo. Three weeks later, a secondary immunization was performed with the same dose of the antigen with incomplete Freund's adjuvant (Difco Laboratories). After two additional immunizations of the antigen at intervals of three weeks, a booster injection was given two days before the splenocytes were harvested.

The Ethics Review Committees for Animal Experimentation of Rakuno Gakuen University (Approval Numbers VH15A13 and VH16A22) and Hokkaido University (Approval Number 14-0023) approved all animal experiments.

Generation of hybridoma cells

The splenocytes (1×10^8 cells) isolated from the immunized mice were fused with mouse myeloma SP2 cells (2×10^7 cells; Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer Tohoku University, Sendai, Japan) in the presence of polyethylene glycol solution (Sigma-Aldrich, St. Louis, MI, USA). Hybridoma cells were selected using the serial dilution method in GIT medium (Wako, Osaka, Japan), which was supplemented with 5% Briclone (Archport, Dublin, Ireland) and hypoxanthine, aminopterin, and thymidine media (Sigma-Aldrich). The culture supernatants were stored at 4°C and 0.02 % sodium azide was added until purification.

Purification of anti-CD163 monoclonal antibodies

The isotype of the monoclonal antibodies that accumulated in the culture media was determined using an IsoStrip mouse monoclonal antibody isotyping kit (Sigma-Aldrich). The media containing anti-CD163 monoclonal antibodies were concentrated using Amicon Ultra-15 Centrifugal Filter Units (Merck Millipore, Darmstadt, Germany), and

were further purified with Protein G GraviTrap (GE Healthcare, Buckinghamshire, UK) according to the manufacturers' protocols.

Western blot analysis

Western blot analysis was performed as previously described with minor modifications (Kitamura *et al*., 2017). Briefly, the spleen samples isolated from the cattle and mice were homogenized in RIPA buffer (50mM Tris/HCl, 150mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA) containing a protease inhibitor cocktail (Nacalai) using an MM300 (Retsch, Haan, Germany). The concentration of each lysate was determined using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The lysates were boiled in Laemmli sample buffer [120mM Tris HCl (pH 6.8), 4% SDS, 20% glycerol, 0.02% bromophenol blue] in the presence or absence of 5% 2-mercaptoethanol for 5 min, and were then separated by SDS-PAGE using a 6% acrylamide gel. The electrophoresed samples were transferred to a PVDF membrane (Merck Millipore) with a semi-dry blotter, HorizeBLOT 2M-R (ATTO, Tokyo, Japan). After blocking with Blocking One solution (Nacalai, Kyoto, Japan), the membranes

were reacted with anti-CD163 antibodies, ABM-1A9 (0.5 μg/mL), ABM-2D6 (0.5 μg/mL), AM-3K (1.0 μg/mL; KT013, Trans Genic, Kobe, Japan), or anti-α-tubulin rabbit antibody (Cell Signaling Technology, Danvers, MA) in 5% Blocking One solution containing Tris buffered saline with 0.1% Tween20. The membranes were then treated with a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (A16072, Thermo Fisher Scientific) as a secondary antibody. The immunoreactive bands were visualized with Chemi-Lumi One Super reagent (Nacalai) and monitored using a LuminoGraph I imaging system (ATTO, Tokyo, Japan).

Immunohistochemistry

Formalin-fixed bovine paramammary lymph nodes were dehydrated in a graded series of ethanol and embedded in paraffin according to conventional pathological methods. Sections 5 μm thick were deparaffinized and blocked with Block Ace (DS Pharma Biomedical, Osaka, Japan) for 60 minutes. The sections were then incubated with mouse anti-CD163 antibodies, ABM-1A9, ABM-2D6 (1.0 μg/mL), AM-3K (10 μg/mL, Trans Genic), or rabbit anti-Iba1 antibody (1:1,000; 019-19741, Wako) in phosphate

buffered saline (PBS) overnight. Sections were then incubated with biotinylated anti-mouse or anti-rabbit IgG (1:200; BA9200 and BA1000, Vector Laboratories, Burlingame, CA, USA) for 1 hour and subsequently stained using a Vectastain ABC Elite kit (Vector Laboratories) for 1 hour. The signals were visualized with 0.01% 3,3'-diaminobenzidine in 0.05M Tris-HCl buffer (pH 7.6) containing 0.003% H_2O_2, counterstained with hematoxylin, and observed under a light microscope (BX51; Olympus, Tokyo, Japan).

For dual immunohistochemistry, the sections were incubated with mouse anti-CD163 (clone 1A9; 0.5 μg/mL) overnight, then incubated with AlexaFluor 488-conjugated anti-mouse IgG (1:200; A21202, Thermo Fisher Scientific) for 2 hours. After washing with PBS, the sections were blocked with Block Ace for 1 hour and incubated with rabbit anti-Iba1 antibody (1:500) overnight. They were then incubated with AlexaFluor 594-labeled anti-rabbit antibody (1:200; A21207, Thermo Fisher Scientific) for 2 hours and observed under a confocal laser microscope (FV300; Olympus, Tokyo, Japan).

ELISA for CD163

Synthetic CD163 bovine (molecular weight, 1061.2; COSMO BIO, Tokyo Japan), human (molecular weight, 1132.5; COSMO BIO), or mouse (molecular weight, 1115.4; COSMO BIO) peptides and 10-fold diluted serum samples were incubated in microplates overnight at 4°C and blocked with 1% ovalbumin (Wako) at 37°C for 1 hour. One μg/mL of ABM-2D6, MCA1853 (Bio-Rad), AM-3K, or normal IgG1 (Sigma Aldrich, M5284) was added to each well and incubated at 37°C for 90 minutes. After washing the plates, HRP-conjugated anti-mouse IgG (1: 5000; A16072, Thermo Fisher Scientific) and 0.1% 3,3',5,5'-tetramethylbenzidine (TMB; Sigma-Aldrich) in 0.05M phosphate-citrate buffer (pH 5.0) containing 0.006% H_2O_2 were sequentially added to each well while avoiding exposure to light. The color reaction with H_2SO_4 was measured at an optical density wavelength of 450 nm using an iMark plate reader (Bio-Rad Laboratories, Hercules, CA, USA). The CD163 peptide detection limit in this assay was 0.5 pmol/mL.

Measurement of serum C-reactive protein (CRP) levels

Serum CRP level, a well-known inflammatory marker in cattle (Lee *et al*., 2003), was also measured using a sandwich ELISA kit for bovine CRP (Cloud-Clone Corp, Houston, TX, USA) according to the manufacturer's instructions.

Statistical analysis

Welch's t-test was performed using Prism version 6 (GraphPad Software, San Diego, CA, USA). Pearson's correlation test was performed to evaluate the relationship between soluble CD163 and CRP levels in the serum samples.

3. Results

3.1. Establishment of ABM-1A9 and ABM-2D6 as novel anti-CD163 antibodies and validation of their specificity and sensitivity

In order to obtain monoclonal antibodies against bovine CD163, we selected the peptide sequence for immunization as follows: 1) avoided the transmembrane region, 2) avoided ambiguous N- and C-terminals, 3) avoided N- and O-glycosylation sites, and 4) chose a sequence that has cysteine residue for KLH conjugation (Fig. 1A). The resultant

CGLALEAGKEA sequence was located 62-73 amino acids on the N-terminal side of the transmembrane region, and did not show any sequence similarity with human and mouse CD163 sequences (Fig. 1B).

Both monoclonal antibodies ABM-1A9 and ABM-2D6 were IgG1 isotypes composed of a kappa light chain. We conducted Western blot analysis in order to determine their specificity. Both antibodies detected CD163 at approximately 150 kDa when more than 1 μg of the splenic protein lysate isolated from cattle with mastitis was loaded (Fig. 2A). Since human CD163 has been reported to be detected around 150 kDa by AM-3K (Komohara *et al*., 2006), it is likely that ABM-1A9 and ABM-2D6 can successfully detect bovine CD163. In addition, the antibodies also presented a weak band (around 120 kDa), which may be a non-specific signal or a cleavage product signal. Similar results were obtained using lung samples (data not shown).

When we examined the detection sensitivity of ABM-1A9 and ABM-2D6 with more than 5.0 ng/mL of ABM-1A9, a significant CD163 band was observed on conventional Western blot membranes when 10 μg of the spleen sample was loaded (Fig. 2B). Sensitivity of ABM-2D6 (detectable concentration greater than 5.0 ng/mL)

was comparable to that of ABM-1A9. In contrast, the commercially available anti-human CD163 antibodies, AM-3K, hardly detected CD163 in the same sample (Fig. 2C), even though a two-fold higher concentration (10 ng/mL) was used. Therefore, the detection sensitivity of ABM-1A9 and ABM-2D6 to bovine CD163 is relatively high using Western blot analysis.

We also assessed the cross-reactivity of the antibodies against mouse CD163. As predicted by the sequence analysis, both ABM-1A9 and ABM-2D6 failed to detect CD163 in mouse spleen, although a significant amount of α-tubulin was detected in the samples (Fig. 2D). Similarly, ELISA using ABM-1A9 and ABM-2D6 (1.0μg/mL) gave intense immune signals against a synthetic peptide corresponding to the surface region of bovine CD163, while neither gave a significant signal against the human and mouse peptides. Conversely, MCA1853 and AM-3K did not recognize the bovine, human, or mouse CD163 peptides (Fig. 3). Collectively, these results indicate that ABM-1A9 and ABM-2D6 antibodies recognize the surface portion of the bovine CD163 peptide and that the epitope of the antibodies is different from those of the commercially available monoclonal antibodies MCA1853 and AM-3K.

Previously, Komohara *et al*. reported a difference in electrophoretic mobility of purified human CD163 in the presence or absence of a reducing agent. To evaluate whether reducing conditions affect the electrophoretic mobility of bovine CD163, we checked the molecular size of bovine CD163 in non-reduced spleen lysate. Instead of a major 150 kDa band like that observed in the reducing condition, only a single band at 130 kDa was observed (Fig. 4).

3.2. Detection of $CD163^+$ cells in formalin-fixed paraffin-embedded sections using ABM-1A9 and ABM-2D6

Application of antibodies to formalin-fixed paraffin-embedded sections in order to make a clinical diagnosis is desirable. Thus, we subjected ABM-1A9 and ABM-2D6 on paraffin-embedded bovine lymph node samples using immunohistochemistry techniques. Antibodies against the pan-macrophage marker Iba-1 gave strong signals, especially in the lymph node (Fig. 5A). Iba-1^+ macrophages displayed a variety of morphological forms: large macrophages engulfing leukocytes and small macrophages with condensed nuclei. On the other hand, ABM-1A9 (1.0 μg/mL) and ABM-2D6 (1.0

μg/mL) mostly stained macrophages phagocytosing up to three leukocytes (Figs. 5B, C, E-G). The immunoreactive signals of AM-3K were scarcely observed and the stained cells did not exhibit the engulfment of leukocytes (Fig. 5D). Given that phagocytosis is a well-documented feature of $CD163^+$ and $CD206^+$-macrophages (A-Gonzalez *et al.*, 2017), the use of ABM-1A9 and ABM-2D6 could be applied to selectively stain $CD163^+$ and $CD206^+$ macrophages in formalin-fixed paraffin-embedded bovine sections.

3.3. Measurement of circulating soluble CD163 with ABM-2D6

Møller (2012) demonstrated that soluble CD163 is a useful index of the inflammatory state in human blood tests. To examine this, we assessed whether ABM-2D6 could discriminate different blood-soluble CD163 concentrations in healthy cattle and in cattle with mastitis. Indirect ELISA using ABM-2D6 (1.0 μg/mL) showed a significant increase in circulating soluble CD163 in cattle with mastitis compared to healthy cattle (Fig. 6A). A similar increase was also observed in serum CRP levels using the same samples (Fig. 6B). Pearson correlation analysis showed that serum CD163 levels were

significantly correlated with serum CRP levels (Fig. 6C). Therefore, ABM-2D6 is useful in the diagnosis of inflammatory disease in cattle using blood samples.

4. Discussion

In this study, we generated two novel monoclonal antibodies, ABM-1A9 and ABM-2D6, to detect bovine CD163. These antibodies enabled us to detect CD163 at 130 kDa in non-reduced bovine tissue lysate by conventional Western blot analysis. This result is consistent with previous results using LND37A and LND68A (Elnaggar *et al*., 2016). On the other hand, Komohara *et al*. reported that purified human CD163 was observed at 150 kDa under reducing conditions in an SDS-PAGE analysis (Komohara *et al*., 2006). Our results also indicate that bovine CD163 is detected as 150 kDa under reducing conditions. Collectively, these results indicate that the three-dimensional structure of bovine CD163 critically affects electrophoretic mobility. Since the putative molecular weight of bovine CD163 is 122 kDa based on amino acid sequence, the size of the detected band (150 kDa) under reducing conditions is much larger. A high degree of glycosylation of bovine CD163 might increase actual molecular weight in

experimental samples. In support of this idea, deglycosylation of human CD163 revealed a band at approximately 130 kDa, although prior to deglycosylation the size of the protein was around 150 kDa (Komohara *et al*., 2006). AM-3K, a commercially available antibody, presented only a 70 kDa band in a Western blot analysis when samples from humans, rabbits, guinea pigs, cats, and dogs were tested (Yamate *et al*., 2000; Zeng *et al*., 1996). Given that the size of mature CD163 is about 150 kDa, AM-3K might only detect a cleavage product.

Both ABM-1A9 and ABM-2D6 stained cells engulfing leukocytes in the lymph node sections. The staining pattern seems reasonable because CD163 is a molecule participating in phagocytosis of the hemoglobin-haptoglobin complex, cellular debris, and neutrophils (A-Gonzalez *et al*., 2017; Kristiansen *et al*., 2001). Thus, ABM-1A9 and ABM-2D6 are useful to detect phagocytic, anti-inflammatory macrophages. Notably, both antibodies could detect engulfing macrophages in formalin-fixed paraffin-embedded sections while AM-3K did not consistently detect bovine CD163 in formalin-fixed samples (Fig. 5, also described by Yamate *et al*., 2000). Since formalin fixation and paraffin embedding are widely used in conventional pathological tests,

ABM-1A9 and ABM-2D6 have the potential to provide improved knowledge about inflammation from conventional clinical samples.

The accumulation of antibody data facilitates researchers to select antibodies suitable to their demand. Elnaggar *et al*. (2016) recently established monoclonal antibodies against ruminant CD163 that are suitable for FACS analysis. Moreover, MCA1853, an anti-human CD163 antibody, clearly detected an increase in macrophage-like cells in inflamed lung tissue (Fry *et al*., 2016). At least, commercial available antibodies, AM-3K and MCA1853, were raised against recombinant full length or native human CD163. Thus, both of them can be applied for detecting CD163 in multiple species. On the other hand, our antibodies were raised against regions that show low similarity in the first structure between ruminants and other mammalian species. The epitopes that our antibodies bind to are therefore likely to be different from those of anti-human CD163 antibodies. The presence of several monoclonal antibodies that recognize different epitopes of the same protein helps in the generation of various diagnostic tools such as sandwich ELISA and bead array systems (Kingsmore, 2006).

Phagocytic macrophages with high expression of $CD163^{+}$ and $CD206^{+}$ receptors

form a distinct population of canonical M2 macrophages in mice (A-Gonzalez *et al*., 2017). $CD163^+$ and $CD206^+$ phagocytic macrophages also exhibit an anti-inflammatory phenotype showing decreased expression of IL-1β. Thus, taking advantage of our new antibodies together with other macrophage antibodies, knowledge of cellular mechanisms underlying bovine inflammatory diseases can be improved. The ratio between inflammatory and anti-inflammatory macrophages in adipose tissue is a determinant of metabolic disorders, including Type 2 diabetes mellitus and adiposity (Kitamura *et al*., 2013; Olefsky and Glass, 2010). Assessment of anti-inflammatory macrophages using ABM-1A9 and ABM-2D6 therefore may lead to a deeper understanding of the cellular basis of metabolic disorders in cattle, such as lipomobilization syndrome and adiponecrosis.

Increasing literature indicates that circulating levels of soluble CD163 is useful as a biomarker of macrophage activation and inflammation in humans. For example, the blood concentration of soluble CD163 is associated with the pathology of rheumatoid arthritis (Matsushita *et al*., 2002), pulmonary tuberculosis (Knudsen *et al*., 2005), chronic HIV and hepatitis C infection (Kazankov *et al*., 2014; Subramanian *et al*., 2012),

and periodontitis (Detzen *et al*., 2017). Moreover, soluble CD163 is elevated in patients suffering from metabolic disorders, including Type 2 diabetes mellitus (Parkner *et al*., 2012). This index is also applicable to dogs with leishmaniasis (Almeida *et al*., 2017), indicating that soluble CD163 usefulness is not limited to human samples. In the current study, we demonstrated that cattle with mastitis exhibited a significant increase in circulating soluble CD163, parallel with an increase in CRP levels. It is noteworthy to mention that there are differences in the roles of CD163 and CRP as inflammatory markers; CRP is produced in the liver by circulating inflammatory cytokines such as IL-1, IL-6, and TNF-α, during systemic inflammation, while CD163 is released from macrophages that accumulate in lesions by an ADAM17/10-mediated mechanism (Zhi *et al*., 2017). Soluble CD163 may thus be advantageous in the diagnosis of local inflammation. Further studies are necessary to clarify the effectiveness of soluble CD163 measurements in the prognosis and diagnosis of various diseases in cattle.

5. Conclusion

We established two novel anti-bovine CD163 monoclonal antibodies, ABM-1A9 and

ABM-2D6. Concentrations greater than 5.0 ng/mL of both antibodies specifically detected CD163 in bovine tissue lysate by conventional Western blot analysis. These antibodies are also applicable in the immunohistological detection of engulfing macrophages using formalin-fixed paraffin-embedded sections. Moreover, the antibody ABM-2D6 can be used in indirect ELISA tests to detect soluble CD163 in blood samples. Taken together, ABM-1A9 and ABM-2D6 are potentially useful in the clinical diagnosis of various bovine diseases.

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Figure Captions

Figure 1. The amino acid sequence of CD163. A: The amino acid sequence of CD163 in cattle (NCBI accession number NP_001156885.1), with the synthetic peptide sequence used as an antigen in this study highlighted in grey. The keyhole limpet hemocyanin conjugation site, Cysteine (C), is shown in red; predicted O-glycosylation sites, Serine (S) and Threonine (T), are shown in blue; and the N-glycosylation site, Asparagine (N), is shown in green. The predicted transmembrane region is double-underlined. **B**: The amino acid sequence alignment of CD163 in cattle, humans, and mice. The amino acid sequences of mouse and human CD163 were obtained from the NCBI with accession numbers NP_004235.4 and NP_001163866.1, respectively. The numbers on the right show the coordinates of the proteins. The framed region shows the amino acid sequence of the synthetic peptide used in this study. Different residues are highlighted in grey. Sequence identities in this region were 64% (between cattle and mouse) and 73% (between cattle and human).

Figure 2. Western blot analysis using reduced spleen lysate prepared from a cow

with mastitis. A: Specific bands immunoreacted with the antibodies ABM-1A9 (left) and ABM-2D6 (right) according to the protein content detection limit (the concentration of both antibodies used for detection was 0.5 μg/mL). The reduced spleen lysate was subjected to SDS-PAGE followed by Western blot. The respective amounts of protein applied to each lane are as follows: Lane M, molecular marker; 1, 10 μg; 2, 5.0 μg; 3, 2.5 μg; 4, 1.0 μg; 5, 0.5 μg; and 6, 0.1 μg. **B**: Determination of the detectable concentration required for Western blot analysis with 10 μg of reduced bovine spleen lysate applied to each lane. After SDS-PAGE and transblotting to a PVDF membrane, the membrane strip of each lane was cut and immunoreacted with different concentrations of the monoclonal antibodies ABM-1A9 (left) and ABM-2D6 (right). Lane M, molecular marker; 1, 0.5 μg/mL; 2, 0.1 μg/mL; 3, 0.05 μg/mL; 4, 0.01 μg/mL; 5, 5.0 ng/mL; and 6, 1.0 ng/mL. **C**: Detection sensitivity comparison of anti-CD163 monoclonal antibodies, with 10 μg of reduced bovine spleen lysate applied into each lane. The membrane strip of each lane was cut and immunoreacted with ABM-1A9 (0.5 μg/ml), ABM-2D6 (0.5 μg/ml), or AM-3K (1.0 μg/ml). After stripping the monoclonal antibodies, these membrane strips were immunoreacted with anti-α-tubulin (TUBA)

antibody as an internal control. Lane M, molecular marker; 1, ABM-1A9; 2, ABM-2D6; 3, AM-3K; and 4, AM-3K (overexposed for 20 minutes). **D**: Cross-reactivity of ABM-1A9 and ABM-2D6 with 10 µg of bovine (lane 1) or 20 µg of mouse (lane 2) reduced spleen lysate. Each membrane was immunoreacted with 0.5 µg/ml of ABM-1A9 (left) or ABM-2D6 (right).

Figure 3. Immunoreactivity of monoclonal antibodies with synthetic CD163 peptides using indirect ELISA. Synthetic CD163 bovine (black bar: CGLALEAGKEA), human (grey bar: CGQAVKALKEA) and mouse (dark grey bar: CGPALKAFKEA) peptides were used as antigen at a content of 0.3 pmol. Data are shown as the optical density at 450 nm (OD 450) ± standard deviation (SD). The horizontal axis shows the antibodies used. The concentration of all antibodies used was 1 µg/mL. Normal IgG1 was used as an isotype control. The assay was performed in triplicate.

Figure 4 Comparison of electrophoretic patterns of reduced and non-reduced

spleen lysate prepared from a cow with mastitis. Effect of the reducing agent on electrophoretic mobility in Western blot. Ten μg of reduced (lane 1) or non-reduced (lane 2) bovine spleen lysate were applied to each lane. Each membrane was immunoreacted with 0.5 μg/ml of ABM-1A9 (left) or ABM-2D6 (right).

Figure 5. Immunohistochemistry of formalin-fixed paraffin-embedded sections of paramammary lymph nodes from a cow with mastitis. A: Immunohistochemistry using anti-Iba-1 antibody. Macrophages immunoreacted with anti-Iba-1, emitting a strong signal (arrow) and displaying a variety of morphological forms. **B** and **C**: Immunohistochemistry using ABM-1A9 and ABM-2D6. $CD163^{+}$ cells immunoreacted with ABM-1A9 (arrows) and ABM-2D6. Most of these cells were large and engulfing leukocytes (asterisks). **D**: Immunohistochemistry using AM-3K. AM-3K detected hardly any macrophages despite using a 10-fold higher concentration (10 μg/mL) of antibody than that of the ABM antibodies. **E-G**: Fluorescent immunohistochemistry using ABM-1A9 and an anti-Iba-1 antibody. Cells that immunoreacted with ABM-1A9 (E) and an anti-Iba-1 antibody (F) emit green and red fluorescence, respectively. G:

Merging E and F (arrows) indicates the existence of CD163-positive cells among Iba-1-positive macrophages.

Figure 6. Serum levels of inflammatory markers in cattle. A: Serum CD163 levels and **B**: Serum CRP levels in healthy cattle and cattle with mastitis. Data are shown with box plots using samples from healthy cattle (n = 18) and cattle with mastitis (n = 3). The assay was performed in duplicate. Welch's t-test was used for data analysis with *** showing statistical significance ($P < 0.001$). **C**: Correlation between serum CD163 levels and serum CRP levels in healthy cattle and cattle with mastitis. Number of samples is the same as in Figs. 6A and 6B. Pearson's correlation test was performed and the correlation coefficient r was 0.82 ($P < 0.001$).

Highlights

- Western blot analysis using ABM-1A9 and ABM-2D6 detected bovine CD163 with high sensitivity.
- ABM-1A9 and ABM-2D6 stained phagocytic macrophages in formalin-fixed sections.
- ELISA using ABM-2D6 is applicable in measuring circulating soluble bovine CD163.

Table 1. Individual details of cattle used in this study with serum and pathology samples collected.

Samples		Normal	Mastitis
Serum		n = 18	n = 3
	Breed	Holstein	Holstein
	Age (month)	54.8 range (30 - 73)	126 range (88 - 158)
	Lactation cycle	Lactating period（15）, Dry period (3)	Dry period (3)
Spleen and lymph node			n = 1
	Breed		Holstein
	Age (month)		48
	Lactation cycle		Lactating period

A

MVLHDNSGSAGFKRCSVHFGPFTLAVVSVLYACLITSALGGTDKELRLVAGQTKCSGRVE
VKVQEEWGTVCNTGWDLAAVSVVCKQLGCPSVIKATGWTNSSAGTGRIWMDHVSCRGNES
ALWDCKHEGWGKHNCTHQQDVGVTCSDGSDLEMRLMNGGNRCSGRIEIKFQGQWGTVCDD
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NCDHAEDAGVICLEGADLSLRLVDGVTKCSGRLEVRFQGEWGTVCDDGWDSDDAAVACQQ
LGCPTAITAVGRVNASEGTGHIWLDSVSCQGHESAVWQCRHHEWGKHYCNHNEDAGVTCS
DGSDLELRLKGGGSRCAGTVEVEIQKLIGKVCDRSWGLKEADVVCKQLGCGSALRTSYQV
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CSGRVEVKHGDTWGTICDSDFSLESASVLCRELECGSVVSILGGAHFGEGNGQIWAEEFQ
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KCSEIAESKGSVKAAGHSSTVALGILGVILLAFLIATLLWIQRRRQRQRLAVSSRGENSV
HEIQYREMNSCLKADDLDLYNSSGLWVLRGSIALGFRLVTAAEAERHST

B

Cattle	RQLGCGLALE AGKEAAFGQG	981
Mouse	KQLGCGQAVK ALKEAAFGPG	988
Human	QQLGCGPALK AFKEAEFGQG	987

Figure 1 Shimamoto *et al.*

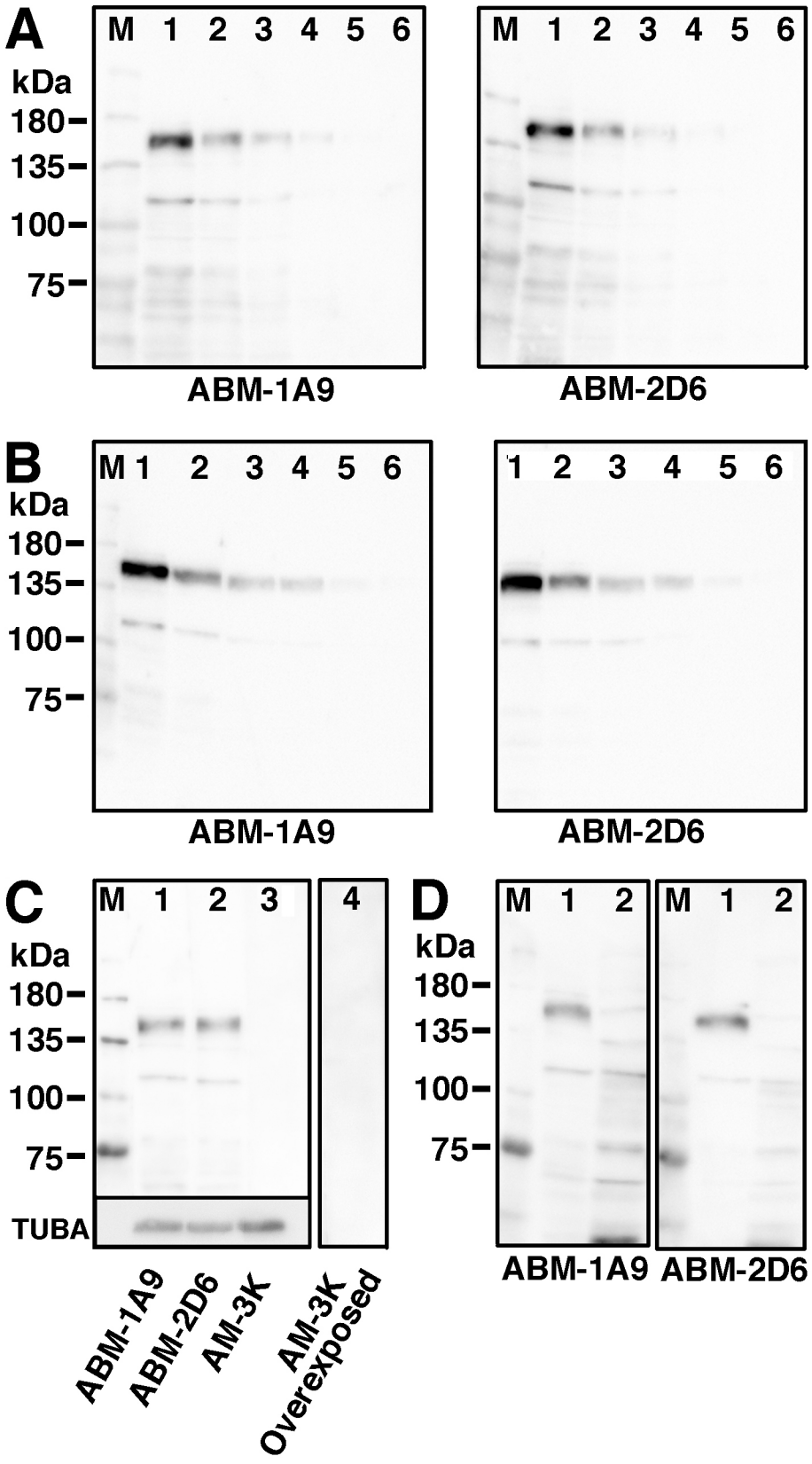


Figure 2 Shimamoto *et al.*

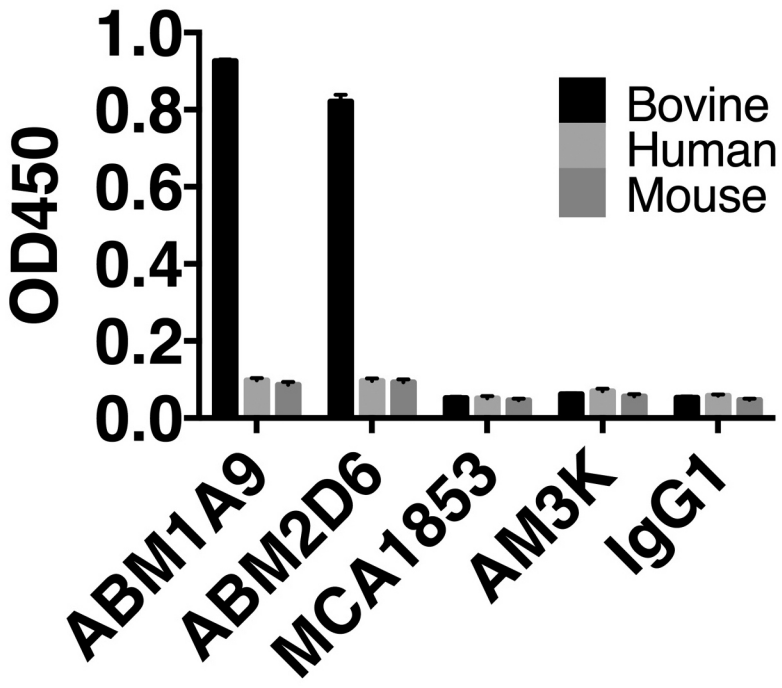
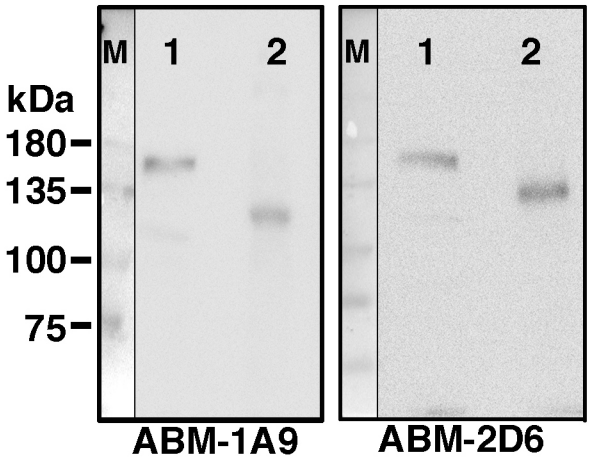


Figure 3 Shimamoto *et al.*



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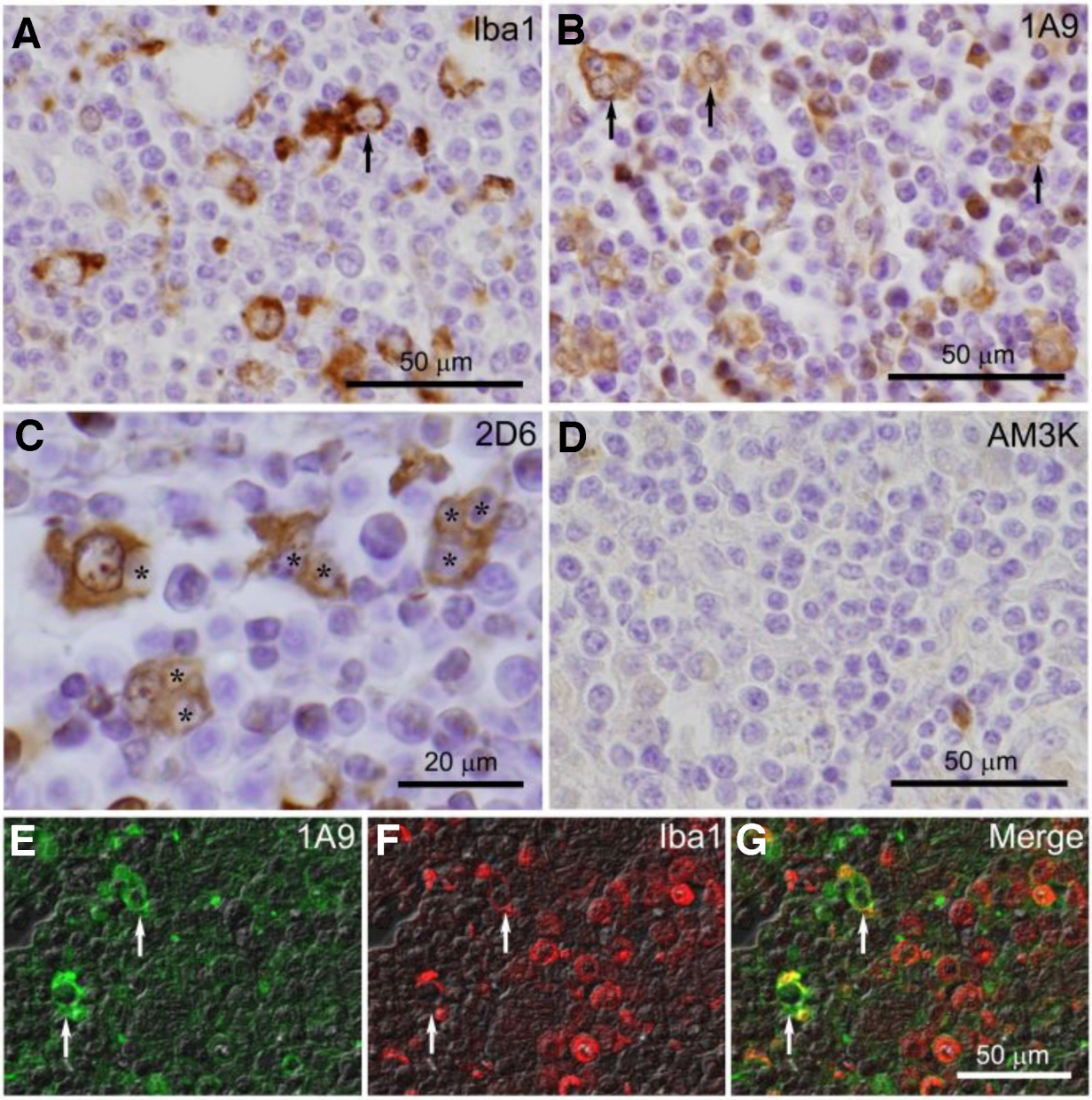

Figure 5 Shimamoto *et al.*

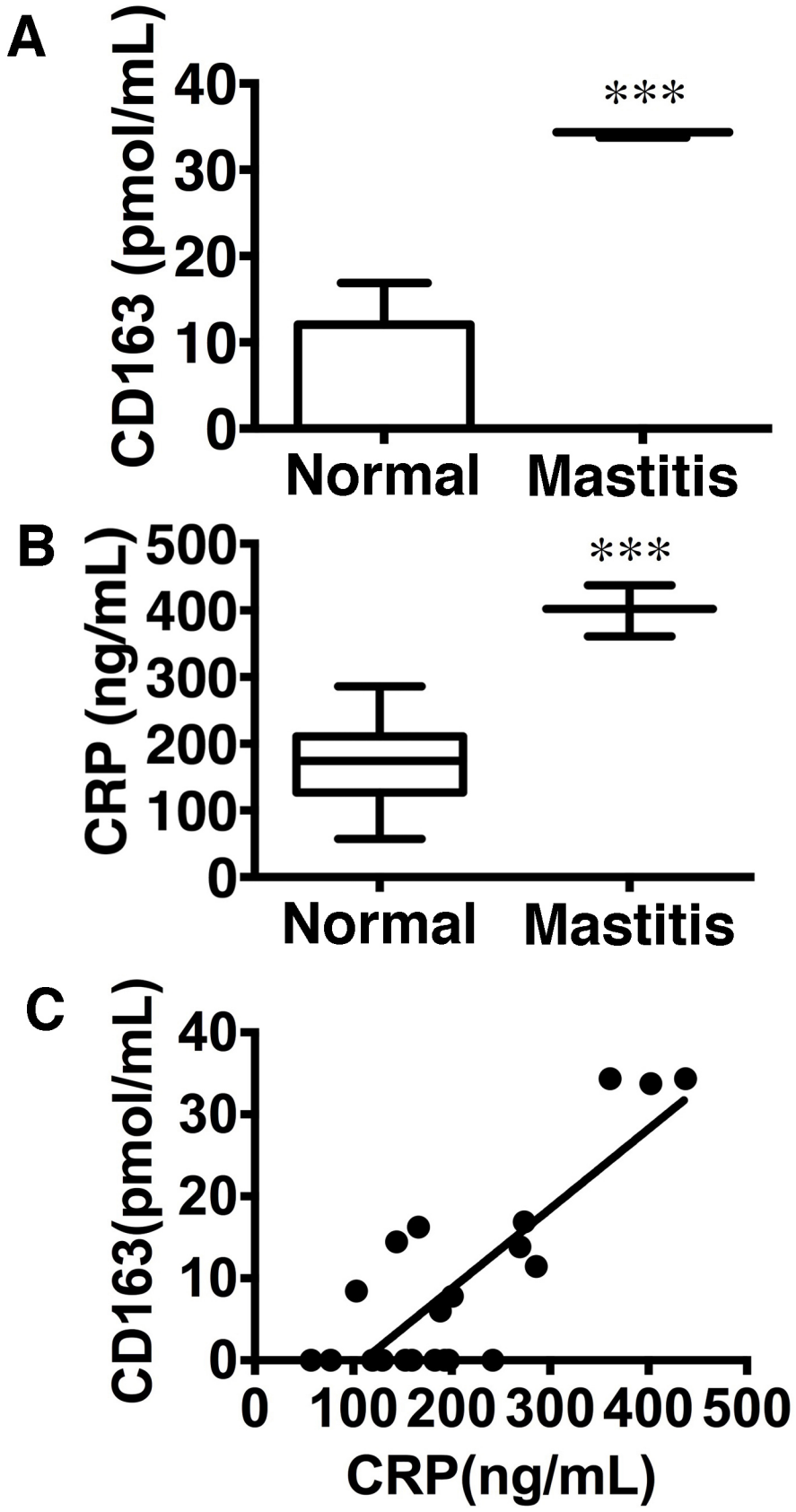

Figure 6 Shimamoto *et al.*