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A non-recoverable hybridoma limits the production of monoclonal antibodies against bovine trophoblast non-classical *NC3*00101* protein

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The non-classical MHC class I (*MHC-Ib*) proteins are important modulators of immune system during pregnancy favoring survival of the fetus. Contrary to ubiquitously expressed classical *MHC-I* proteins, *MHC-Ib* proteins are oligomorphic, and expressed in specific cell/tissue types thus minimizing maternal immune-mediated rejection of fetal-allograft and a successful pregnancy. A unique characteristic of *MHC-Ib* glycoproteins is expression of surface and soluble isoforms due to alternative splicing phenomenon. Bovine fetal trophoblast cells, during the third trimester of pregnancy, express non-classical bovine leukocyte antigen class Ib (*BoLA-Ib*) antigens. *BoLA-NC3*00101*, is a non-classical class I allele from cattle AH11 haplotype and is expressed as cell surface isoform. However, lack of monoclonal antibody (mAb) hinders the development of specific assay to detect the soluble/secreted *BoLA*-I antigens released by fetal trophoblast cells. The objective of this study is to synthesize mAb specific to *NC3*00101* molecule to develop an isotype-matched ELISA to assess *BoLA-I* protein(s). We demonstrated that majority of *NC3*00101* hybridoma-supernatants were low-titer and showed inappreciable reactivity in ELISA and flow cytometry assays. We identified a clone of hybridoma (NC3-3*2) that secreted mAb specific to *BoLA-NC3*00101* as demonstrated with flow cytometry. NC3-3*2 clone secreted supernatant that captured *BoLA-NC3*00101* protein in ELISA. Unfortunately, despite several efforts we could not recover the cryopreserved hybridoma. Non-recoverability and instability, thus, limited production of *NC3*00101*-mAbs. This study provides the evidence that mice immunized with bovine class Ib proteins elicit a specific immunogenic response and warrants future studies into generation of stable hybridoma secreting antibodies against *BoLA-Ib* proteins using robust methods of fusion and cryopreservation of hybridomas.

Keywords: Bovine leukocyte antigen (*BoLA*), Non-classical *NC3*00101* protein, Monoclonal antibodies, Hybridoma, Trophoblast, P815 cell line

The major histocompatibility complex (MHC) genetic region encodes the *MHC-I* and -II transplantation antigens[1]. Classical *MHC-I* (*MHC-Ia*) molecules are extremely polymorphic and ubiquitously expressed and present intracellular foreign or non–self-pathogen-derived peptides to CD8+ T lymphocytes. Consequently, they are involved in the acceptance or rejection of tissue grafts based on the degree of similarity of these proteins among the donor and recipient cells. An allogeneic fetus must remain unharmed by the mother's potentially hostile immune system throughout the term for a successful pregnancy. Discovery of non-classical *MHC-I* proteins (*MHC-Ib*/class Ib), such as human leukocyte antigen (HLA)-E, -F, and -G[2] in humans and Qa-2 in mice[3,4], led to a new paradigm of fetal immune tolerance. They play an immune-modulatory role and possess the following major characteristics, which make them uniquely different from *MHC-Ia* proteins[5]: (a) *MHC-Ib* proteins are oligomorphic or monomorphic; (b) Their expression is conditional and/or tissue specific; and (c) They are produced as surface and/or soluble isoforms as a result of alternative or differential splicing.

One major function of class Ib proteins is that they mediate inhibitory and activating stimuli and inhibit the lytic properties of uterine natural killer cells contributing to fetomaternal tolerance[6]. Notably, HLA-G is a potent immunomodulatory antigen expressed by invasive cytotrophoblast cells and is

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encoded as surface and soluble isoforms. Soluble HLA-G is present in maternal blood during pregnancy, and a low concentration of it has been found to be associated with pregnancy failure[7]. Moreover, soluble HLA-G is secreted by human embryos before implantation and is associated with a higher implantation rate, uninterrupted gestation, and more live births resulting from in vitro fertilization[8]. Expression of HLA-G increases the cleavage rate of human embryos and plays an important role in embryonic development[9].

The bovine MHC region (bovine leukocyte antigen [*BoLA*]) is divided into three regions—I, II, and III—each of which encodes proteins with different functions[10]. The *BoLA-Ia* locus encodes at least six classical class I genes, which are expressed in a number of different combinations[11]. Transcripts from *BoLA-Ib* loci have been attributed to the *BoLA*-NC1, *BoLA*-NC2, *BoLA*-NC3, and *BoLA*-NC4 glycoproteins. Analogous to other class Ib proteins, *BoLA-Ib* proteins have putative amino acid motifs in their exons and are expressed specifically in trophoblast cells. *BoLA*-NC1 also is expressed as a surface isoform (NC1*00501) and a soluble/secreted isoform (NC1*00101)[12].

Evaluation of soluble and/or secreted *BoLA-Ib* proteins released by embryos and trophoblast culture supernatant is vital to understanding the role of class Ib proteins in bovine embryonic development and the establishment of pregnancy. However, unavailability of a monoclonal antibody to *BoLA-Ib* proteins is a caveat to developing a quantitative enzyme-linked immunosorbent assay (ELISA). Monoclonal antibody production technology discovered by Kohler and Milstein[13] not only enables researchers to isolate a specific antibody from a complex mixture of polyclonal antibodies present in the in vivo immunization response; it also allows scientists to culture hybridomas and produce antibodies in large quantities in vitro when required. By virtue of their specificity to a single epitope, monoclonal antibodies decrease background noise and cross-reactivity with nonspecific antigens. Monoclonal antibodies have been used in various diagnostic tests to detect minute quantities of antigens such as hormones, enzymes, drugs, and toxins. For instance, human chorionic gonadotropin is detected in urine or serum to diagnose pregnancy in humans; acquired immune deficiency syndrome is diagnosed using the ELISA test[14-16].

In this study, we used the *BoLA-NC3*00101* protein to immunize mice to generate monoclonal antibodies and thus to develop an ELISA that can be used to detect secreted or soluble *BoLA-NC3*00101* and possibly other secreted or soluble bovine *MHC-Ib* proteins produced from trophoblast cells and embryos. Detection of the *BoLA-NC3*00101* protein in serum/trophoblast supernatant is instrumental to understand the association between pregnancy success rate and the expression level of this protein.

Materials and Methods

Cell line and transfection with bovine NC3*00101 cDNA

Murine mastocytoma P815 cells were used to express *NC3*00101* protein. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% bovine calf serum at 37°C and 5% CO_2. The cell line was obtained from American Type Culture Collection (ATCC, USA). Coding sequence for *BoLA-NC3*00101* was amplified from complementary deoxyribonucleic acid (cDNA) using specific primers as below:

Forward primer (BoC1FP-E1B)
ACCATGGGGCCGCGAACCCTC

Reverse primer (BoC1RP-E7A)
TTTAGGAACCGTGAGAGACACATC

The reverse primer was designed to produce clones expressing a 3' 6x histidine tag and V5 epitope. The pcDNA3.1™D/V5-His-TOPO® directional mammalian expression vector (Invitrogen) was used for cloning the cDNA and expressing the protein in mammalian P815 cells. Positive clones were sequenced and a perfect *NC3*00101* clone was selected for transfection. Cells were transfected as per the method described earlier[17]. The transfected cells were analyzed with flow cytometer to check initial expression. Transfectants were selected with Geneticin (G418) antibiotic and stably transfected cells were sorted using fluorescence activated cell sorter (FACS). The sorted high-expressing cells were grown in T75 tissue culture flasks until they reached a sufficient number to seed the pitched-blade bioreactor[17].

Flow cytometry and isolation of protein

The *NC3*00101*-trasnefected P815 cells were stained for flow cytometry using the protocol described earlier[17]. Briefly, cells were resuspended in fluorescence buffer (FB) (PBS with 0.1% sodium azide, 1% bovine serum albumin) and incubated with

H1A antibody (15 µg/mL) for 15 min. Cells were washed twice with FB and then incubated with secondary antibody for 15 min. All incubations were performed at 4°C. Cells were washed twice and fixed in PBS with 1% of 37% formaldehyde or paraformaldehyde. One million cells were stained for each sample. An irrelevant antibody ColiS169A was used as a negative control. Cells were analyzed using a Becton-Dickinson FACS (Fluorescence Activated Cell Sorter) Aria II flow cytometer equipped with FACS Diva software. The FACS-sorter was used to sort and enrich the high expressing cells using fluorescence buffer without sodium azide in it. For hybridoma testing, we used supernatants as the primary antibodies. *NC3*00101*-transfected cells were stained with serum on flow cytometer. Untransfected P815 cells were used as negative control. Sorted cells were grown in large scale using a pitched-blade bioreactor and protein was isolated using affinity-column chromatography as explained earlier. Specific *NC3*00101* protein band was identified with anti-V5 antibody on Western blot and purified with affinity column chromatography as explained earlier[17].

ELISA screening of hybridoma supernatant

ELISA plates (CoStar Inc.) were coated with 16 µg/mL of *NC3*00101* antigen diluted in ELISA-AP binding buffer (0.05M Tris, pH 9.5). The plate was incubated overnight at 4°C. Plate was washed 4X with the ELISA wash buffer (phosphate buffered saline, 0.1% sodium azide, 0.05% Tween-20) and blocked for 2 h at 37°C with blocking buffer (0.05M Tris, 0.3% BSA) to block the non-specific protein binding sites. After 4X washes, antibodies H1A, W6/32, H6A, H11A and hybridoma supernatants were added to the wells. The antibody used per well was 1 µg. Antibodies were diluted in dilution buffer-I. Plates were incubated at 37°C for 1 h and washed 4x. The secondary antibody W6/32-biotin (Novus Biologicals) diluted in dilution buffer-II was added and incubated for 1 h at 37°C. After washing the plate four times, substrate buffer (50 mM K_2CO_3, 1 µM $MgCl_2.6H_2O$) was added and incubated for 1 h at 37°C. Finally, *para*-nitro phenylphosphate (pNPP) solution (1 tablet of Sigma 104 phosphatase substrate in 5 mL of substrate buffer) was added to the wells and incubated at 37°C. End point reading was measured at OD 405 nm at every 15 min interval up to an hour. Data were analyzed by plotting line graphs taking time on X axis and OD on Y axis. We used diluted (1:100) and neat (1:1) *NC3*00101*-hybridoma culture supernatants in ELISA.

For Sandwich ELISA the procedure was same as mentioned above except that the plate was coated with antibody or supernatant overnight at 4°C and after blocking the plate, antigen was added. Sandwich ELISA was intended to detect soluble bovine *MHC-Ib* proteins. In other studies, a capture (sandwich) ELISA procedure had been used to detect the soluble class-I proteins[7,8,18-20].

Purification of NC3*00101 protein

The method is detailed previously[17]. Briefly, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl with 1 mM mammalian protease inhibitor Cocktail (Sigma), 1 mM PMSF) per 1×10^7 cells/mL of lysis buffer. After an hour of incubation at 4°C, the supernatant (lysate) was collected by centrifugation at 10000 g for 15 min at 4°C.

Specific histidine-tagged protein was purified using His-GraviTrap Columns (GE Healthcare). Eluates which showed clear and high-intensity bands on Western blots were pooled and resultant purified protein solution was dialyzed against phosphate buffer solution (20 mM sodium phosphate, 500 mM NaCl, pH 7.4) using 20 KDa MWCO Slide-A-Dialysis cassettes (Pierce). Dialysates were centrifuged at maximum speed for 10 min at 4°C and the supernatant was recovered in a fresh tube. The dialysates were concentrated using Vivaspin-20 concentrators with 30 KD MWCO membranes (Vivaproducts Inc., USA). The concentration of purified protein was measured using a BCA protein assay kit (Thermo Scientific).

Monoclonal antibody production

Immunization

Antibodies to *NC3*00101* protein were produced at Washington State University (WSU) Monoclonal Antibody Center following the protocol described by Hamilton and Davis[21]. A total of three batches of immunizations were conducted for the *NC3*00101*. Each batch, four mice were hyperimmunized with purified antigen in Ribi's Adjuvant (Sigma-Aldrich). Each mouse was given three immunizations (d0, d14, and d21) each with 50 µg antigen. Mice were bled before each immunization and serum was collected. Mouse serum ELISA was performed after d21 to determine titer and a final booster dose of the antigen without adjuvant was administered to mice intravenously through the tail three days prior to fusion of spleen cells and myeloma.

Fusion

For each fusion, mice were euthanized and spleen cells (plasma cells or B-lymphocytes) were collected aseptically and pooled from four mice. Approximately, 1×10^8 spleen cells were fused with 4×10^7 X63 Ag8.653 myeloma cells (Kearney *et al*.[22]) following the protocol described earlier[21]. Fused cells were resuspended in growth medium containing hypoxanthine aminopterin and thymidine (HAT medium) and cultured in ten 96-well culture plates.

Initial screening of Hybridoma

With the help of indirect ELISA and flow cytometry, hybridoma supernatants were tested for immunoglobulin of interest[23]. Positive hybridomas were cloned or expanded in 12-well culture plates. Two ampoules of hybridomas per culture were collected and cryopreserved following expansion. Additional supernatants were collected and characterized with ELISA, flow cytometry and Western Blotting.

Culture of hybridoma

We obtained live cultures of *NC3*00101*-hybridoma (WSU, Antibody Center, Pullman) and IL-A88 and IL-A19 hybridomas (Sigma-Aldrich). They were cultured in RPMI with 1% Zap Hybridoma (InVitria Inc., USA). Cells were enumerated and cultured in a 6-well plate at the seeding concentration 1×10^6 cells/ mL of medium. After 1 week of culture, cells were transferred to T25 flask. When cells were ~90% confluent, they were moved to T75 flasks. Multiple T75 flasks were grown @ 5 million cells/50 mL and grown for a week until cells died. The exhausted supernatant containing antibodies was collected by centrifugation at 1500 RPM for 10 min[22].

Antibody isotyping of supernatants

All *NC3*00101* supernatants were characterized for their isotypes using a radio immune assay (RIA) antibody isotyping kit (Pierce). H1A, W6/32 and IL-A88 were used as positive controls.

Results

Flow cytometric analysis and Isolation of *NC3*00101* protein from FACS-sorted cells

Flow cytometric analysis of newly transfected *NC3*00101*-P815 cells revealed <20% expression of the protein, which increased to ~50% following 2 weeks of G418 selection. Following fluorescence-activated cell sorting (FACS)-sorting of transfected cell line with a pan-bovine MHC class I monoclonal antibody (H1A), a high level of stable transgene expression, usually >90% positive cells, was achieved. An appreciable protein concentration (1.9 mg/mL) was achieved following purification, yielding a sufficient amount of protein for immunization of mice (~600 µg for the immunization of three mice) and for screening of initial sera, supernatants, and hybridomas. The purified *NC3*00101* protein (~minimum of 600 µg for immunization of three mice) was used for immunization of mice and initial screening of the hybridoma.

Screening of hybridoma supernatants

We screened the initial hybridomas using ELISA and flow cytometry from three fusions performed at WSU (Pullman, WA).

ELISA screening

First fusion

The *NC3*00101* hybridomas were initially screened with ELISA at the WSU Antibody Development Center. Twelve positive NC3 hybridoma colonies were created from the first fusion. NC3-1*4 and NC3-1*9 appeared to have a slightly positive reaction in ELISA using 8 µg/mL of antigen to coat the ELISA plate. The hybridomas NC3-1*4 and NC3-1*9 were slightly positive in ELISA. None of the 12 sera was positive from the first fusion.

Second fusion

From the second fusion of spleen cells with myeloma cells, one positive hybridoma (NC3-2*2) was selected and sent to our laboratory for further expansion and screening. However, we could not identify positive reactivity of the NC3-2*2 supernatant in ELISA. By the time of the second fusion, 12 clones derived from the first fusion were identified as negative.

Third fusion

The NC3-3*7A and NC3-3*7B supernatants were not positive when tested on ELISA. The reactivity pattern of the *NC3*00101* supernatants are summarized in Table 1. Because the antibody titer was low in all NC3-supernatants, we used neat supernatants. The NC3-3*2 hybridoma supernatant showed a strong reactivity, which was more obvious in ELISA performed with neat supernatants than those with 1:100 dilution (Figs. 1 and 2). We endeavored to clone the cryopreserved NC3-3*2 hybridoma, but we failed to recuperate it. Consequently, we lost our indefinite source of *NC3*00101* monoclonal antibodies. We also tested the NC3 hybridoma

Table 1 — The summary of ELISA results of NC3 supernatants

mAb	*N*01701*	*N*01802*	*NC3*00101*
H1A	-	++++	+++
W6/32	-	+++	-
IL-A88 (1:1)	++++	++++	++++
IL-A88 1:10	++++	++++	++++
NC3-1*1	-	-	-
NC3-1*2	-	-	-
NC3-1*3	-	-	-
NC3-1*4	+	+	+
NC3-1*5	-	-	-
NC3-1*6	-	-	-
NC3-1*7	-	-	-
NC3-1*8	-	-	-
NC3-1*9	++++	++	++++
NC3-1*10	-	-	-
NC3-1*11	-	-	-
NC3-1*12	-	-	-
NC3-2*1	+	+	+
NC3-2*9	+	+	+
NC3-3*1	-	-	-
NC3-3*2	-	-	++++
NC3-3*3	-	-	-

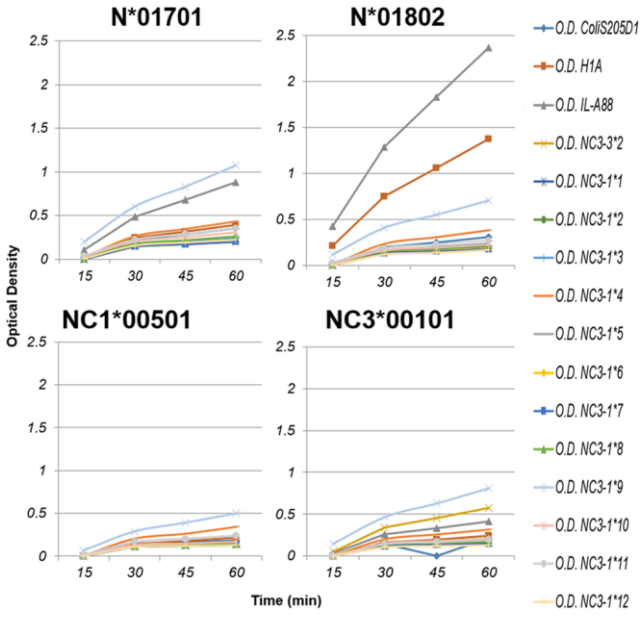

Fig. 1 — ELISA screening of *NC3*00101* supernatants (1:100 dilution). [NC3-hybridoma culture supernatants were tested with ELISA and ODs were recorded at four time intervals of 15, 30, 45 and 60 min. Two bovine classical N*01701 and N*01802 and another non-classical NC1*00501 protein to test specificity of *NC3*00101* supernatants. H1A and IL-A88 are bovine MHC-specific antibodies (broad). ColiS205D1 was used as a negative control. All *NC3*00101*-supernatants were negative or not above background except the NC3-3*2 hybridoma supernatant that had positive reactivity with NC3-00101 antigen. X-axis: Time points (15, 30, 45 and 60 min). Y-axis: Optical Density measured at time points]

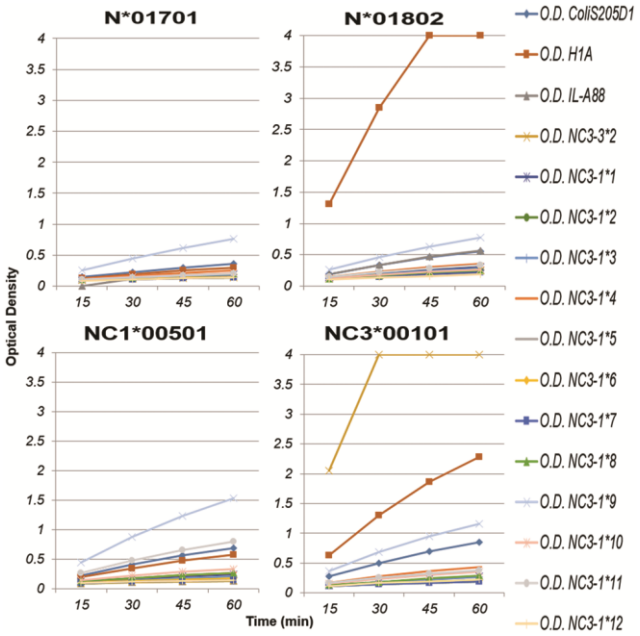

Fig. 2 — ELISA screening of *NC3*00101* supernatants when used neat (1:1). [End points (Optical Density) were measured at four time intervals of 15, 30, 45 and 60 min. Bovine classical class I *N*01701* and *N*01802* and non-classical *NC1*00501* antigens were used as a control for H1A and IL-A88 antibodies. At 1:1 dilution NC3-3*2 hybridoma supernatant had positive reactivity with NC3-00101 antigen. X-axis: Time points (15, 30, 45 and 60 min). Y-axis: Optical Density measured at time points]

supernatants to capture antigens in sandwich ELISA, which did not yield promising results. We plan to perform more fusions in the future using modified protocols for immunization and cloning of hybridomas.

Flow cytometry screening

First fusion

None of the twelve sera was positive from the first fusion.

Second fusion

In flow cytometry screening of the hybridomas NC3-2*1 and NC-2*9, supernatants reacted with all the bovine *MHC-I* proteins, showing weaker reactivity to untransfected P815 cells, which suggests that these supernatants reacted with nonspecific cell cycle antigen on mouse P815 cells. However, NC3-2*1 stained more strongly with the *NC3*00101* protein than the other proteins. We did not detect any specific reactivity in the NC3-2*9 supernatant. Consequently, we decided not to clone NC3-2*1 and NC3-2*9 hybridomas further, and the third fusion was performed using the frozen splenocytes.

Third fusion

Initial flow cytometric screening from the third fusion produced 10 NC3-positive clones, but 2 of them

were expanded and cryopreserved. We received the supernatants NC3-3*1, NC3-3*2, and NC3-3*3, which were tested in flow c ytometry using 1:1 (neat) and 1:100 dilutions. The NC3-3*2 supernatant showed specific reactivity with *NC3*00101* transfectants (Fig. 3) and thus was cloned. At WSU we attempted twice to recuperate the NC3-3*2 hybridoma cell line, but we failed to clone and rescue it. The third fusion was done, but all the clones had nonspecific reactivity. Additional supernatants (NC3-3*4, NC3-3*5, NC3-3*8, NC3-3*9, NC3-3*10, and NC3-3*11) were tested, but none of them were positive on transfected cells (Fig. 4). From the third fusion we cultured four cell lines and tested them on flow cytometry: NC3-3*2, NC3-3*4, NC3-3*7, and NC3-3*8. The NC3-3*7A and 7B supernatants reacted strongly with the NC3 protein, but they also showed a nonspecific reaction with untransfected P815 cells with the same intensity.

Antibody isotyping of supernatants

We characterized the classes and subclasses of immunoglobulins secreted by *NC3*00101* hybridomas. Positive controls H1A, W6/32 and IL-A88 were of IgG2a isotypes and possessed a kappa (κ) light chain. All tested *NC3*00101* hybridomas secreted antibodies of IgG1 isotypes with Kappa (κ) light chain (Table 2).

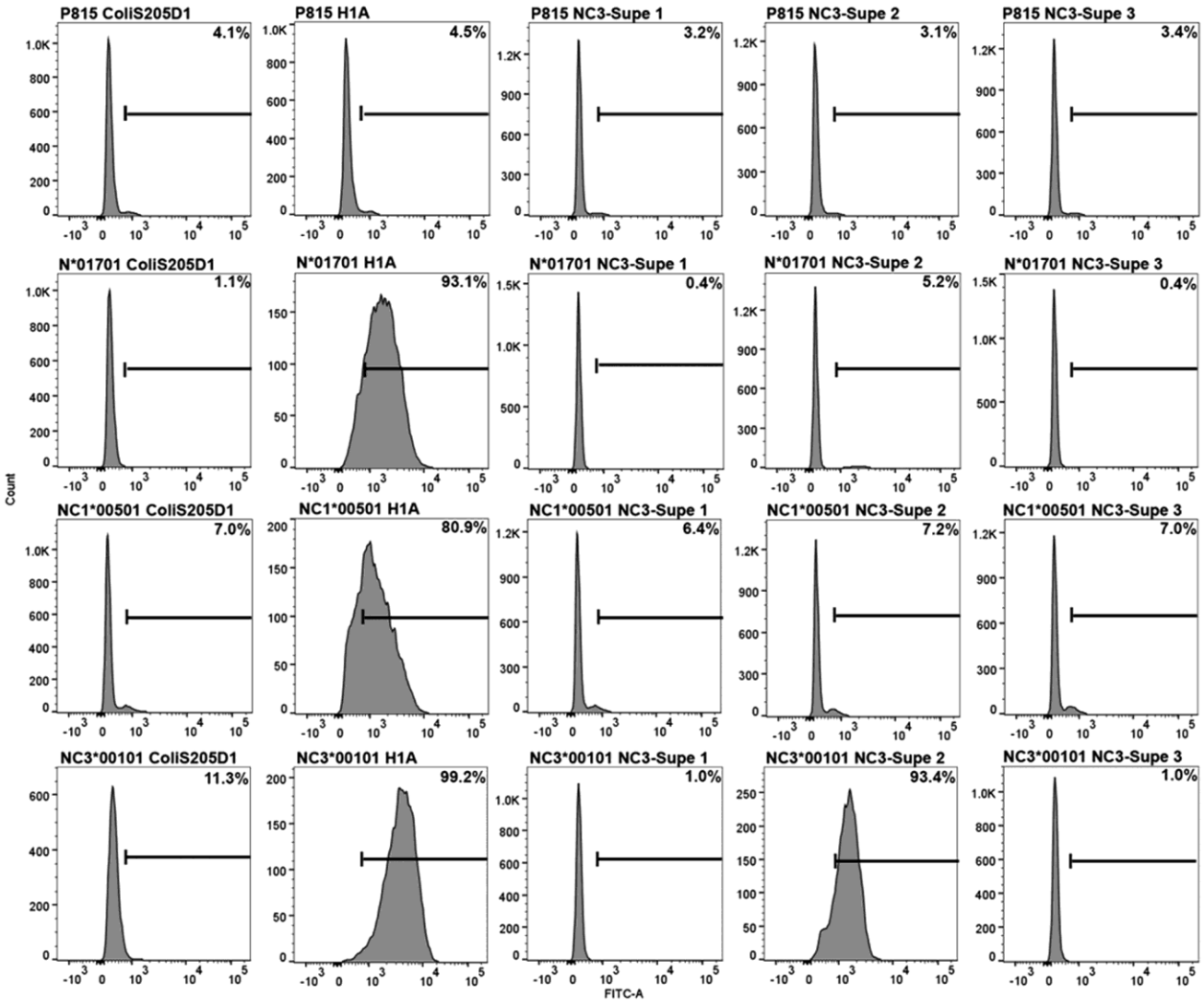

Fig. 3 — Flow cytometry staining of *NC3*00101* hybridoma supernatants from the third fusion. [*BoLA-NC3*00101*-transfected P815 cells were stained for flow cytometry and incubated with NC3-3*1, NC3-3*2 and NC3-3*3 supernatants. *BoLA-NC3*00101*-NC3-Supernatant 2 neat had specific positive response without reacting with the classical N*01701- and non-classical NC1*00501–transfected P815 cells. Untransfected P815 cells were used as negative control in the experiment. H1A is a positive control antibody which stains the surface-expressed *BoLA*-I proteins. An irrelevant isotype-matched ColiS205D1 antibody served as negative control]

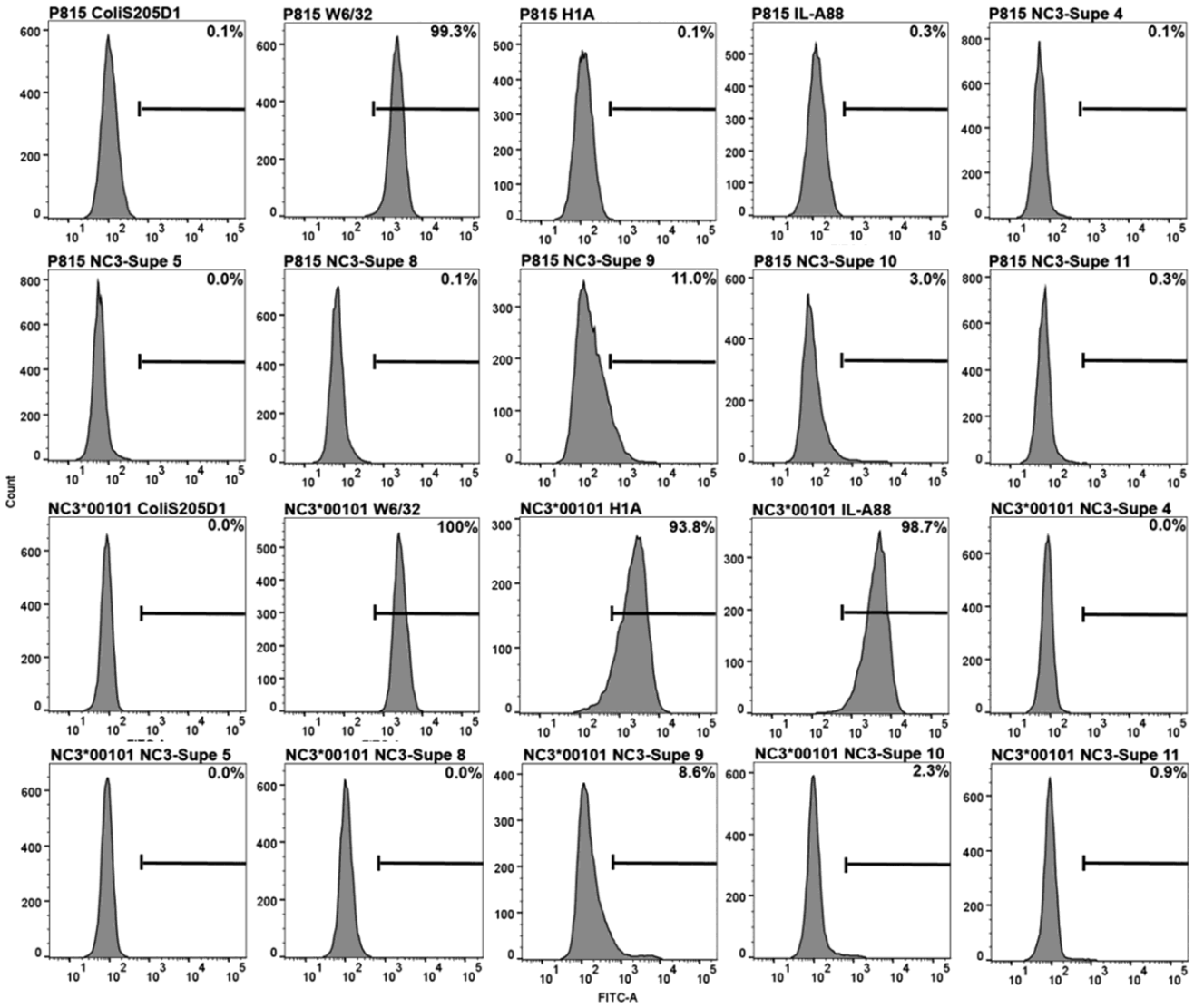

Fig. 4 — Flow cytometry staining of *NC3*00101* hybridoma supernatants (NC3-3*4, NC3-3*5, NC3-3*8, NC3-3*9, NC3-3*10, and NC3-3*11) from the third fusion. [Untransfected (negative control) P815 (top two panels) and *NC3*00101*-transfected P815 cells (bottom two panels) were stained with the hybridoma culture supernatants 4, 5, 8, 9, 10, and 11 from the third fusion. As shown, none of the NC3* hybridoma culture supernatants was positive and none reacted specifically with the *NC3*00101* protein. ColiS205D1 antibody was used as a negative control. Anti-MHC class I antibody W6/32, H1A, and IL-A88 were used as positive control antibodies]

Table 2 — Summary of isotyping of representative *NC3*00101* supernatants

mAb	Isotype	Light Chain
H1A	IgG2a	Kappa
IL-A88	IgG2a	Kappa
W6/32	IgG2a	Kappa
NC3-3*2	IgG1	Kappa
NC3-1*5	IgG1	Kappa
NC3-1*6	IgG1	Kappa
NC3-1*9	IgG1	Kappa
NC3-1*10	IgG1	Kappa
NC3-1*11	IgG1	Kappa

Discussion

There is no specific monoclonal antibody to bovine non-classical *MHC-I* proteins. The first report of bovine monoclonal antibody to red blood cell antigen[24]. Bovine *MHC-I* specific monoclonal antibodies IL-A88 and IL-A19 were produced at International Laboratory for Research on Animal Diseases (ILRAD), Kenya. To develop ELISA to detect secreted and/or soluble *BoLA-Ib* proteins from the trophoblast cell culture supernatant, we used a purified *BoLA-NC3*00101* protein to immunize mice with non-classical *BoLA-NC3*00101* protein to synthesize monoclonal antibodies. Almost none of the

hybridoma supernatants tested on ELISA and flow cytometry were positive. Nonetheless, we identified a hybridoma cell line (NC3-3*2) which secreted *NC3*00101*-specific antibodies which not only was specific to *NC3*00101*-transfected cells on flow cytometry but also captured purified *NC3*00101* protein in ELISA when culture supernatant was used as capture antibody. Unfortunately, despite several efforts, we failed to recover the cryopreserved positive NC3-3*2 hybridoma cell line.

A surface expressed MHC glycoprotein molecule structurally possesses a heavy chain which is covalently linked with β2-microglobulin or light chain expressed endogenously. During culturing of mouse cells transfected with bovine *MHC-I* heavy chain cDNA bovine *MHC-I* protein is expressed combined initially with the endogenously derived murine β2-M that is swapped or exchanged with bovine β2-M from bovine calf serum or fetal bovine serum used in the culture media. Davies *et al*. 1987[25] in their study identified that the *MHC-I* specific antibodies recognized the heavy chain of *MHC-I* proteins and not β2-M. They discovered that WSU-VMRD mAbs (H1A, H6A, H11A, and PT-85A) did not work on western blot, but in non-reducing conditions, they all reacted with the light chain (β2-M) as reported by Li *et al*.[26]. It is possible that these antibodies only recognized the cattle *MHC-I* proteins in which the murine β2-M is replaced by bovine β2-M from the serum used. These antibodies, could not be used to specifically detect bovine *MHC-Ia* and –Ib proteins in culture supernatant. We require an antibody which can detect a single epitope conserved in secreted classical and non-classical bovine *MHC-I* proteins and provide a way to quantitate these proteins released from cells and tissues during pregnancy.

Despite the non-recoverability of the only positive hybridoma in the present study, we have observed some interesting findings in this study project. Importantly, mice immunized with bovine non-classical class I protein *NC3*00101* elicits an immunogenic response. A research group used transfected cells to immunize host to achieve the production of immunoglobulins[27]. We also observed that except the NC3-3*2 hybridoma, all hybridomas showed positive staining with untransfected cells which raises questions about the use of appropriate host and/or purified protein derivative to immunize mice. Non-positive hybridomas and lack of ability to recover cryopreserved clonal cells beg us to rethink on choosing a more suitable form of immunogen for the host. Transfected cells can be better immunogens than protein to avoid background and non-specificity. Murine P815 cells are broad host cell line for transfection studies used by various research groups. These cells express endogenous murine *MHC-I* which may interfere with binding of bovine *MHC-I* protein expression. An *MHC-I* null cell line such as human K562 can also be used for expressing bovine class I transgenes. It may also be worth choosing an alternative host animal for immunization.

Hybridoma stability is affected by various factors such as mutations, chromosome losses and the monoclonal antibody process variables[28]. Moreover, selection of adjuvants, cryopreservation of polyclonal and monoclonal hybridomas and their limiting dilution are the parameters of consideration for generation of antibodies[29,30]. In future, we intend to perform repeated cloning and fusions or a combination of thereof. In flow cytometric and ELISA screens performed on hybridomas and culture supernatant respectively, a typical non-specific reactivity was observed except one specific NC3-3*2 hybridoma clone that was non-recoverable. Thus, data indicate that we had very low-titer/non-specific antibodies in supernatants and a rigorous cloning of cryopreserved hybridomas is required to produce a stable clone of hybridoma cell line.

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