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# Expression of bovine non-classical major histocompatibility complex class I proteins in mouse P815 and human K562 cells

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ABSTRACT

Major histocompatibility complex class I (MHC-I) proteins can be expressed as cell surface or secreted proteins. To investigate whether bovine non-classical MHC-I proteins are expressed as cell surface or secreted proteins, and to assess the reactivity pattern of monoclonal antibodies with non-classical MHC-I isoforms, we expressed the MHC proteins in murine P815 and human K562 (MHC-I deficient) cells. Following antibiotic selection, stably transfected cell lines were stained with H1A or W6/32 antibodies to detect expression of the MHC-I proteins by flow cytometry. Two non-classical proteins (BoLA-NC1*00501 and BoLA-NC3*00101) were expressed on the cell surface in both cell lines. Surprisingly, the BoLA-NC4*00201 protein was expressed on the cell membrane of human K562 but not mouse P815 cells. Two non-classical proteins (BoLA-NC1*00401, which lacks a transmembrane domain, and BoLA-NC2*00102) did not exhibit cell surface expression. Nevertheless, Western blot analyses demonstrated expression of the MHC-I heavy chain in all transfected cell lines. Ammonium-sulfate precipitation of proteins from culture supernatants showed that BoLA-NC1*00401 was secreted and that all surface expressed proteins where shed from the cell membrane by the transfected cells. Interestingly, the surface expressed MHC-I proteins were present in culture supernatants at a much higher concentration than BoLA-NC1*00401. This comprehensive study shows that bovine non-classical MHC-I proteins BoLA-NC1*00501, BoLA-NC3*00101, and BoLA-NC4*00201 are expressed as surface isoforms with the latter reaching the cell membrane only in K562 cells. Furthermore, it demonstrated that BoLA-NC1*00401 is a secreted isoform and that significant quantities of membrane associated MHC-I proteins can be shed from the cell membrane.



## 1. Introduction

The major histocompatibility complex (MHC) in cattle is known as the bovine leukocyte (or lymphocyte) antigen (BoLA) complex (Davies et al., 1994a, b, 1997). The Immuno Polymorphism Database (IPD-MHC) is the official database for MHC nomenclature (Ellis et al., 2006; Hammond et al., 2012; Robinson et al., 2005). There are two subclasses of MHC class I (MHC-I) proteins: classical MHC-I and non-classical MHC-I. Classical MHC-I (MHC-Ia) proteins are membrane-bound isoforms that are expressed in all nucleated cells of the body. Bovine MHC-I haplotypes code for one to three expressed MHC-Ia proteins at a minimum of six distinct loci (*BoLA-1* through *BoLA-6*) (Birch et al., 2006; Codner et al., 2012; Ellis and Ballingall, 1999; Hammond et al., 2012; Schwartz and Hammond, 2015). These proteins present intracellular pathogen-derived peptides or autologous peptides to CD8 T cells (Goddeeris et al., 1986a; Goddeeris et al., 1986b; Graham et al., 2008; Guzman et al., 2008; Splitter et al., 1988). With the discovery of human leukocyte antigen HLA-G (Ellis et al., 1986), another category of MHC-I proteins, referred to as non-classical MHC-I (MHC-Ib), was recognized. MHC-Ib molecules are less polymorphic, exhibit tissue specific expression patterns, possess specific molecular motifs in their transmembrane domains, and/or contain premature stop codons. MHC-Ib proteins are important immunomodulatory molecules that interact with inhibitory and activating receptors on leukocytes. The MHC-Ib proteins have been shown to induce immunotolerance to tumors and play an important role in maternal tolerance to the conceptus during human pregnancy (Garziera and Toffoli, 2014; Hunt et al., 2005; Ishitani et al., 2006). Humans have three MHC-Ib genes – *HLA-E*, *HLA-F* and *HLA-G* – and mice have two MHC-Ib gene – *Qa-1* and *Qa-2*

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(Comiskey et al., 2003; Djurisic and Hviid, 2014; Kochan et al., 2013; Vance et al., 1999; Zeng et al., 2012). There are four well characterized bovine MHC-Ib genes: *BoLA-NC1*, *BoLA-NC2*, *BoLA-NC3* and *BoLA-NC4* (Birch et al., 2008a; Davies et al., 2006).

A unique characteristic of some MHC-Ib proteins is that they undergo alternative splicing (differential splicing) to generate membrane and soluble isoforms. By virtue of this phenomenon, *HLA-G* produces membrane-associated isoforms, HLA-G1, -G2, and -G3, and soluble isoforms, HLA-G5 and -G6 (Ellis et al., 1986; Hunt et al., 2005). Similarly, murine *Qa-2* encodes two soluble isoforms S1 Qa-2 and S2 Qa-2 (Comiskey et al., 2003), *Mamu-AG* in rhesus monkeys encodes membrane-bound isoforms Mamu-AG1, -AG2 and -AG3 (Boyson et al., 1997), and *Paan-AG* in baboons encodes four membrane isoforms, Paan-AG1, -AG2, -AG3 and -AG4, and a soluble isoform, sPaan-AG1 (Langat et al., 2002). One of the bovine non-classical MHC-I loci, *BoLA-NC1*, has also been shown to encode multiple, alternatively spliced transcripts (Araibi et al., 2006; Birch et al., 2008a; Davies et al., 2006) (Davies and Schmidt, unpublished). However, splice variants encoded at the other bovine MHC-Ib loci – *BoLA-NC2*, *-NC3*, and *-NC4* – have not been identified.

In humans, membrane bound and secreted non-classical class I molecules interact with inhibitory receptors expressed by natural killer (NK) cells, T lymphocytes, and antigen presenting cells (APC) to inhibit these immune cells (Bainbridge et al., 2000; Braud et al., 1998a; Ellis et al., 1990; Ellis et al., 1986; Hunt et al., 2006; Hunt and Langat, 2009; Hunt et al., 2005; Le Bouteiller, 2000; Park et al., 2004). The human MHC-Ib molecule HLA-G interacts with leukocyte inhibitory receptors, including leukocyte immunoglobulin-like receptors LILRB1 and LILRB2 and the killer immunoglobulin-like receptor KIR2DL4, to induce immunosuppression (Clements et al., 2007; Comiskey et al., 2003; Rajagopalan et al., 2006; Shiroishi et al., 2003). HLA-G also upregulates expression of LILRB1, LILRB2, LILRB4, and KIR2DL4 in APC, NK cells and T cells, which helps protect HLA-G expressing tissues from immune cell attack (LeMaoult et al., 2005). HLA-E and its murine counterpart Qa1 are ligands for activating and inhibitory members of the CD94/NKG2 receptor family, which are encoded in the natural killer complex (Braud et al., 1998a; Kelley et al., 2005; Vance et al., 1999; Zeng et al., 2012). Cattle have genes encoding a wide variety of immunoglobulin-like (LILR and KIR) and lectin-like (CD94/NKG2 receptor family) NK cell receptors (Allan et al., 2015; Birch and Ellis, 2007; Boysen et al., 2006; Dobromylskyj and Ellis, 2007; Govaerts and Goddeeris, 2001; Guethlein et al., 2007; Hogan et al., 2012; McQueen et al., 2002; Storset et al., 2004; Storset et al., 2003). It has been shown that the cattle NKG2D receptor recognizes proteins encoded by two MHC class I chain-related (*MIC*) genes: MIC1 and MIC4 (Birch et al., 2008b; Guzman et al., 2010). However, nothing is known about the ligands for the other bovine activating and inhibitory NK cell receptors or if any of these receptors recognize classical or non-classical MHC-I proteins.

It is likely that bovine non-classical MHC-I proteins are expressed as both cell surface and secreted proteins. To investigate this hypothesis, and to characterize monoclonal antibodies that react with different non-classical MHC-I isoforms, bovine MHC-I proteins were expressed in the murine mastocytoma cell line P815 and the human MHC-I deficient cell line K562. Cell-surface expression and the reactivity pattern of MHC-I specific monoclonal antibodies were analyzed by flow cytometry. Secreted proteins in culture supernatants were precipitated using ammonium sulfate and then detected using Western blots.

## 2. Materials and methods

### 2.1. Samples

Full-length MHC-I cDNAs were reverse transcribed from interplacentomal trophoblast RNA, cloned in the pCRII-TOPO® vector (Invitrogen), and stored at −80 °C as part of a previous study (Davies et al., 2006). Two classical (BoLA-2*01802 and BoLA-3*01701) and five non-classical (BoLA-NC1*00401, BoLA-NC1*00501, BoLA-NC2*00102, BoLA-NC3*00101 and BoLA-NC4*00201) MHC-I isoforms were expressed for this study. The two classical MHC-I proteins have previously been expressed by other investigators and were included as positive controls for cell surface expression (Ellis et al., 1999). We have previously shown that the five non-classical isoforms are transcribed in bovine trophoblast cells (Davies et al., 2006). All of these isoforms were normally spliced isoforms except for the BoLA-NC1*00401 isoform, which was a splice variant that lacked the transmembrane domain.

### 2.2. Subcloning of classical and non-classical MHC class I genes

To facilitate expression in mammalian cell lines the MHC-I cDNAs were subcloned from the pCRII-TOPO® sequencing vector (Invitrogen) into the pcDNA3.1™D/V5-His-TOPO® directional mammalian expression vector (Invitrogen). Fifty microliter PCR reactions were prepared by combining 1 U Platinum *Pfx* proofreading DNA Polymerase (Invitrogen), 0.8 μM each of a forward and a reverse primer (listed below), 2.0 mM MgSO4, 0.2 mM dNTPs, 1× optimized PCR buffer, and 2 μl of diluted (1:1000) pCRII-TOPO® plasmid. A single forward PCR primer was used with two different reverse primers:

| | |
|---|---|
| Forward primer (BoC1FP-E1B) | ACCATGGGGCCGCGAACCCTC |
| Reverse primer (BoC1RP-3′A) | GATGAAGCATCACTCAGTCCCC |
| Reverse primer (BoC1RP-E7A) | TTTAGGAACCGTGAGAGACACATC |

The two reverse primers were used so that clones with the normal stop codon (reverse primer BoC1RP-3′A) and clones expressing a 3′ 6× histidine tag and V5 epitope (reverse primer BoC1RP-E7A) could be produced. PCR amplification was carried out using an Eppendorf Mastercycler® with the following parameters: 1 min 30 s at 94 °C; 25 cycles of 30 s at 94 °C, 15 s at 60 °C and 90 s at 68 °C; 10 min at 68 °C; hold at 4 °C. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and product size was confirmed using 1% agarose gel electrophoresis. Purified DNA was ligated into the pcDNA3.1™D/V5-His-TOPO® expression vector for 5 min at room temperature, transformed into TOP10F′ One Shot Competent *Escherichia coli* (Invitrogen), and plated on *LB* agar containing 100 μg/ml ampicillin. Insert size in isolated colonies was checked by PCR amplification of lysed bacteria with T-7 forward and BGH reverse sequencing primers, which bind to sites within the vector, followed by agarose gel electrophoresis. Only clones with inserts of the expected size were considered for further evaluation.

### 2.3. Sequencing of subclones

Multiple subclones with the correct size insert were sequenced using T-7 forward and BGH reverse sequencing primers. Plasmids were purified using a QIAprep spin Miniprep Kit (Qiagen), and sequenced in both directions using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and ABI Prism® 3100 DNA Analyzer (Applied Biosystems). Sequence analysis was done using Lasergene SeqMan™ II and Meg-Align™ software (DNASTAR, Inc.). Subclones with perfect sequences were selected for expression in the mammalian cell lines.

### 2.4. Cell lines and transfection

P815 cells (ATCC TIB-64) are derived from a mastocytoma that developed in a mouse (*Mus musculus*) of the DBA/2 strain. The majority of the mastocytoma cells grow in suspension (<5% adherent). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Caisson Laboratories) with 10% bovine calf serum (Hyclone), 2 mM L-glutamine (Hyclone), 100 units/ml penicillin and 100 μg/ml streptomycin (Hyclone) at 37 °C, 5% $CO_2$.

The K562 cell line (ATCC CCL-243) is a MHC-I deficient cell line derived from a person with chronic myelogenous leukemia. These cells

grow in suspension and were grown in Iscove's Modified Dulbecco's Medium (IMDM; Fisher Scientific) supplemented with 10% bovine calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Hyclone) at 37 °C, 5% $CO_2$. Both cell lines were obtained from American Type Culture Collection (ATCC, USA).

Cells were transfected with either a classical or non-classical MHC-I gene in the pcDNA3.1 vector using Lipofectamine 2000 (Invitrogen) transfection reagent according to the manufacturer's protocol. As a positive control P-815 cells were transfected with a plasmid encoding beta-galactosidase supplied with the vector kit. Transfected cells were incubated for 24 to 48 h prior to selection of stable transfectants by addition of G418 (Invivogen) antibiotic (500 μg/ml). After two weeks in selective medium transfectants were screened by flow cytometry.

### 2.5. Flow cytometry

The following monoclonal antibodies were used in the analysis. The anti-bovine MHC-I monoclonal antibody H1A was used for screening and sorting the transfected cell lines. Other anti-bovine MHC-I antibodies that were also used included: H6A, H11A, H58A, PT85A (Davis et al., 1987) and IL-A88 (Toye et al., 1990). The anti-human MHC-I monoclonal antibody W6/32, which recognizes MHC-I heavy chains associated with human or bovine beta-2-microglobulin (β2m), was used as a positive control for mouse P815 cells as these cells express mouse MHC-I proteins that associate with bovine β2m present in the culture medium (Bernabeu et al., 1985; Kahn-Perles et al., 1987). With the MHC-I deficient human K562 cells, W6/32 was negative on untransfected cells but reacted with the bovine MHC-I proteins expressed by the transfected cells. ColiS205D1 (IgG2a), which is specific for an *Escherichia coli* antigen, was used as an isotype negative control for both cell lines. All of the monoclonal antibodies except for IL-A88 were obtained from the Monoclonal Antibody Center at Washington State University, Pullman, WA. The IL-A88 hybridoma was purchased from Sigma-Aldrich. Fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG antibody (Kirkegaard & Perry Laboratories) was used as a secondary antibody. For staining, cells were resuspended in fluorescence buffer (PBS with 0.1% Sodium Azide, 1% bovine serum albumin) and incubated with primary antibody (15 μg/ml) for 15 min. Cells were washed twice with fluorescence buffer and then incubated with secondary antibody for 15 min. Cells were washed twice and fixed in PBS with 1% formaldehyde. All incubations were performed at 4 °C. One million cells were stained for each sample. Cells were analyzed using a BD Biosciences FACSAria II fluorescence activated cell sorter (FACS) equipped with FACS Diva software. The FACSAria II was also used to sort the transfected cells to enrich for high expressing cells. Flow cytometry figures for publication were created using FlowJo software (FlowJo, LLC).

### 2.6. Purification of recombinant histidine tagged proteins

Transfected P815 and K562 cells were grown in T75 cell culture flasks (Corning). From each flask approximately $100 \times 10^6$ cells were harvested by centrifuging at 1500 RPM for 10 min at room temperature. Cells were washed in ice cold PBS, pH 7.2 and lysed in lysis buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 1% Nonidet P-40). One micromole protease inhibitor cocktail (Sigma) for mammalian cell extracts and 1 mM PMSF (Phenyl Methane Sulfonyl Fluoride) were also added to the suspension. Lysates were centrifuged at 10,000 RPM for 10 min at 4 °C and the supernatants were filtered using 0.2 μM filters and stored in sterile tubes at −20 °C until purified.

HisGraviTrap Columns (GE Healthcare) were used to purify the histidine-tagged proteins. The manufacturer's recommended purification procedure was followed. Purified eluates were stored at −20 °C. Eluates for each protein that had a strong band on a Western blot were pooled and dialyzed against phosphate buffer solution (20 mM Sodium Phosphate, 500 mM NaCl, pH 7.4) using 20 kD molecular weight cut-off (MWCO) Slide-A-Dialysis cassettes (Pierce). To prevent precipitation and to maximize the stability of the protein, 50 mM charged amino acids L-Arg and L-Glu were added to all purification buffers and dialysis buffers (Golovanov et al., 2004). Dialysates were centrifuged at maximum speed (28,000*g*) in an Eppendorf 5804R centrifuge for 10 min at 4 °C and the supernatant was recovered to a fresh tube. The dialysates were concentrated using vivaspin-20 concentrators with 30 kD MWCO membranes (Vivaproducts, Inc.) and protein concentrations were measured using a BCA protein assay kit (Thermo Scientific).

### 2.7. Western blots

The high sensitivity Western Breeze Chemiluminiscence Kit (Invitrogen) with Alkaline Phosphatase (AP) conjugated anti-mouse secondary antibody was used to perform Western blots. The Magic Mark ladder (Invitrogen) was used for size determination. Ten microliters of cell lysate or purified dialysate were added to 5 μl 4× LDS sample buffer (Invitrogen) and 5 μl deionized water. Twenty microliters of each sample were heated at 70 °C for 10 min and 15 μl was loaded on a NuPAGE® Novex 4–12% Bis-Tris Gel (Invitrogen). After 30 min of electrophoresis at a constant voltage of 200 V, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen) using an XCell SureLock® Mini-Cell and XCell II™ Blot Module Kit (Invitrogen). Transfer was performed for 80 min at a constant voltage of 30 V. Membranes were blocked with blocking buffer provided with the Western Breeze Kit and stained with anti-V5 antibody and an AP conjugated secondary antibody (Invitrogen) as per the instructions provided by the manufacturer. Chemiluminescence was detected by exposing Blue X-ray film (ISC Bioexpress) to the blots for different exposure times.

### 2.8. Ammonium sulfate precipitation

Thirty percent ammonium sulfate (AS) was used to precipitate proteins in culture supernatants from the transfected cells. Fifty milliliters of supernatant was used for each protein. The required quantity of ammonium sulfate was calculated using the EnCor Biotechnology Inc. webpage (http://www.encorbio.com/protocols/AM-SO4.htm). Salt was added slowly while stirring the supernatant. Precipitation was performed at 4 °C for 1 h. The suspension was centrifuged at 10,000*g* for 10 min at 4 °C to pellet the precipitated material. The supernatant was poured off and the pellet was dissolved in a mixture of 50% phosphate buffer with 50 mM L-Arg and L-Glu, pH 7.2 and 50% DMSO.

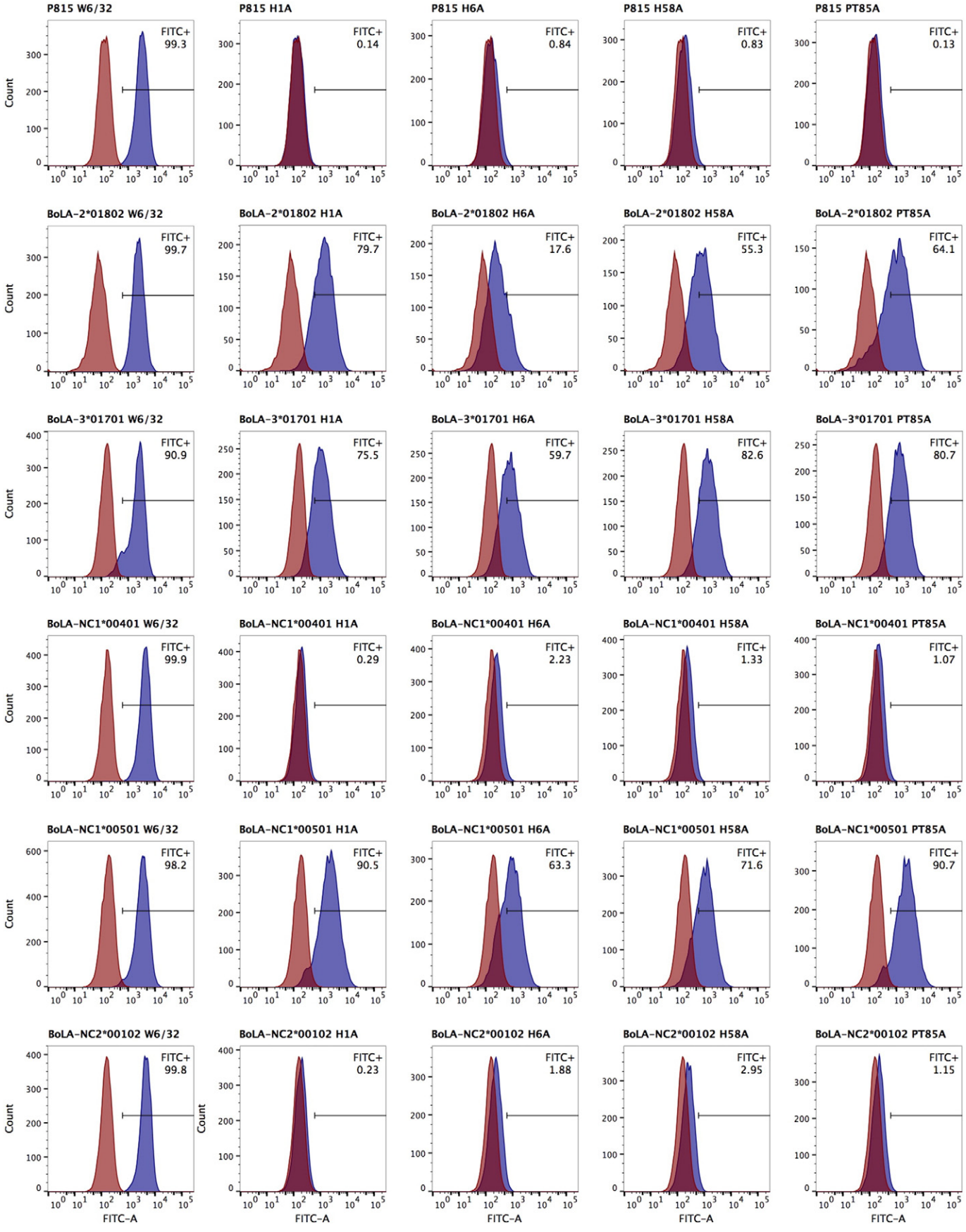
## 3. Results

### 3.1. Subcloning of MHC-I cDNAs

Most subclones had inserts with the correct sequences in the correct orientation and contained the C-terminal 6× Histidine tag and V5 epitope in-frame. The following MHC-I isoforms from the AH11 haplotype were expressed in the two cell lines: BoLA-2*01802, BoLA-3*01701, BoLA-NC1*00401 (splice variants with no transmembrane domain), BoLA-NC1*00501 (full length protein with transmembrane domain), BoLA-NC2*00102, BoLA-NC3*00101 and BoLA-NC4*00201 (Davies et al., 2006).

### 3.2. Evaluation of cell surface expression by flow cytometry

Transfected cell lines were analyzed by flow cytometry to determine which bovine MHC-I proteins were expressed on the cell surface. Twenty-four hours post-transfection <20% of cells expressed the bovine MHC-I transgenes. Expression of the transgenes increased to about 40% after selection with G418 for two weeks. Following FACS-sorting of cell lines with cell surface expression of bovine MHC-I proteins a high level of stable transgene expression, usually >90% positive cells, was achieved. Due to lack of surface expression of the BoLA-


P815 W6/32
FITC+ 99.3
P815 H1A
FITC+ 0.14
P815 H6A
FITC+ 0.84
P815 H58A
FITC+ 0.83
P815 PT85A
FITC+ 0.13
BoLA-2*01802 W6/32
FITC+ 99.7
BoLA-2*01802 H1A
FITC+ 79.7
BoLA-2*01802 H6A
FITC+ 17.6
BoLA-2*01802 H58A
FITC+ 55.3
BoLA-2*01802 PT85A
FITC+ 64.1
BoLA-3*01701 W6/32
FITC+ 90.9
BoLA-3*01701 H1A
FITC+ 75.5
BoLA-3*01701 H6A
FITC+ 59.7
BoLA-3*01701 H58A
FITC+ 82.6
BoLA-3*01701 PT85A
FITC+ 80.7
BoLA-NC1*00401 W6/32
FITC+ 99.9
BoLA-NC1*00401 H1A
FITC+ 0.29
BoLA-NC1*00401 H6A
FITC+ 2.23
BoLA-NC1*00401 H58A
FITC+ 1.33
BoLA-NC1*00401 PT85A
FITC+ 1.07
BoLA-NC1*00501 W6/32
FITC+ 98.2
BoLA-NC1*00501 H1A
FITC+ 90.5
BoLA-NC1*00501 H6A
FITC+ 63.3
BoLA-NC1*00501 H58A
FITC+ 71.6
BoLA-NC1*00501 PT85A
FITC+ 90.7
BoLA-NC2*00102 W6/32
FITC+ 99.8
BoLA-NC2*00102 H1A
FITC+ 0.23
BoLA-NC2*00102 H6A
FITC+ 1.88
BoLA-NC2*00102 H58A
FITC+ 2.95
BoLA-NC2*00102 PT85A
FITC+ 1.15
Count
FITC-A

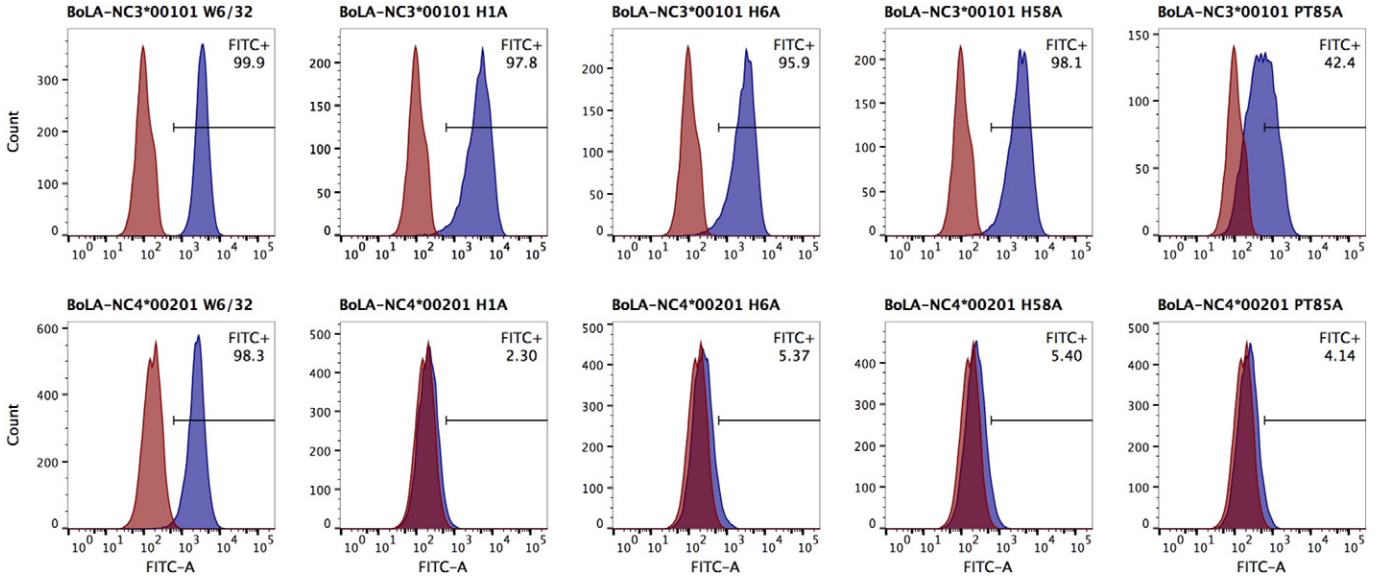



**Fig. 1.** Flow cytometric analysis of FACS sorted murine P815 cells transfected with bovine MHC-Ia and MHC-Ib transgenes. Data for four anti-bovine MHC-I monoclonal antibodies are shown: H1A, H6A, H58A and PT-85A (blue histograms). ColiS205D1 (red histograms) and W6/32 (blue histograms), which reacts with mouse MHC-I on the P815 cells, were used as negative and positive antibody controls, respectively. Untransfected P815 cells served as a negative control for transgene expression (first line). The MHC-Ia proteins BoLA-2*01802 and BoLA-3*01701, and MHC-Ib proteins BoLA-NC1*00501 and BoLA-NC3*00101 exhibited cell membrane expression on P815 cells. The BoLA-NC1*00401, BoLA-NC2*00102 and BoLA-NC4*00201 proteins did not exhibit cell surface expression. With these three MHC-Ib proteins and the untransfected P815 cells the histograms for the antibody control, ColiS205D1, and MCH-I specific antibodies are superimposed (dark red).

NC1*00401 and BoLA-NC2*00102 non-classical MHC-I proteins, it was not possible to sort the cell lines expressing these transgenes.

The BoLA-2*01802, BoLA-3*01701, BoLA-NC1*00501 and BoLA-NC3*00101 isoforms were expressed on the cell membrane in P815 cells (Fig. 1). All the monoclonal antibodies – H1A, H6A, H11A, H58A, PT85A and IL-A88 – reacted with all of the bovine MHC-I proteins (Table 1). However, antibody affinity varied considerably with the different MHC-I isoforms. In transfected K562 cells the BoLA-2*01802, BoLA-3*01701, BoLA-NC1*00501, BoLA-NC3*00101 and BoLA-NC4*00201 isoforms were expressed on the cell surface (Fig. 2). Reactivity of the H1A, H6A, H11A, H58A and PT85A antibodies on K562 cells varied depending on the specific bovine MHC-I isoform expressed by the transfected cell line (Table 2). For instance, the H6A and H11A antibodies reacted very weakly with K562 cells expressing the BoLA-2*01802 and BoLA-NC1*00501 proteins. Similarly, the H1A and PT-85A antibodies did not react with the BoLA-NC4*00201 protein.

### 3.3. Detection of protein expression by Western blotting

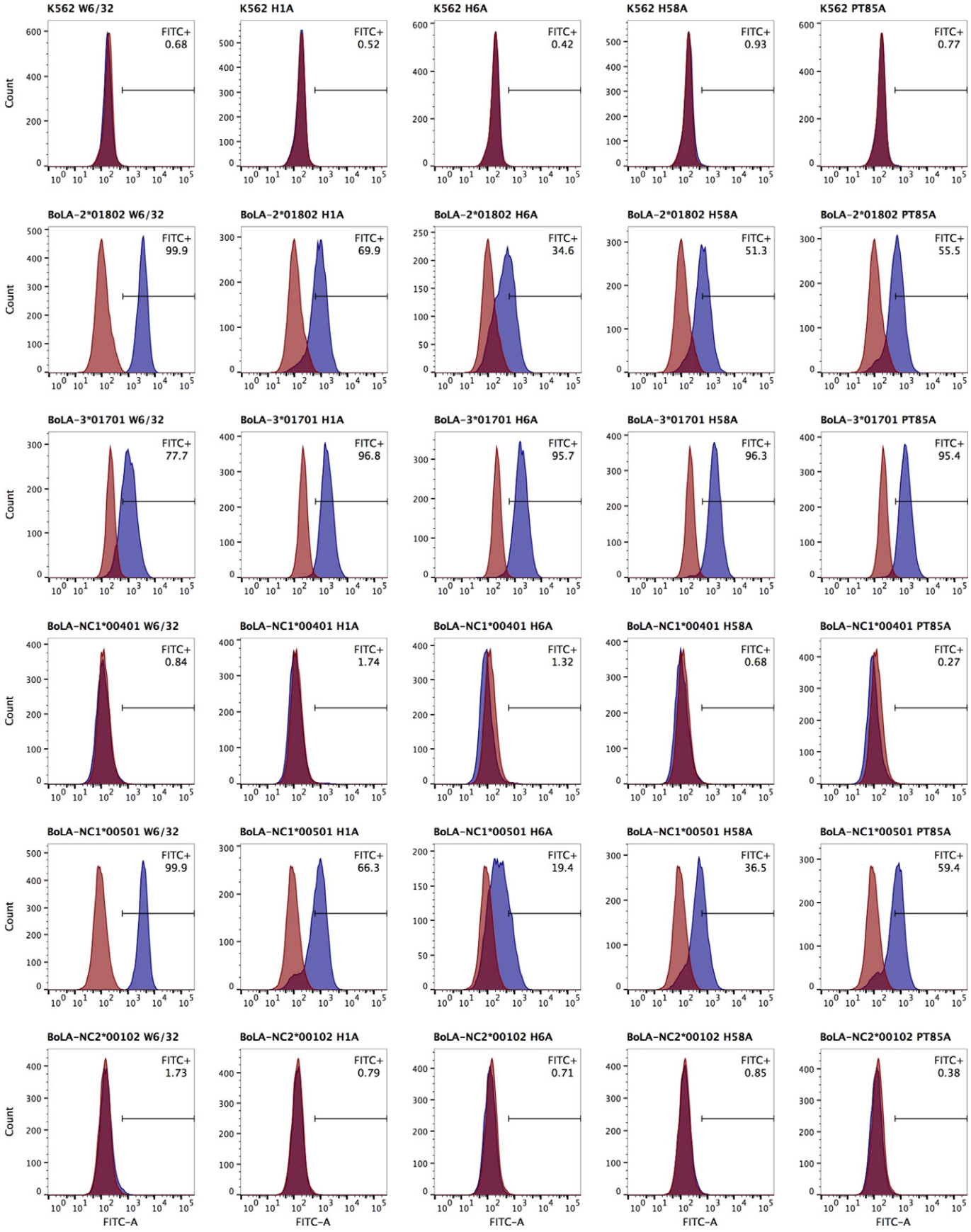
To confirm that the transgenes were translated in the host cells, we performed Western blotting of the crude cell lysates using an anti-V5 antibody (Invitrogen), which recognizes the V5 epitope in the C-terminal peptide. We also tested an antibody directed against the C-terminal His tag (Invitrogen) but this antibody did not work. A cell line transfected with a plasmid encoding β-galactosidase, a 120 kD protein, was used as a positive control. A lysate from untransfected cells was used as a negative control. Lysates from all of the MHC-I transfected cell lines contained proteins of approximately 45 kD that were bound by the anti-V5 antibody. The BoLA-NC1*00401 protein, which has a complete deletion of the transmembrane domain, was slightly smaller than the other MHC-I proteins. Sometimes there were non-specific protein bands seen in the crude lysates from the transfected cells but these bands disappeared after purification of the proteins with His GraviTrap Columns (GE Healthcare; Figs. 3 and 4).

### 3.4. Assessment of protein secretion by ammonium sulfate precipitation

MHC-I proteins in the culture supernatants were detected by ammonium sulfate precipitation followed by Western blotting with anti-V5 antibody (Figs. 5 and 6). Strong bands were present for all of the surface expressed MHC-I proteins: BoLA-2*01802, BoLA-3*01701, BoLA-NC1*00501, BoLA-NC3*00101, and BoLA-NC4*00201 (only in K562 cells). This suggests that the MHC-I proteins were being shed from the cell membrane at a significant rate. There was a clear but fairly weak band for BoLA-NC1*00401, which lacks the transmembrane domain, in the supernatant from K562 cells suggesting that this may be a secreted isoform. The BoLA-NC2*00102 protein apparently remained trapped inside the cells as it was not detected in the culture supernatants.

**Table 1**
Reactivity patterns of monoclonal antibodies on P815 cells transfected with cattle MHC-I genes.

| P815 cell line | Monoclonal antibody | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | ColiS205 | W6/32 | H1A | H6A | H11A | H58A | PT85A | IL-A88 |
| Untransfected | − | ++++ | − | − | − | − | − | − |
| BoLA-2*01802 | − | ++++ | +++ | + | + | ++ | ++ | +++ |
| BoLA-3*01701 | − | ++++ | +++ | ++ | ++ | +++ | +++ | +++ |
| NC1*00401 | − | ++++ | − | − | − | − | − | − |
| NC1*00501 | − | ++++ | ++++ | ++ | ++ | +++ | ++++ | +++ |
| NC2*00102 | − | ++++ | − | − | − | − | − | − |
| NC3*00101 | − | ++++ | ++++ | ++++ | ++++ | ++++ | ++ | ++++ |
| NC4*00201 | − | ++++ | − | − | − | − | − | − |


K562 W6/32
FITC+ 0.68
K562 H1A
FITC+ 0.52
K562 H6A
FITC+ 0.42
K562 H58A
FITC+ 0.93
K562 PT85A
FITC+ 0.77
BoLA-2*01802 W6/32
FITC+ 99.9
BoLA-2*01802 H1A
FITC+ 69.9
BoLA-2*01802 H6A
FITC+ 34.6
BoLA-2*01802 H58A
FITC+ 51.3
BoLA-2*01802 PT85A
FITC+ 55.5
BoLA-3*01701 W6/32
FITC+ 77.7
BoLA-3*01701 H1A
FITC+ 96.8
BoLA-3*01701 H6A
FITC+ 95.7
BoLA-3*01701 H58A
FITC+ 96.3
BoLA-3*01701 PT85A
FITC+ 95.4
BoLA-NC1*00401 W6/32
FITC+ 0.84
BoLA-NC1*00401 H1A
FITC+ 1.74
BoLA-NC1*00401 H6A
FITC+ 1.32
BoLA-NC1*00401 H58A
FITC+ 0.68
BoLA-NC1*00401 PT85A
FITC+ 0.27
BoLA-NC1*00501 W6/32
FITC+ 99.9
BoLA-NC1*00501 H1A
FITC+ 66.3
BoLA-NC1*00501 H6A
FITC+ 19.4
BoLA-NC1*00501 H58A
FITC+ 36.5
BoLA-NC1*00501 PT85A
FITC+ 59.4
BoLA-NC2*00102 W6/32
FITC+ 1.73
BoLA-NC2*00102 H1A
FITC+ 0.79
BoLA-NC2*00102 H6A
FITC+ 0.71
BoLA-NC2*00102 H58A
FITC+ 0.85
BoLA-NC2*00102 PT85A
FITC+ 0.38
Count
FITC-A

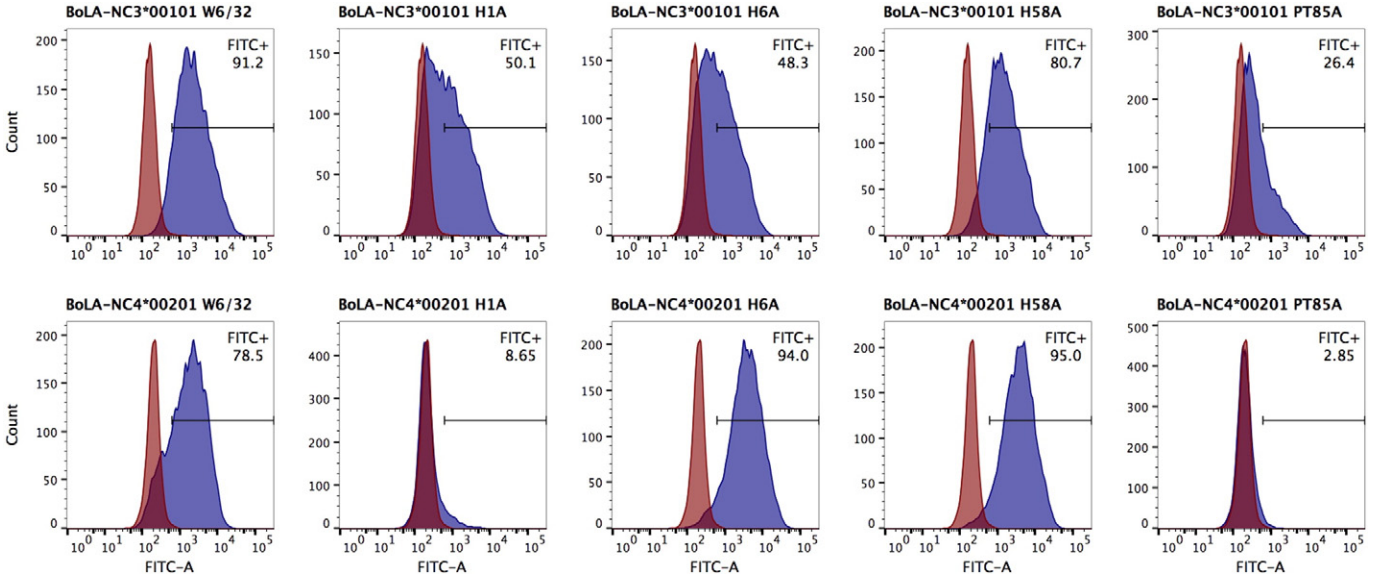



**Fig. 2.** Flow cytometric analysis of MHC-Ia and MHC-Ib expression on transfected human MHC-I deficient K562 cells. Reactivity of the W6/32, H1A, H6A, H58A and PT-85A monoclonal antibodies with the bovine MHC-I proteins is shown (blue histograms). The ColiS205D1 monoclonal antibody was used as an antibody control (red histograms). Untransfected K562 cells served as a negative control for transgene expression (first line). The MHC-Ia proteins BoLA-2*01802 and BoLA-3*01701, and MHC-Ib proteins BoLA-NC1*00501, BoLA-NC3*00101 and BoLA-NC4*00201 were expressed on the surface of transfected K562 cells. However, the BoLA-NC1*00401 and BoLA-NC2*00102 MHC-Ib proteins did not exhibit cell surface expression. With these two proteins and the untransfected K562 cells the histograms for the antibody control, ColiS205D1, and MCH-I specific antibodies are superimposed (dark red).

## 4. Discussion

This study investigated the expression of bovine MHC-I isoforms encoded at two MHC-Ia and four MHC-Ib loci in two different cell lines, a murine cell line (P815) and a human cell line (K562). In addition, we characterized the reactivity pattern of a panel of anti-MHC-I monoclonal antibodies on the expressed MHC-I proteins. We found that the two classical isoforms BoLA-2*01802 and BoLA-3*01701 and the non-classical isoforms BoLA-NC1*00501, BoLA-NC3*00101 and BoLA-NC4*00201 were expressed as membrane associated proteins (Figs. 1 and 2). Other researchers have previously demonstrated expression of classical bovine MHC-I proteins and the non-classical BoLA-NC1*00101 protein (originally called N*50001) on the surface of transfected P815 cells (Araibi et al., 2006; Ellis et al., 1999; Ellis et al., 2005). It has also been reported that expression of BoLA-NC3*00101 was documented in the PhD thesis of N. Barker (Barker, 1996; Birch et al., 2008a). A novel finding in our study was that the BoLA-NC4*00201 heavy chain remained intracellular in P815 cells but exhibited surface expression on K562 cells (Figs. 1 and 2). It is likely that human β2m can associate with the BoLA-NC4*00201 heavy chain thereby allowing peptide binding and cell surface expression, while murine β2m does not form a functional heterodimer with this bovine MHC-I heavy chain. The percent amino acid identity for the mature β2m peptides is 75.8% for bovine (GenBank Acc. # XM_001251107 and X69084) and human (GenBank AF072097), 67.7% for bovine and murine (GenBank NM_009735), and 69.7% for human and murine β2m. It seems plausible that this level of variation could influence pairing with the MHC class I heavy chain and/or the tertiary structure of the heterodimer. An alternative explanation is that murine P815 cells don't contain an appropriate peptide ligand for BoLA-NC4*00201.

Two MHC-Ib proteins, the BoLA-NC1*00401 protein (formerly called BoLA-N*50501), which was expressed as a *BoLA-NC1* splice variant that lacks the transmembrane domain (Davies et al., 2006), and the BoLA-NC2*00102 protein were expressed as intracellular proteins in transfected cells (Figs. 3 and 4) but were not present on the cell membrane (Figs. 1 and 2). Ellis and colleagues (Ellis et al., 1996) attempted to express a different *BoLA-NC2* allele (*BoLA-NC2*00101* referred to as HD15 in this paper) in P815 cells and failed to detect any protein expressed at the cell surface even when they co-transfected the cells with bovine β2m. We agree with Birch et al. (2008a) that intracellular retention of the heavy chain in multiple cell lines suggests that BoLA-NC2*00101 may require a specific peptide for cell surface expression, as is the case with HLA-E (Braud et al., 1998b; Lee et al., 1998; Sala et al., 2004). The BoLA-NC2*00102 protein has an intact transmembrane domain (Davies et al., 2006), suggesting that this is a membrane associated rather than a secreted protein. Furthermore, we found no evidence for secretion of the BoLA-NC2*00101 heavy chain in our ammonium sulfate precipitation experiments (Figs. 5 and 6). Further research is needed to determine the requirements for surface expression of BoLA-NC2 and whether the *BoLA-NC2* gene is an orthologue of HLA-E.

**Table 2**
Reactivity patterns of monoclonal antibodies on K562 cells transfected with cattle MHC-I genes.

| K562 cell line | Monoclonal antibody | | | | | | |
|---|---|---|---|---|---|---|---|
| | ColiS205 | W6/32 | H1A | H6A | H11A | H58A | PT85A |
| Untransfected | − | − | − | − | − | − | − |
| BoLA-2*01802 | − | ++++ | +++ | + | + | ++ | ++ |
| BoLA-3*01701 | − | +++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| NC1*00401 | − | − | − | − | − | − | − |
| NC1*00501 | − | ++++ | ++ | + | + | + | ++ |
| NC2*00102 | − | − | − | − | − | − | − |
| NC3*00101 | − | ++++ | ++ | ++ | ++ | +++ | + |
| NC4*00201 | − | +++ | − | ++++ | ++++ | ++++ | − |

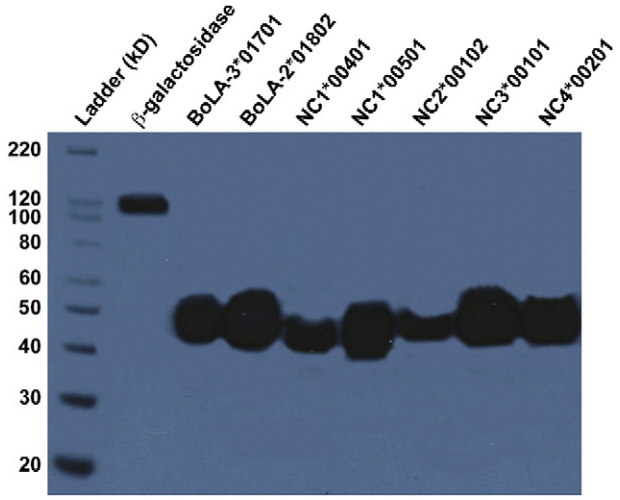



**Fig. 3.** Expression of recombinant MHC-Ia and MHC-Ib proteins in mouse P815 cells. Purified proteins were detected on a Western blot with anti-V5 antibody. Beta-galactosidase was expressed as a positive transfection control.

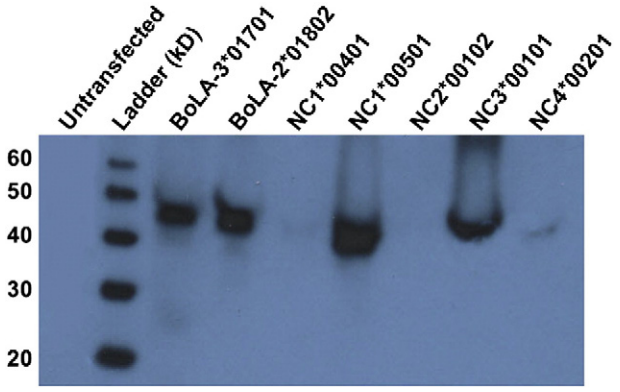



**Fig. 5.** Identification of bovine MHC-Ia and MHC-Ib proteins released in P815 cell culture supernatants. Ammonium sulfate-precipitated proteins from transfected murine P815 cell culture supernatants were detected by Western blot with anti-V5 antibody.

As far as we know, we are the first to investigate secretion of bovine MHC-I proteins. Immunoblotting experiments with proteins precipitated from culture supernatants showed that all of the surface expressed MHC-I proteins were shed or released from the cell membrane (Figs. 5 and 6). An anti-V5 monoclonal antibody was used for the Western blots to detect the bovine MHC-I proteins. Consequently, only full-length proteins that were shed from the cell membrane and retained their cytoplasmic tail with its 3′ histidine tag and V5 epitope were detected. MHC-I proteins that were enzymatically cleaved from the cell membrane would not be detected with this antibody.

The presence of multiple *BoLA-NC1* splice variants is reminiscent of what has been observed with the *HLA-G* and *Qa-2* genes (Comiskey et al., 2003; Davies et al., 2006; Ellis et al., 1986; Hunt et al., 2005). Because we used a splice variant of the *BoLA-NC1*00401* gene that lacks a transmembrane domain, we predicted that this transgene would encode a secreted protein. Although there was noticeable secretion of the BoLA-NC1*00401 protein by transfected K562 cells (Fig. 6) the level of secretion was fairly low compared to the level of shedding of membrane associated MHC-I proteins. One explanation for the low level of secretion is that this cell line was not enriched for high expressing cells using fluorescence activated cell sorting. It is also possible that secretion of *BoLA-NC1* splice variants is more efficient in certain specialized cell types such as trophoblast cells.

Identification of monoclonal antibodies that distinguish different MHC isoforms, particularly the products of different MHC-I genes, is important for identification of specific isoforms on the surface of specialized cells such as trophoblast cells and for the detection of secreted or shed MHC-I proteins that could act as immunomodulatory molecules. The monoclonal antibodies that were tested varied in their affinity for the different MHC-I isoforms and in some cases reacted slightly differently in the two cell lines. For the two classical MHC-I proteins there were some clear differences in antibody reactivity (Tables 1 and 2). All of the antibodies, with the exception of W6/32, had very high affinity for the BoLA-3*01701 protein expressed on K562 cells. The percentage of cells staining positive for BoLA-3*01701 was somewhat lower in P815 cells but it is likely that this was due to a lower level of protein expression on the cell surface rather than lower antibody affinity. The BoLA-2*01802 protein stained strongly with W6/32, moderately well with H1A and IL-A88, relatively poorly with H58A and PT85A, and very weekly with H6A and H11A in both cell lines. Two of the non-classical MHC-I proteins, BoLA-NC1*00501 and BoLA-NC3*00101 were expressed at a much higher level in P815 cells than in K562 cells. In contrast, the BoLA-NC4*00201 isoform was expressed at a high level in K562 cells and not at all in P815 cells. The BoLA-NC1*00501 protein was bound with very high affinity by W6/32 in the K562 cells, which had relatively low protein expression, and showed strong reactivity with H1A and PT85A, particularly in the P815 cells. The BoLA-NC3*00101 protein was recognized particularly strongly by W6/32 in K562 cells and by H58A in both cell lines, but H1A, H6A, H11A and IL-A88 also reacted strongly with this MHC-I protein on P815 cells. The BoLA-NC4*00201 protein was recognized strongly by H6A, H11A and H58A and somewhat less strongly by W6/32. Interestingly, neither the H1A or PT85A antibodies recognized this protein (Fig. 2).

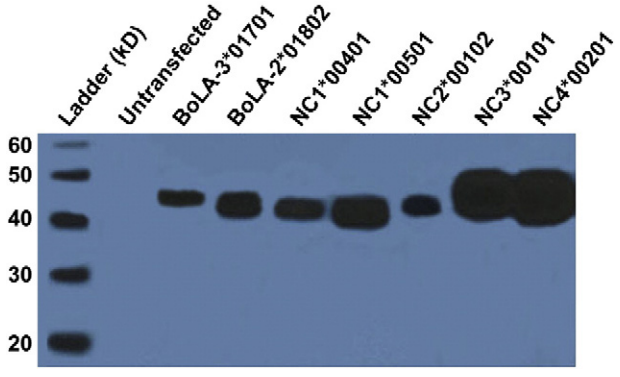



**Fig. 4.** MHC-Ia and MHC-Ib proteins were expressed in transfected K562 cells. Purified recombinant proteins were detected on a Western blot with anti-V5 antibody. Untransfected K562 cells were included as a negative control.

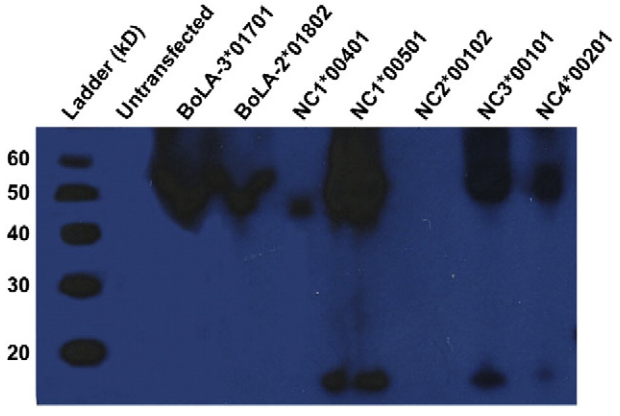



**Fig. 6.** Detection of bovine MHC-Ia and MHC-Ib proteins released in K562 cell culture supernatants. Proteins were precipitated using $(NH4)_2\ SO_4$ and detected with anti-V5 antibody on a Western blot.

In conclusion, this comprehensive study demonstrated cell surface expression of three non-classical MHC-I proteins – BoLA-NC1*00501, BoLA-NC3*00101 and BoLA-NC4*00201 – but found no evidence for cell surface expression of two other MHC-Ib proteins: BoLA-NC1*00401 and BoLA-NC2*00102. The BoLA-NC1*00401 isoform that was used in our experiments is a *BoLA-NC1* splice variant lacking a transmembrane domain. Consequently, we had hypothesized that this construct would code for a secreted protein. Our data support secretion of BoLA-NC1*00401, and also shedding and/or secretion of all membrane associated MHC-I proteins tested. Lack of surface expression of BoLA-NC2*00102 is particularly interesting. It appears that this highly conserved, virtually monomorphic, MHC-Ib protein has special requirements for cell surface expression such as the presence of a particular peptide ligand. This is intriguing because it implies that the *BoLA-NC2* gene could be a functional orthologue of HLA-E. Future objectives include: (1) identification of the receptors that bind bovine non-classical MHC-I proteins, (2) determination of the requirements for cell surface expression of the BoLA-NC2 protein, and (3) measurement of the level of secretion or shedding of soluble MHC-I proteins during bovine pregnancy.

## Conflict of interest

The authors have no conflict of interest to declare.

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