



Research paper

Monoclonal antibodies to equine CD23 identify the low-affinity receptor for IgE on subpopulations of IgM⁺ and IgG1⁺ B-cells in horses

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ABSTRACT

CD23, also called FcεRII, is the low-affinity receptor for IgE and has first been described as a major receptor regulating IgE responses. In addition, CD23 also binds to CD21, integrins and MHC class II molecules and thus has a much wider functional role in immune regulation ranging from involvement in antigen-presentation to multiple cytokine-like functions of soluble CD23. The role of CD23 during immune responses of the horse is less well understood. Here, we expressed equine CD23 in mammalian cells using a novel IL-4 expression system. Expression resulted in high yield of recombinant IL-4/CD23 fusion protein which was purified and used for the generation of monoclonal antibodies (mAbs) to equine CD23. Seven anti-CD23 mAbs were further characterized. The expression of the low-affinity IgE receptor on equine peripheral blood mononuclear cells was analyzed by flow cytometric analysis. Cell surface staining showed that CD23 is mainly expressed by a subpopulation of equine B-cells. Only a very few equine T-cells or monocytes expressed CD23. CD23⁺ B-cells were either IgM⁺ or IgG1⁺ cells. All CD23⁺ cells were also positive for cell surface IgE staining suggesting *in vivo* IgE binding by the receptor. Two of the CD23 mAbs detected either the complete extracellular region of CD23 or a 22 kDa cleavage product of CD23 by Western blotting. The new anti-CD23 mAbs provide valuable reagents to further analyze the roles of CD23 during immune responses of the horse in health and disease.

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1. Introduction

The molecular structure and function of CD23 have been extensively studied in humans and mice. Besides IgE, ligands of CD23 comprise CD21, MHC class II and the integrin α-chains CD11b and CD11c. CD23 is a type II membrane protein. The N-terminal part of CD23 is composed of the intracellular and transmembrane regions, while the extracellular region contains a C-terminal C-type lectin domain and three membrane-proximal repeats of 21 amino acids. Repeats form a coiled α-helical structure that results in formation of the receptor timer at the cell surface.

The C-type lectin domain is responsible for IgE, CD21 and integrin binding. The MHC class II binding region is located in close proximity to the cell membrane. Proteolytic cleavage of membrane-bound CD23 generates a soluble form (sCD23) of 37 kDa and a range of degradation forms of lower molecular weight. All of these retain their lectin head group, can bind IgE and have pleiotropic cytokine-like activities (reviewed by Acharya et al., 2010).

In mice and humans, CD23 is expressed on subsets of B-cells, monocytes (Melewicz et al., 1982) and more weakly on other hematopoietic cells. The latter include T-cells, follicular dendritic cells, eosinophils, NK-cells, Langerhans cells and platelets (Armitage et al., 1989; Joseph et al., 1986; Grangette et al., 1989). In addition, intestinal epithelial cells (Yu et al., 2003) and bone marrow stroma cells (Fourcade et al., 1992) express the receptor. Alternative splicing at the transcriptional level results in two isoforms of the receptor, CD23a and CD23b, which differ in the first nine amino

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acids of the N-terminal cytoplasmic region (Yokota et al., 1988). CD23a is constitutively expressed on resting B-cells and is majorly involved in IgE regulation (Yokota et al., 1988). CD23b plays a key role during transcytosis of IgE and IgE complexes in intestinal epithelial cells (Yu et al., 2003; Montagnac et al., 2005). CD23 expression is regulated by various stimuli such as IL-4, IL-13, IL-5, IL-9, GM-CSF, IFN- γ and CD40L or by infection with Epstein-Barr virus (Wang et al., 1991; Ewart et al., 2002; Rosenwasser and Meng, 2005).

Because of its multiple ligands CD23 plays important roles in the regulation of IgE production, B-cell survival and growth, T-cell and myeloid cell differentiation, as well as in antigen presentation (Gustavsson et al., 2000; Acharya et al., 2010; Platzer et al., 2011). IgE binding to membrane bound CD23 can inhibit the production of IgE in B-cells by a negative feedback loop (Gould and Sutton, 2008; Acharya et al., 2010). Similarly, the interaction of monomeric sCD23 with IgE decreases IgE synthesis in B-cells, while trimeric sCD23 simultaneously bound to IgE and CD21 enhances IgE synthesis by activated B-cells (Hibbert et al., 2005; McCloskey et al., 2007). Other cytokine-like activities mediated by CD23 binding to CD21 or integrins affect B-cell compartments by sustaining growth of activated mature B-cells, promoting differentiation of plasma cells and increasing B-cell precursor survival. In addition, sCD23 promotes differentiation of myeloid precursors, thymocytes and bone marrow CD4⁺ cells (reviewed by Acharya et al., 2010 and Platzer et al., 2011). Finally, binding of sCD23 to CD11b/CD18 and CD11c/CD18 on monocytes increases the production of inflammatory cytokines and activates nitric oxide production (Platzer et al., 2011). Because of its roles in lymphocyte survival and cytokine release, sCD23 has been associated with chronic lymphocytic leukemia (Hallek and Pflug, 2010) and autoimmune inflammatory conditions in humans, such as systemic lupus erythematosus (Bansal et al., 1992) and rheumatoid arthritis (Bansal et al., 1994; Massa et al., 1998). Consequently, CD23 has been a target as diagnostic marker in rheumatoid arthritis (Huissoon et al., 2000; Ribbens et al., 2000) and a humanized monoclonal anti-CD23 antibody was tested for therapeutic interventions in patients with leukemia or atopic disorders (Poole et al., 2005; Byrd et al., 2010).

Much less is known about CD23 expression and its regulatory functions on the immune system of the horse. The nucleotide sequence of equine CD23 was described previously (Watson et al., 2000) and mRNA transcripts were found in PBMC and alveolar macrophages (Jackson et al., 2004). CD23 was assumed to be expressed on a subpopulation of equine B-cells because cell surface bound IgE was detected on peripheral B-cells of adult horses (Wagner et al., 2003).

Here, our goal was to produce monoclonal antibodies (mAbs) to equine CD23 and to identify the cell populations in peripheral blood that express the low-affinity IgE receptor in horses. Because of the unusual orientation of the receptor with its C-terminal part representing the extracellular region, we used a novel IL-4 fusion protein system to express equine CD23 in eukaryotic cells. IL-4, formerly known as B-cell stimulating factor (Paul, 1987), initiates B-cell differentiation and immunoglobulin class switching

which then results in formation of antigen-specific memory B-cells and IgG-secreting plasma cells (Siebenkotten et al., 1992). We assumed that an IL-4/CD23 fusion protein would boost the immune response to CD23 during immunization and increase the number of equine CD23-specific hybridoma clones during mAb production.

2. Material and methods

2.1. IL-4 expression vector

An expression vector containing equine IL-4 (411 bp) and a sequence encoding an enterokinase digestion site (EK; 24 bp) was generated using the mammalian expression vector pcDNA3.1 (–)/Myc-His, version B (Invitrogen, Carlsbad, CA, USA). The IL-4 expression vector allowed cloning of the gene of interest into the multiple cloning site (MCS) downstream of the IL-4/EK sequence for expression of rIL-4 fusion proteins (Fig. 1A). The complete equine IL-4 gene (Genbank Accession GU139701) without stop codon was amplified in two steps with (1) primers containing a *NotI* restriction site (forward – 5'-gcgccgcgcatgggtctcactaccaactg-3') and the partial EK sequence (reverse – 5'-cgctgtacagatcacactgtggagtattctcttc-3') and (2) the same forward primer together with a primer for the complete EK sequence with a *BamHI* restriction site (reverse 2 – 5'-ccggtatcctatctgtcatctgtacagatc-3').

2.2. Cloning of equine CD23

For the initial PCR to amplify the complete coding region of the equine CD23 gene (Genbank Accession AF141931, bases 199–1179) from PBMC primers flanking the 981 bp coding region of equine CD23 were used (forward – 5'-atggaggacatgcatactcag-3'; reverse – 5'-tcagcacgtgaccagccgggtc-3'). The resulting PCR product was cloned into pCR4 TopoBlunt (Invitrogen, Carlsbad, CA, USA) as previously described (Wagner et al., 2005). In a second PCR, the CD23/pCR4 plasmid was used as template to amplify the 831 bp extracellular region of CD23 without the stop codon (bases 346–1176 of AF141931). Primers for the second PCR (forward – 5'-ggcggatccggagactgtgcagaagctgaaac-3', reverse – 5'-cgcgaaagcttgggcacgtgaccagccgggtcacac-3') contained *BamHI* and *HindIII* restriction sites (underlined) for cloning of the extracellular region of the CD23 gene into the IL-4 expression vector (Fig. 1B). A partial digestion step was used for expression cloning of the 831 bp CD23 fragment because an internal *BamHI* restriction site. The sequence of the cloned extracellular region of the CD23 gene was 100% homologous to Genbank Accession AF141931 and contained the complete coding sequence for the C-terminal lectin domain responsible for CD23 ligand binding.

2.3. Transfection and protein purification

Transfection of Chinese Hamster Ovary (CHO) cells and generation of a stable rIL-4/CD23 transfectant were performed as previously described for other fusion proteins (Wagner et al., 2005). Expression of rIL-4/CD23 was

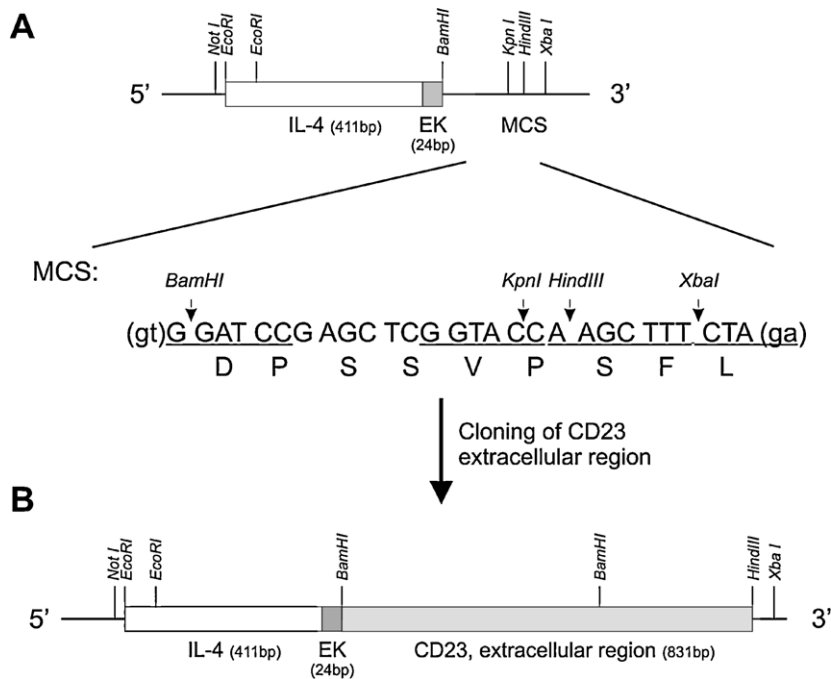


Fig. 1. IL-4 expression system for 3' cloning of equine CD23 and expression of IL-4/CD23 fusion protein. (A) IL-4 expression vector containing the equine IL-4 gene (GU139701), a sequence encoding an enterokinase digestion site (EK), and a multiple cloning site (MCS). (B) IL-4/CD23 expression vector: the gene encoding the extracellular region of equine CD23 was cloned into the MCS.

detected within transfected cells by flow cytometric analysis or by ELISA using an anti-equine IL-4 mAb (Wagner, 2006). The same mAb (clone 13G7) was also used to generate an IL-4 affinity column using CNBr activated sepharose 4B as previously described (Wagner et al., 2003). The affinity column was then used for purification of the complete rIL-4/CD23 fusion protein from serum-free supernatant of a stable transfectant. Purification was performed on a Fast Protein Liquid Chromatography (FPLC) system using an ÄKTA FPLC instrument (GE Healthcare, Piscataway, NJ) equipped with the anti-IL-4 affinity column. The rIL-4/CD23 fusion was eluted from the column by using 0.1 M glycine, pH 3.0 and the solution was neutralized immediately using 1 M Tris, pH 8.0. The protein solution was dialyzed against PBS. The concentration of the purified protein was determined in a BCA assay (Pierce, Rockford, IL). Purification of a liter of supernatant resulted in 2.3 mg rIL-4/CD23 fusion protein.

2.4. Immunization, cell fusions and identification of CD23 and IL-4 specific hybridomas

The immunization of one Balb/C mouse and the fusion procedure to produce hybridoma cell lines secreting mAbs was performed as described previously (Wagner et al., 2003, 2008a,b). Hybridoma supernatants containing antibodies to equine CD23 or IL-4 were identified by two ELISAs. First, ELISA plates (Immunoplate Maxisorp, Nalge Nunc Int., Rochester, NY, USA) were coated with the purified rIL-4/CD23 fusion protein (1 µg/ml) in carbonate buffer (15 mmol Na₂CO₃, 35 mmol NaHCO₃, pH 9.6) and incubated overnight at 4°C. The coating solution was

discarded and plates were blocked with PBS, containing 0.5% (w/v) BSA and 0.02% (w/v) NaN₃ for 30 min at room temperature. Plates were then washed five times with phosphate buffer (2.5 mmol NaH₂PO₄, 7.5 mmol Na₂HPO₄, 145 mmol NaCl, 0.1% (v/v) Tween 20, pH 7.2) and the hybridoma supernatants were applied undiluted and incubated for 90 min at room temperature. Plates were washed again with phosphate buffer and a peroxidase conjugated goat anti-mouse IgG (H+L) antibody (Jackson ImmunoResearch Lab., West Grove, PA) was used to detect antibodies that bound to the rIL-4/CD23 fusion protein. After a last washing step with phosphate buffer, substrate solution composed of substrate buffer (33.3 mmol citric acid, 66.7 mmol NaH₂PO₄, pH 5.0) supplemented with 130 µg/ml 3,3',5,5'-tetramethylbenzidine (TMB, Sigma, St. Louis, MO) and 0.01% (v/v) hydrogen peroxide (Sigma, St. Louis, MO) was added to the plates. Substrate solution was incubated for 20 min in the dark and the reaction was stopped by adding one volume of 0.5 mol H₂SO₄. Plates were evaluated in an automatic microplate reader (Synergy HT, Bio Tek Instruments, Winooski, VT) at 450 nm absorbance.

All supernatants were also tested in a second ELISA using a goat anti-horse IgG (H+L) antibody (Jackson ImmunoResearch Lab., West Grove, PA, USA) for coating of the ELISA plates. These plates were then incubated with equine rIL-4/IgG1 fusion protein as described previously (Wagner et al., 2005). Afterwards, hybridoma supernatants followed by all steps described above were added. All hybridomas that detected rIL-4/IgG1 were declared IL-4-specific, while those that were positive in the rIL-4/CD23 ELISA only were considered as CD23-specific.

Table 1

Number of positive supernatants obtained by hybridoma development using rIL-4/CD23 fusion protein and that detected CD23 or IL-4 by initial ELISA screening.

mAb specificity	Number of positive supernatants ^a	Number of clones selected for characterization	mAbs detecting native protein	Different epitope specificities
CD23	>260	26	19	At least 3
IL-4	42	12	3	1

^a A supernatant corresponded to one initial well in 24 well plates with 1–4 hybridoma clones per well. A total of 480 supernatants were tested.

2.5. Purification and conjugation of monoclonal antibodies

Purification of anti-CD23 mAbs from cell culture supernatants was performed by FPLC using Protein G affinity columns (GE Healthcare, Piscataway, NJ). Protein concentrations were determined by the BCA assay. Aliquots of the purified antibodies were conjugated to the Alexa fluorochromes A488 or A647 (Molecular Probes, Invitrogen, Eugene, OR). All procedures were performed according to the manufacturer's instructions. Murine isotypes of the anti-CD23 mAbs were determined by a mouse isotype ELISA (Sigma, St. Louis, MO).

2.6. Flow cytometric analysis

Equine leukocytes were isolated as previously described (Wagner et al., 2003). PBMC were isolated from heparinized blood of four clinically healthy, adult Thoroughbred mares by density gradient centrifugation (Ficoll-Paque™ Plus, GE Healthcare, Piscataway, NJ).

For CD23 cell surface staining the leukocytes were incubated *ex vivo* with the CD23 mAbs in PBS, supplemented with 0.5% (w/v) BSA and 0.02% (w/v) NaN₃, for 15 min at room temperature. Double staining to characterize the CD23⁺ cell population was performed with mAbs to equine CD4 (HB61A, VMRD, Pullman, WA), CD8 (CVS8; Lunn et al., 1998) to label T-cells, IgM (clone 1-22, Wagner et al., 2008b) for detection of B-cells, MHC class II (cz.11, Barbis et al., 1994), IgE (clone 176, Wagner et al., 2003), CD14 (clone 105, Kabitche et al., 2010), IgG1 (IgGa; clone CVS45), IgG4/7 (IgGb; clone CVS39), or IgG3/5 (IgG(T); clone CVS40; all Lunn et al., 1998). All antibodies were conjugated to Alexa dyes (A488 or A647) as previously described (Wagner et al., 2008a). In addition, a fluorescein conjugated anti-human CD23 mAb (clone 9P25, Beckman-Coulter/Immunotech, Indianapolis, IN) was used for comparison. This mAb was previously reported to possibly cross-react with equine CD23 (Mérant et al., 2003). All antibodies used here were mouse IgG1 and an IgG1 isotype control was included for staining and flow cytometric analysis.

Tri-color staining was performed with anti-CD23-A488, CVS45-A647 and biotinylated anti-IgM-1-22 followed by staining with streptavidin PE (Jackson ImmunoResearch Lab., West Grove, PA, USA). Cells were measured in a FACS Canto II flow cytometer (BD Biosciences, San Diego, CA).

For intracellular IL-4 staining of CHO transfectants, a total of 3×10^6 cells were washed in PBS and fixed in 2% formaldehyde for 20 min at room temperature. Intracellular staining was performed with the Alexa fluorochrome conjugated anti-IL-4 (Wagner, 2006) or the

fluorescein conjugated anti-human CD23 mAb in saponin buffer (PBS, supplemented with 0.5% (w/v) BSA, 0.5% (w/v) saponin and 0.02% (w/v) NaN₃) for 20 min in the dark. Cells were washed twice in saponin buffer, resuspended in PBS/BSA and measured as described above.

2.7. SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were performed as previously described (Wagner et al., 2003, 2008b). The rIL-4/CD23 fusion protein was separated on a 12% gel under reducing conditions. The blot membranes were incubated with different anti-CD23 clones, followed by incubation with peroxidase conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Lab., West Grove, PA, USA).

3. Results

3.1. Expression of equine rIL-4/CD23 fusion protein

CHO cells transiently transfected with the IL-4/CD23 expression vector were subsequently tested for protein expression and contained a clear population of IL-4 positive cells. An anti-human CD23 mAb that was previously described to possibly cross-react with equine CD23 did not detect the putative IL-4/CD23 positive cells (Fig. 2A). The anti-human CD23 antibody was subsequently used for staining of equine leukocytes and did not stain any cell population using our protocols (Fig. 2B). We concluded that the anti-human CD23 mAb was unlikely to detect CD23 positive cells in horses and continued with rIL-4/CD23 protein expression and the production of mAbs specific to equine CD23.

A stable IL-4/CD23 transfectant was generated and equine rIL-4/CD23 fusion protein was purified using an anti-IL-4 affinity column. The predicted rIL-4/CD23 fusion protein (422aa) was composed of IL-4 (145aa including EK) linked to the extracellular region of CD23 (277aa) corresponding to a calculated molecular weight of the fusion protein of 47 kDa. The amino acid sequence of IL-4 and the extracellular region of CD23 contain a total of four N-glycosylation sites that contribute to the molecular weight after expression in mammalian cells. The purified protein was separated on a SDS-gel and had the expected molecular weight of a complete, glycosylated rIL-4/CD23 fusion protein (Fig. 2C). The fragments of lower molecular weight likely represent degradation fragments of the fusion protein.

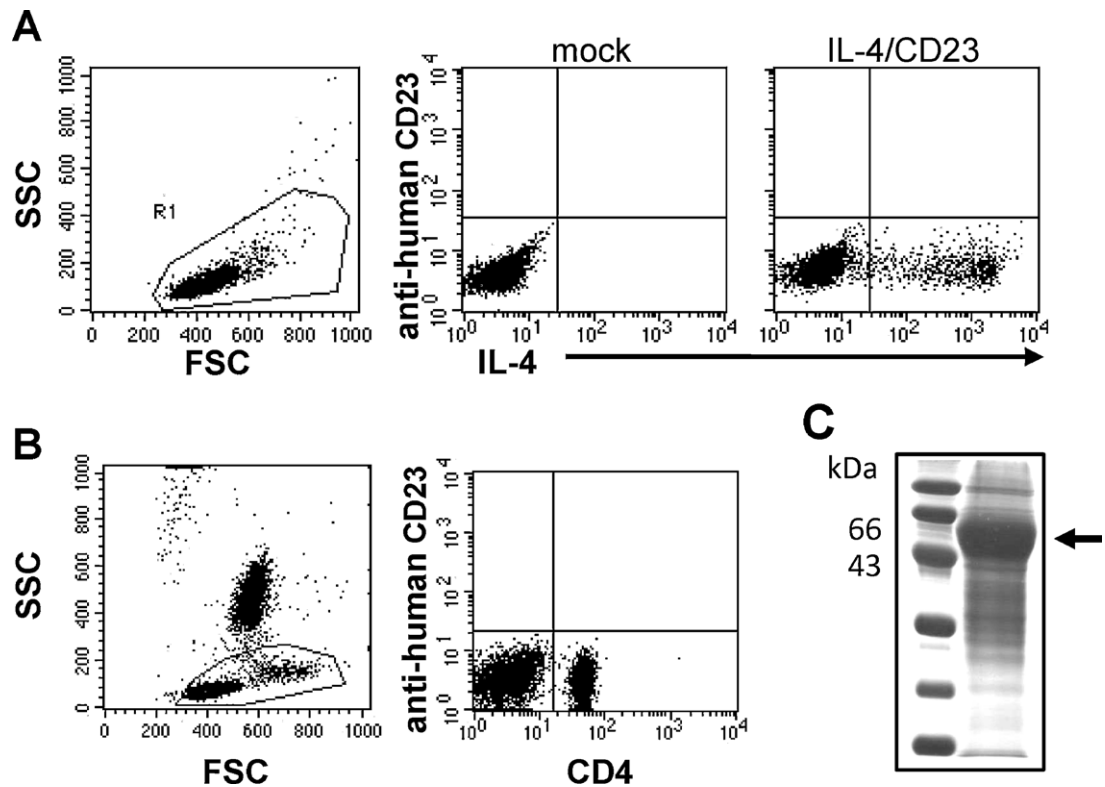


Fig. 2. Expression and purification of rIL-4/CD23 fusion protein. (A) Transiently transfected CHO cells were stained for intracellular IL-4/CD23 expression with anti-equine IL-4 and anti-human CD23 mAbs; left panel, CHO cell morphology plot with cell gate for analysis; middle panel, mock transfection; right panel, staining of CHO cell that were transiently transfected with the IL-4/CD23 expression vector. (B) Cell surface staining of equine peripheral blood leukocytes with anti-human CD23. Left panel, cell morphology and PBMC gate used for analysis; right panel, staining with anti-human CD23 and anti-equine CD4. (C) Purified rIL-4/CD23 fusion protein. The protein was purified using an anti-IL-4 affinity column and was separated on a 12% SDS gel under reducing conditions. The gel was stained with Coomassie Brilliant Blue. The arrow points to the complete rIL-4/CD23 fusion protein of approximately 47 kDa.

3.2. Hybridomas producing antibodies to CD23

Antibodies were produced using the whole rIL-4/CD23 fusion protein. Hybridoma supernatants were screened initially by two ELISAs for antibodies detecting CD23 or IL-4. The initial screening showed that more than 50% of all supernatants (>260) detected CD23 and a total of 42 supernatants recognized IL-4 (Table 1). These numbers suggested that equine IL-4, which was used in this system as a tag for protein detection and purification, did not dominate the immune response of the mouse as sometimes reported for other expression tags. Although antibodies to equine IL-4 were induced during immunization the total number of initially positive clones to CD23 was more than six-fold higher.

3.3. Characterization of mAbs to CD23 by flow cytometry

Out of all hybridomas detecting CD23 by ELISA, 26 clones were randomly selected and subsequently tested by cell surface staining of equine leukocytes and flow cytometric analysis. Nineteen of these clones detected a subpopulation of equine IgM⁺ B-cells and some IgM negative cells as shown in Fig. 3A. Neutrophils were not detected by the CD23 mAbs (data not shown). Additional cell

surface staining was performed with seven selected equine CD23 mAbs (Table 2) and various equine cell surface markers on non-stimulated equine leukocytes. All seven CD23 mAbs resulted in similar detection pattern by flow cytometric analysis as shown for the representative clone 51-3 in Fig. 3. Cell surface staining indicated that the putative equine CD23 molecule was expressed on small lymphocytes expressing high levels of MHC class II (Fig. 3B). CD23 is the low-affinity receptor for IgE and double staining with anti-CD23 and anti-IgE showed that all CD23⁺ cells are also positive for cell surface IgE (Fig. 3C). CD23 was almost not

Table 2

Anti-equine CD23 clones, mouse isotypes, and applications the mAbs have been tested for.

CD23 mAb	Mouse isotype	Flow cytometric analysis, equine leukocytes or PBMC	Western blotting
51-3	IgG1	All mAbs detect a subpopulation of equine B-cells	No
53-2	IgG1		No
54-3	IgG1		Yes ^a
57-1	IgG1		No
60-1	IgG1		No
62-2	IgG1		No
63-2	IgG1		Yes

^a This mAb detected a 22 kDa CD23 degradation fragment.

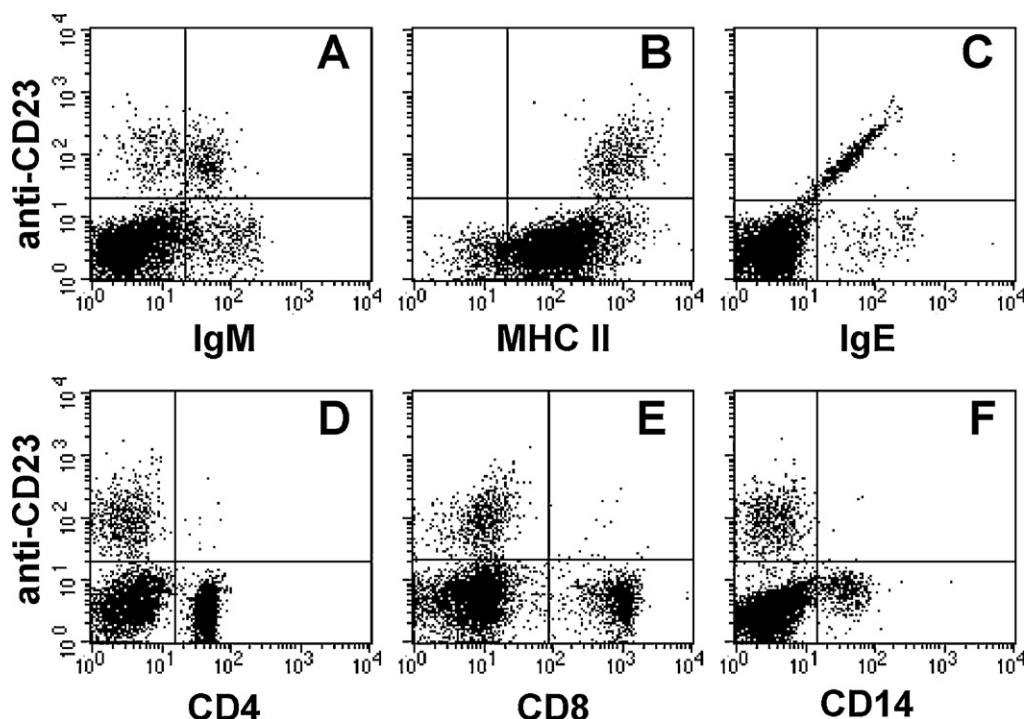


Fig. 3. Characterization of anti-equine CD23 mAbs by flow cytometry. Equine leukocytes were isolated and cell surface double staining was performed with anti-equine CD23 (clone 51-3) and various equine cell surface markers. For analysis a gate was set on PBMC. The plots show double staining of PBMC with anti-CD23 and markers for (A) IgM; (B) MHC class II; (C) IgE; (D) CD4; (E) CD8; and (F) CD14.

identified on equine peripheral blood T-cells or monocytes (Fig. 3D–F).

Equine PBMC from four horses were then used to perform additional staining for CD23 and various immunoglobulin subclasses (Fig. 4A). A median of 2.5% CD23⁺ cells (range 0.9–4.5%) was found in PBMC. Approximately half of the CD23⁺ cells were also positive for IgM (median 1.0%, range 0.3–2.3%). A similar percentage was found for CD23⁺/IgG1⁺ cells (median 0.9%, range 0.3–1.2%). No IgG4/7 (IgGb) or IgG3/5 (IgG(T)) positive cells were observed in the CD23⁺ PMBC fraction (Fig. 4A and B). Cells from all four horses were then stained for CD23, IgM and IgG1. This showed that the CD23⁺/IgG1⁺ cells express no or very low levels of IgM on their surface, while most of the CD23⁺/IgG1⁺ cells are IgM⁺ (Fig. 4C). In summary, the population of CD23⁺ PBMC identified by the new CD23 mAbs is primarily composed of membrane IgM⁺ or IgG1⁺ B-cells.

3.4. Western blotting using CD23 mAbs identified three different epitope recognition pattern

The CD23 mAbs were also tested for detection of rIL-4/CD23 by Western blotting under reducing conditions (Fig. 5). Only two mAbs recognized CD23 by this method. MAbs 63-2 recognized the complete CD23 fusion protein of 47 kDa, while mAb 54-3 detected a 22 kDa degradation fragment of rIL-4/CD23 and recognized the complete 47 kDa fusion protein only very weakly. Although the detection of the 22 kDa degradation product by mAb 54-3 is likely an artificial result related to storage of the rIL-4/CD23, the recognition pattern of all seven mAbs obtained by

Western blotting suggested that they detect at least three different epitopes on the extracellular region of equine CD23 (Table 2). Two of these epitopes are maintained by the reducing conditions of Western blotting and likely represent linear epitopes of CD23. The epitope detected by mAb 54-3 is accessible in the native receptor, as shown by flow cytometric analysis, and on the reduced 22 kDa cleavage product of CD23 but not on other reduced cleavage products of CD23.

4. Discussion

The new mAbs to equine CD23 identified the low-affinity receptor for IgE on equine PBMC. In non-stimulated PBMC, CD23 was constitutively expressed on a subpopulation of equine B-cells which is similar to its expression on human and mouse B-cell subsets (Acharya et al., 2010). In addition, T-cells, monocytes and NK-cells were described to express the receptor in humans and mice depending on their stimulation status. In equine non-stimulated PBMC, CD23 expression on monocytes and T-cells was almost absent. Equine CD23⁺ B-cells had a phenotype of mature, naïve B-cells, i.e. they were either cell surface IgM⁺ or IgG1⁺. Interestingly, almost half of the equine CD23⁺ B-cells were IgG1⁺ in all horses investigated. IgG1, also known as IgGa, is produced during B-cell and antibody responses of the horse to a variety of pathogens, including viruses (Nelson et al., 1998; Goodman et al., 2006; Goehring et al., 2010), intra- and extracellular bacteria (Lopez et al., 2003), or parasites (Dowdall et al., 2002; Mealey et al., 2012), although IgG1 was never reported to be the dominating isotype of any

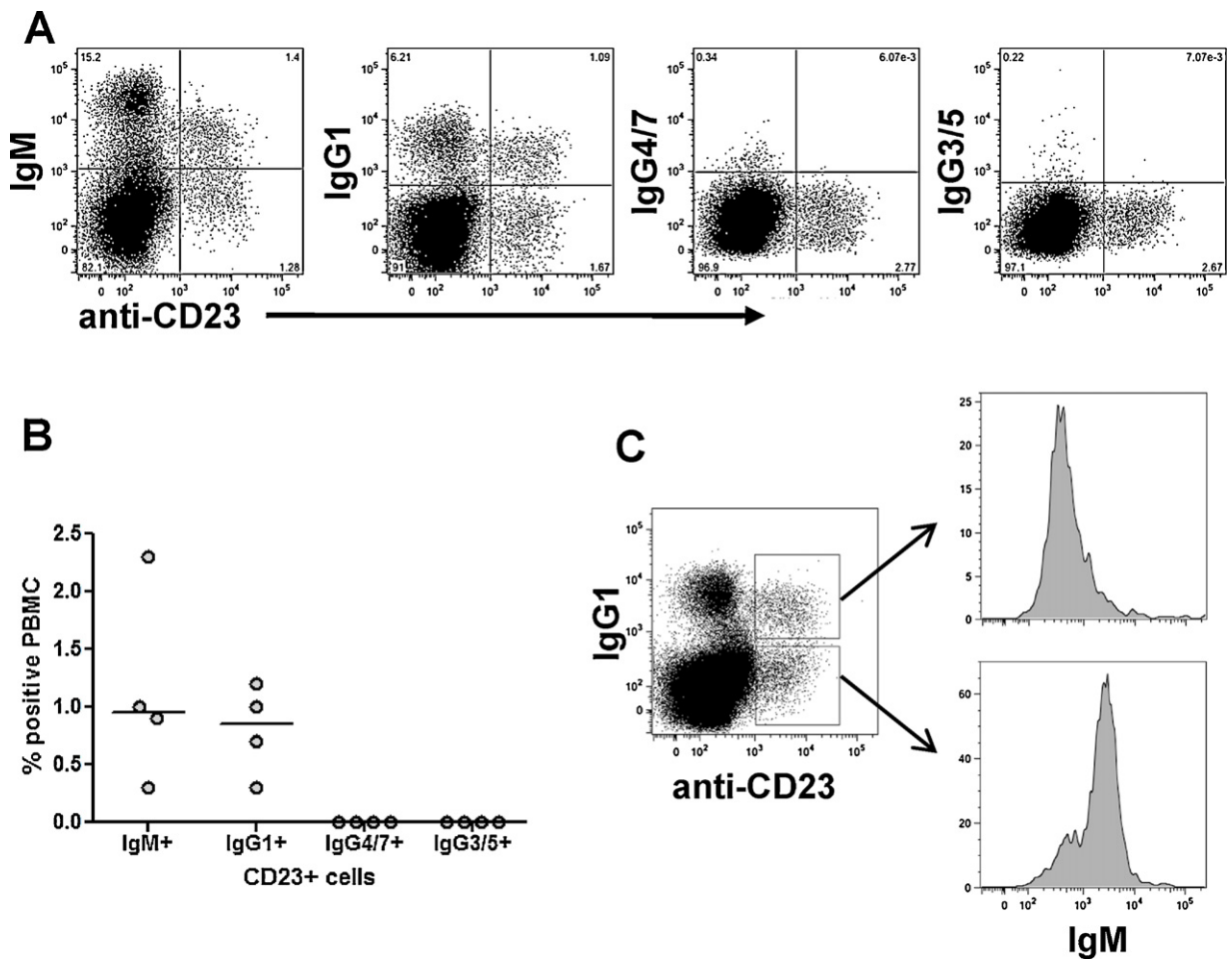


Fig. 4. Flow cytometric analysis of PBMC from four horses with anti-equine CD23 and markers for different immunoglobulin subclasses. (A) Representative analysis of PBMC from one horse. Double staining for cell surface CD23 (clone 51-3) and IgM, IgG1 (IgGa), IgG4/7 (IgGb), or IgG3/5 (IgG(T)). (B) Distribution of surface IgM⁺ and IgG⁺ cells within the population of CD23⁺ PBMC. Each dot represents one horse. (C) Representative tri-color staining for CD23, IgG1 (dot plot) and IgM from one horse. The histograms show cell surface IgM staining of CD23⁺/IgG1⁺ (upper histogram) and CD23⁺/IgG1⁻ cells (lower histogram), respectively.

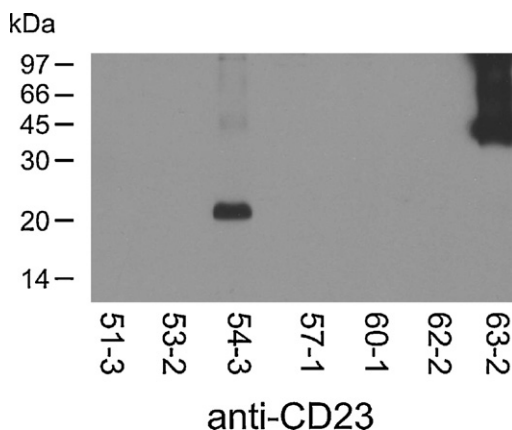


Fig. 5. Immunoblotting using seven anti-equine CD23 mAbs. Equine rIL-4/CD23 was separated on a 12% SDS-gel under reducing conditions and transferred to PVDF membranes by Western blotting. The membranes were stained with different anti-CD23 mAb clones followed by detection with a peroxidase conjugated goat anti-mouse antibody.

of these immune responses. Nevertheless, IgG1 is the only IgG isotype that can consistently be found in serum of newborn foals before colostrum uptake (Sheoran et al., 2000) indicating its production *in utero* and in the absence of environmental stimuli. On the genetic level, the gene encoding IgG1 (IGHG1) is located downstream of the IGHM/IGHD genes and is thus the most 5' located IGHG gene in the equine IGH-locus (Wagner, 2006). These observations on IGHG1 gene orientation and IgG1 expression support the assumption that IgG1 expression may be a result of various stimuli and/or that class switching to other IgG isotypes may always initially involve the IGHG1 gene and IgG1 production in horses. The fact that a clear population of circulating IgG1⁺ B-cells, but almost none cell surface IgG⁺ cells of other isotypes, exists in healthy adult horses also suggests that surface IgG1⁺ B-cells may rather function *via* their Ig-receptors and, similar to surface IgM⁺ cells, represent a naïve B-cell phenotype in horses.

CD23 serves as a receptor for various ligands including IgE, CD21, integrins and MHC class II and is involved

in the regulation of IgE production (Acharya et al., 2010; Platzer et al., 2011). We reported previously that equine basophils bind IgE via high-affinity IgE receptors (Fc ϵ RI). In adult horses, subpopulations of monocytes and B-cells also bind IgE to their surface (Wagner et al., 2003, 2010). Here, all cells detected by the CD23 mAbs were also positive for cell surface IgE. Consequently, the small population of IgE⁺/CD23⁻ cells shown in Fig. 3C likely represented cells expressing Fc ϵ RI such as basophils. Because equine monocytes did not show any significant expression of CD23 it can also be concluded that predominately CD23⁺ B-cells maintain the homeostasis of IgE in healthy adult horses. As their names suggest, Fc ϵ RI and CD23 vary in their affinities for IgE. Fc ϵ RI binds IgE with $K_D \sim 1$ nM while monomeric CD23 interacts with IgE with a $K_D \sim 0.1$ – 1 μ M. However the net affinity of the membrane bound trimeric CD23 receptor is close to that of the high-affinity IgE receptor (Acharya et al., 2010). Here, binding of IgE was observed on all peripheral equine CD23⁺ B-cells *ex vivo* and with a fluorescent intensity comparable to that of the IgE⁺/CD23⁻ basophil population. This supported that the equine trimeric cell surface CD23 receptor binds IgE with a net affinity similar to that of Fc ϵ RI on basophils.

Equine CD23 mAbs 54-3 and 63-2 also detected a 22 kDa and a 47 kDa fragment of CD23, respectively, by Western blotting. In humans or mice, CD23 is cleaved from the cell surface by proteases such as the metalloprotease ADAM10 which generates the 37 and 33 kDa forms of sCD23 (Weskamp et al., 2006; Lemieux et al., 2007). Cleavage by Der p1 protease, a major allergen of the house dust mite *Dermatophagoides pteronyssinus*, yields the 16–17 kDa sCD23 fragment (Schultz et al., 1995). Additional proteolytic cleavage products of sCD23 with molecular weights of 25–27 kDa were described in cell culture supernatants and human serum from patients with chronic lymphocytic leukemia and are believed to be degradation intermediates (Platzer et al., 2011). Similarly, the 22 kDa CD23 degradation product detected by anti-equine CD23 mAb 54-3 could be an equine homolog to the 25–27 kDa human sCD23 isoform. In contrast, CD23 mAb 63-2 detected the non-degraded IL-4/CD23 fusion protein which contained the complete extracellular region of CD23. However, mAb 63-2 detected none of the lower molecular weight cleavage products by this method. Thus, the epitope detected by mAb 63-2 is likely located in close proximity to the cell membrane, *i.e.* is not maintained on the lower molecular weight cleavage products. Because all proteolytic cleavage sites are located outside of the lectin head domain, it has been described that all cleavage products of CD23 can still bind IgE and display cytokine-like activities (Acharya et al., 2010; Platzer et al., 2011). Nevertheless, at least the epitope detected by mAb 54-3 using Western blotting is only displayed by the 22 kDa fragment of CD23 but not by the fragments of higher molecular weight, despite the fact that all seven equine CD23 mAbs described here do not differ in their staining pattern of native transmembrane CD23 when analyzed by flow cytometry. It can be concluded that the epitope detected by mAb 54-3 is accessible for the antibody on cell surface expressed CD23 and also on the reduced and denatured 22 kDa CD23 fragment by Western blotting. In contrast, the reducing and denaturing Western blotting

procedure seems to make the epitope detected by mAb 54-3 almost non-accessible on the complete CD23 fusion protein as indicated by its weak recognition on the Western blot.

Besides the development of valuable reagent tools for the detection of equine CD23, the novel equine IL-4 expression system used here showed some clear advantages compared to existing mammalian expression constructs. In particular, recombinant protein expression yielded remarkably high amounts of rIL-4/CD23 fusion protein. Since its first use for equine rIL-4/CD23, the IL-4 expression construct has been used to produce various other equine IL-4 fusion proteins and yielded an average protein amount of 1.3 mg purified IL-4 fusion protein per liter supernatant. A comparable number of equine proteins was expressed and purified using an IgG fusion protein expression system, which uses the identical vector backbone, CHO cells and transfection method (Wagner et al., 2005), and resulted in average of 0.23 mg purified protein from the same volume of cell culture supernatant (Wagner et al., unpublished data).

In addition, the use of the complete rIL-4/CD23 for immunization resulted in a remarkably high number of CD23⁺ hybridomas, while IL-4 was clearly of lower immunogenicity as demonstrated by the comparable small number of IL-4⁺ clones from the same cell fusion. This suggested that equine IL-4, formerly named B-cell stimulating factor (Paul, 1987), can boost the immune response of the mouse towards CD23 antibody development. We hypothesize that the enhanced murine antibody response was a result of simultaneous detection of CD23 by the antigen receptor on CD23-specific murine B-cells and of IL-4 by the IL-4 receptor on the same B-cells. We further assume that this receptor cross-linking on the B-cell surface resulted in enhanced B-cell differentiation and immunoglobulin class switching to produce numerous CD23-specific, IgG antibody-secreting plasma cells available for the cell fusion. Although the mechanisms of boosting the murine immune response by equine IL-4 requires experimental confirmation, the number of CD23⁺ clones obtained from the single cell fusion described here was remarkable compared to fusions using other non-IL-4 fusion proteins (Wagner et al., unpublished results).

In summary, we generated various new mAbs to equine CD23 using rIL-4/CD23 fusion protein. The CD23 mAbs are valuable tools for further analysis of the expression of this multi-functional receptor and its role during immune responses of the horse. The CD23 mAbs can be obtained for research purposes through Cornell University. We also developed a promising novel IL-4 fusion protein system for expression of recombinant proteins in mammalian cells that likely has the potential to advance monoclonal antibody production to small proteins or those of low immunogenicity.

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